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Corticotropin releasing factor influences aggression and monoamines: Modulation of attacks and retreats

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Abstract

Salmonids establish social hierarchies as a result of aggressive social interactions. The establishment of dominant or subordinate status is strongly linked to neuroendocrine responses mediated through the stress axis. In this study, we tested the effects of icv CRF on the behavioral outcome, plasma cortisol and monoamine function in trout subjected to a socially aggressive encounter. Rainbow trout were treated with an icv injection of artificial cerebrospinal fluid (aCSF), 500 or 2000 ng ovine CRF, or not injected. Fish were allowed to interact with a similarly sized conspecific for 15 minutes. Following the behavioral interaction, plasma cortisol and central monoamine concentrations were analyzed. Trout treated with CRF were victorious in approximately 60% of the aggressive encounters against aCSF treated opponents. Trout injected with CRF exhibited a reduction in the total number of attacks and decreased latency to attack. When trout were divided winners and losers, only victorious CRF-treated fish exhibited a reduced latency to attack and fewer retreats. Social stress increased cortisol levels in both winners and losers of aggressive interaction. This effect was enhanced with the additional stress incurred from icv injection of aCSF. However, icv CRF in addition to social stress decreased plasma cortisol in both winners and losers. While aggression stimulated significant changes in serotonergic and dopaminergic activity, the magnitude and direction were dependent on limbic brain region, CRF dose, and outcome of social aggression. With broad effects on aggressive behavior, anxiety, stress responsiveness, and central monoaminergic activity, CRF plays an important role modulating the behavioral components of social interaction.

Keywords

Rainbow trout; aggression; CRF; cortisol; serotonin; dopamine

Rainbow trout (*Oncorhynchus mykiss*) readily exhibit aggressive and socially stressful interactions to form distinct social hierarchies (Øverli et al., 1999). During the formation of

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dominant and subordinate social status, individuals with differing rank exhibit different behavior, neurochemistry and hormone responsiveness (Øverli et al., 2001; Øverli et al., 2004b; Winberg et al., 1993; Winberg et al., 2001; Winberg and Lepage, 1998). Dominant individuals are characterized by a proactive stress coping style, that reflects a low level of trait anxiety, a high level of aggression towards conspecifics, robust growth rates and increased feeding behavior (Øverli et al., 2004a; Øverli et al., 2007). Subordinate individuals show a higher level of trait anxiety characteristic of a more reactive coping style, in which decreased feeding rate, decreased locomotor activity, decreased growth rate and decreased immune response are mediated by the neuroendocrine machinery serving stress responsiveness (Øverli et al., 2002a; Øverli et al., 2004b; Øverli et al., 2004a; Winberg et al., 2001). These coping styles, based on responsiveness to stress, are robust, heritable and predictive of social status (Øverli et al., 2002b; Pottinger and Carrick, 2001).

Prior residence time, prior winning or losing experience, size, rearing environment and genetics (hatchery or wild stock) influence the nature and outcome of aggressive interactions in salmonids (Rhodes and Quinn, 1998). Physiological status of individual fish also plays an important role in determining outcomes of behavioral interactions. Artificially elevated cortisol levels from implants and dietary supplements modify aggressive behavior. In rainbow trout treated with cortisol and subjected to social interaction, treatment significantly decreased locomotion and aggression (Øverli et al., 2002a), and the probability that the fish would become dominant when paired with a smaller (>5% size difference) conspecific (DiBattista et al., 2005). In size matched (<1.5% size difference) pairs of fish, those treated with cortisol became subordinate significantly more often than their non-treated counterpart. Furthermore, co-administration of cortisol and the glucocorticoid receptor antagonist RU486 abolished the effect of the cortisol implant to cause subordination (DiBattista et al., 2005). Recent work has implicated the neuroactive peptide corticotropin releasing factor (CRF) in stimulating the hypothalmo-pituitary-interrenal (HPI) axis in salmonids (Flik et al., 2006), and stimulating anxious locomotion independent of cortisol release (Carpenter et al., 2007).

The peptide CRF is strongly linked to stress and anxiety in vertebrates through neuromodulatory activity in the brain, as well as hormonal activity, stimulating behavioral (Koob and Heinrichs, 1999), neurochemical (Summers et al., 2003a) and endocrine responses to various stimuli. In mammals, CRF neurons in the paraventricular nucleus of the hypothalamus (PVN) are stimulated by stress and control the action of the HPA axis to allow for release of downstream ACTH and cortisol (Balm et al., 1994). Furthermore, social interaction, restraint, hypoxia, and other stressors have been shown to cause a depletion of CRF in the PVN while concurrently stimulating an increase in brain CRF mRNA (Chen et al., 2004; Deschamps et al., 2003; Imaki et al., 1995; Matthews and Challis, 1995). By interacting centrally with monoamine systems such as serotonin (5-HT), dopamine (DA) and norepinephrine (NE), CRF modulates behavioral responses, especially those related to social and conflict situations (Lowry and Moore, 2006). In familiar and social environments, both mammals and fish show CRF-dependent stimulation of behaviors including increased locomotion which may indicate a more anxious state (Campbell et al., 2004; Carpenter et al., 2007; Dunn and Berridge, 1990).

In fish as well as mammals, CRF and its related peptide urotensin I (UI) contribute to stress responsiveness (Lowry and Moore, 2006; Pepels et al., 2004). These peptides also influence appetite in hypoxic rainbow trout (Bernier and Craig, 2005). Intracerebroventricular injections of either CRF or UI in goldfish (*Carassius auratus*) dose-dependently decrease food intake; an effect that is blocked by pretreatment with a CRF receptor antagonist (Bernier and Peter, 2001; De Pedro et al., 1993; De Pedro et al., 1997; De Pedro et al., 1998). Furthermore, icv CRF treatment in Chinook salmon (*Oncorhynchus tshawytscha*) stimulates increased locomotion and downstream migration activity (Clements et al., 2002; Clements and Schreck,

2004). Similar treatment with CRF in rainbow trout stimulates increased locomotion when compared to un-injected and non-injected controls and induces a previously unseen headshaking behavior that may be representative of increased anxiety (Carpenter et al., 2007).

Considering the broad interrelated behavioral influences of CRF in fish, and the evidence that CRF influences aggression in mammals (Gammie et al., 2004), suggests that CRF in the brain may influence the outcome of socially aggressive interactions in rainbow trout. Therefore, we hypothesized that rainbow trout treated icv with CRF, through increased anxiety and heightened threat awareness, will perform an increased number of attacks. As a consequence, we hypothesized that these more anxiously aggressive CRF-treated fish would prevail during socially aggressive interaction when paired with a size matched aCSF-injected control fish. Our results suggest that CRF potentially influences aggression. However, the behavioral manifestations of this treatment are complex. In addition, while we hypothesized that aggression and icv injection would stimulate increased plasma cortisol concentrations, we did not expect the addition of CRF to further stimulate cortisol secretion (Carpenter et al., 2007). Furthermore, either treatment with icv CRF (Carpenter et al., 2007) or exposure to an aggressive encounter (Øverli et al., 2007; Summers and Winberg, 2006) have been shown to stimulate limbic (subpallium and dorsal pallium) and brainstem (raphé) monoaminergic activity in trout. Serotonin has been demonstrated to stimulate the HPI axis in salmonids (Winberg et al., 1997). Therefore we hypothesized that trout treated with icv CRF in agonistic interactions with icv saline treated fish will exhibit increased dopaminergic and serotonergic activities in the subpallium.

Experimental Procedures

Subjects and Housing

Juvenile rainbow trout (*Oncorhynchus mykiss*; raised from eggs) weighing 150 ± 30 g were housed indoors in a six foot diameter circular tank under natural light conditions prior to experimentation (Gavins Point National Fish Hatchery, Yankton, SD). Fish were fed daily with Nelson's Silver Cup sinking trout feed at a rate of 1% body weight per day. All experiments were conducted in a manner that minimized suffering and the number of animals used, in accordance with the Declaration of Helsinki and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), under a protocol approved by University of South Dakota IACUC.

Experimental fish were allowed to acclimate for 10 days in individual compartments of 75 gallon glass aquaria with a flow through water system and aeration. Each tank was individually lit and separated into 4 equal sized compartments by the insertion of 3 opaque barriers. This time frame was chosen to stimulate territorial association with each compartment, while barriers inhibited social interaction between fish.

Experimental design

A cohort of 32 fish ($N = 16$ CRF group, $N = 8$ at each dose; $N = 16$ aCSF group) were anesthetized and injected intracerebroventricularly (icv) with one of the following: 0 (injection control; 2 μ l of aCSF only), 500, or 2,000 ng CRF (ovine corticotrophin releasing factor; Sigma, St. Louis)(Clements et al., 2002) in 2 μ l of artificial cerebrospinal fluid (aCSF). Social interactions were between size matched pairs of rainbow trout, one of which was injected with CRF and one with aCSF. Another group of 16 fish were subjected to the social interaction, but not injection and these fish served as the aggression only control group. Finally, 8 other fish served as completely untreated controls that were assessed for cortisol and monoamine

concentrations but did not receive anesthesia or icv injection, and were not subjected to socially aggressive interaction. Throughout the paper these fish are referred to as “no-fight controls”.

Anesthesia

Fish were anesthetized in 500 mg/l of methane tricane sulfonate (MS222) in a volume of 10 liters. The time to loss of equilibrium was consistent (~18 sec) for each fish. Following anesthesia and treatment, fish were placed back in their individual compartment and allowed to recover (2-5 minutes to recovery of equilibrium). Post-injection behavioral observations were carried out 25 minutes after equilibrium was recovered.

Injections

Previously we validated an icv injection procedure which allowed us to deliver vehicle and drugs directly into the 3rd ventricle of anesthetized rainbow trout (Clements et al., 2002). Prior to experimentation, methylene blue was used as a tracer to determine injection site penetration and assess the accuracy of the icv procedure. Over multiple trials we had greater than 95% success rate in penetrating the 3rd ventricle. Anesthetized fish were injected postorbitally (26 gauge needle), along the midline to a depth of 7 mm into the 3rd ventricle of the brain. To minimize leakage of CRF or aCSF from the injection site, 2 µl of fluid was injected slowly, and 5 seconds was allowed to elapse after the injection before the needle was withdrawn.

Behavioral Analysis

Following injection procedure, fish were returned to their home tank and allowed to recover for 25 minutes following recovery of equilibrium. At 25 minutes, tank barriers were removed and pairs of fish were allowed to interact for 15 minutes. Removal of the barrier merged the two previously distinct territories into one space, without physically disturbing the fish, while still preserving the experience of familiarity with the home tank. Observers recorded latency to first attack, total numbers of attacks and retreats, as well as which individual won the interaction. An attack was scored when one fish swam towards and bit the other individual, either in the side (lateral attack) or in the head (frontal attack). If an individual swam toward the other fish and bluffed an attack, and no physical bite took place, an attack was not recorded. Swimming toward the other fish was recorded as approach rather than attack. At some point in the interaction, one fish would cease all offensive attacks and switch to evasive behaviors. Forsaking offensive aggression for retreat behavior is the criteria we used to determine the loser of the interaction, with the winner being the fish that remained aggressive throughout the entire trial.

During the experiment, observers noticed a head-shaking behavior in rainbow trout injected with CRF. This head shaking is similar to behavior previously observed in fish treated with CRF at the same doses (Carpenter et al., 2007), and was characterized by flaring of the gill covers and violent side-to-side shaking of the entire head several times in succession. Each bout of head shaking lasted approximately two to four seconds, and was recorded as one head shake.

Cortisol Analysis

Immediately following the social interaction and behavioral observation, both fish were anesthetized as previously described, blood samples were taken from the caudal vein (21 gauge needle pretreated with heparin), and the fish were killed by rapid decapitation. Plasma was separated by centrifugation and samples were frozen (-80°C) until analysis. Analysis of plasma cortisol was accomplished by enzyme linked immuno-sorbant assay in a 96 well plate (ELISA; R&D Systems cortisol kit, Minneapolis, MN)(Carpenter et al., 2007). The data were

transformed and checked against control values to determine actual plasma cortisol concentration and values were expressed as ng/ml cortisol.

Analysis of Monoamines

Brains were removed from the fish intact in the cranium, stored at -80°C, and sliced coronally (300 µm) in a temperature controlled (-12°C) cryostat (Leica-Jung 1800, Wetzlar, Germany). Slices were thaw-mounted on glass microscope slides and rapidly refrozen on dry ice. Individual brain areas sampled were microdissected from the appropriate sections using a 500 µm internal diameter cannula (Fig. 1). To examine the role of CRF on monoaminergic activity that mediates aggression, anxiety and locomotion, monoamines were measured in limbic forebrain regions including the dorsolateral pallium (DL), dorsomedial pallium (DM), subpallium (Vc), preoptic area of the hypothalamus (POA) and the caudal hypothalamus (NT) using HPLC with electrochemical detection. The DL is a putative homologue of the mammalian hippocampus (Carruth et al., 2000; Northcutt, 2006). The vertebrate amygdala is a complex structure that includes nuclei derived from both limbic and striatal elements (Laberge et al., 2006). Previous work suggests that the dorsomedial pallium (DM), the commissural (Vc) and supracommissural (Vs) nuclei of the ventral area of the teleost telencephalon are likely to be subregions homologous to mammalian amygdalar nuclei (Northcutt, 2006). Based on recent neuroanatomical work in fish and amphibians, the Vc was chosen for analysis as it represents a nucleus in fish that combines the functions of the mammalian striatum and central amygdala (Laberge et al., 2006; Northcutt and Davis, 1983). The striatum is important for motivated locomotion, and the amygdala is an important site of extrahypothalamic CRF production in mammals. Locations of brain regions were based on a stereotaxic atlas for trout and salmonid maps of brain neurochemistry (Billard and Peter, 1982; Carpenter et al., 2007; Carruth et al., 2000).

Dopamine (DA), norepinephrine (NE), epinephrine (Epi), and the DA catabolite: 3,4-dihydroxyphenylacetic acid (DOPAC); serotonin (5-HT); and its catabolite: 5-hydroxyindoleacetic acid (5-HIAA) were measured using high performance liquid chromatography (HPLC) with electrochemical detection (Emerson et al., 2000; Renner and Luine, 1986; Renner and Luine, 1984). Microdissected samples were expelled into 60 µl of sodium acetate buffer (pH 5.0) containing 6.82×10^{-5} M DHBA (9.4 pg/µl dihydroxybenzylamine; internal standard). Each sample was frozen, then thawed and centrifuged at $15\,000 \times g$ for 3 min. The supernatant was removed and 45 µl was injected into a chromatographic system (Waters Associates, Milford, MA) and analyzed electrochemically with an LC-4B potentiostat (Bioanalytical Systems, West Lafayette, IN). The electrode potential was set at +0.6 V with respect to an Ag/AgCl reference electrode. The tissue pellet remaining from each sample was dissolved in 110 µl 0.4 N sodium hydroxide (NaOH) and protein content was assayed by coulometric absorbance (Bradford, 1976).

Statistics and Calculations

Comparison of the proportion of winners by injection treatment was accomplished using the Fisher Exact test. Hormonal and neurochemical data were compared in two separate ways, by treatment irrespective of social rank, and by treatment including social status. *A priori* hypotheses about CRF treatment alone (not considering social rank) were examined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests with significance accepted at $P < 0.05$. When the data did not meet statistical assumptions required for ANOVA, the data were log transformed. Treatment-only analyses for comparisons of hormone or neurotransmitters include a group that was neither injected nor subjected to the socially aggressive interaction. Comparisons of CRF treatment that consider the outcome of the social interaction were made using a two-way ANOVA corrected model (main effect) which is used to derive independent and interaction factor effects, which for this experiment

includes fight outcome effects, icv injection treatment (no injection, aCSF, 500 ng CRF, 2000 ng CRF) effects, and outcome by injection interaction effects. Comparisons between specific treatment groups following significant two-way ANOVA results were accomplished using Student's t-tests. When comparing treatment groups it is important to keep the experimental design in mind, fish treated with CRF were never paired with other CRF treated conspecifics, but rather with aCSF treated opponents. It must be noted for this statistical comparison, because CRF-treated trout were more often winners than losers, the sample-size for CRF treated losers is very small, diminishing the power for all statistical comparisons. Appropriate comparisons include those between animals with the same treatment (winners vs losers), the same outcome of aggressive interaction (winners vs winners), similar icv injection (CRF vs aCSF), and comparisons with un-injected controls. To facilitate comprehension of the array of possible comparisons, Greek letter notations are included at the base of each bar in select figures to indicate appropriate contrasts (for example: groups labeled with a β can be compared to β_1 and β_2 , groups labeled with γ to γ_1 and γ_2 , β_1 to γ_1 , β_2 with γ_2 , and α with β or γ).

Results

Behavior

Social interaction was initially tested between sized-paired fish, one injected icv with aCSF and the other with CRF (either 500 or 2000 ng doses). A greater percentage of fights were won by fish treated with CRF, regardless of dose (Fisher Exact, $P < 0.022$; Fig. 2). However, the most meaningful results are revealed by examining the effects of CRF on specific types of behavior.

Attacks—As there was no obvious difference in the number of lateral versus frontal attacks in any treatment group, these two attack types were grouped together as “total attacks.” It is important to note that the number of attacks decreased with icv injection (two-way ANOVA main effect: $F_{7,59} = 5.0$, $P < 0.0001$; Fig. 3A, B), suggesting that the icv injection procedure is stressful (two-way ANOVA injection effect: $F_{3,59} = 8.8$, $P < 0.0001$), which is consistent with previous results (Carpenter et al., 2007). Winners attack more than losers in uninjected control pairs ($t_9 = 2.3$, $P < 0.05$; Fig. 3B). This relationship is maintained in aCSF treated trout ($t_{35} = 3.95$, $P < 0.001$; Fig. 3B), even though the total number of attacks is markedly lower in this group (Fig. 3A). However, in CRF-treated trout, this differential expression of attack behavior is not observed. When all combatants (winners + losers) are statistically considered together, fish injected icv with 500 ng CRF exhibited a significant suppression (one-way ANOVA: $F_{3,58} = 4.02$, $P < 0.012$) of attack behavior (Fig. 3A).

Latency to Attack—There was a trend for latency to first attack to decrease in both groups of fish treated with icv CRF compared to aCSF-injected and non-injected control fish ($F_{3,55} = 1.96$, $P < 0.130$) when winners and losers were grouped together (Fig. 4A). When treatment groups were separated by winners and losers, a significant decrease in latency to first attack was seen in winners treated with 500 ng CRF compared to losers ($t_8 = 2.35$, $P < 0.045$) treated with that same dose and compared to aCSF treated winners ($t_{20} = 2.27$, $P < 0.034$) (Fig. 4B). Fish treated with 2000 ng CRF were also significantly ($t_{18} = P < 0.042$) faster to attack than winners treated with aCSF.

Retreats—All groups of fish treated with an icv injection (saline and CRF) showed a significant ($F_{3,63} = 5.88$, $P < 0.001$) decrease in the total number of retreats compared to non-injected control fish (Fig. 5A). There was a significant effect in our two-way model (Two-way ANOVA main effect: $F_{7,62} = 3.69$, $P < 0.002$), which was also significant for the effects of outcome (Two-way ANOVA winner vs loser effect: $F_{1,62} = 8.41$, $P < 0.005$), CRF treatment (Two-way ANOVA CRF effect: $F_{3,62} = 4.14$, $P < 0.01$), and CRF by outcome interaction (Two-

way ANOVA CRF $\times$ winner effect: $F_{3,62} = 2.9$, $P < 0.04$). When these groups were separated into winners and losers, non-injected winners show a trend toward significantly ($t_{16} = 1.84$, $P < 0.085$) fewer retreats than non-injected losers. In addition, icv CRF treated winners at both doses (500 and 2000 ng), showed significantly (500 ng CRF: $t_{11} = 3.99$, $P < 0.002$; 2000 ng CRF: $t_{12} = 2.4$, $P < 0.031$) fewer retreats than non-injected winners, and also significantly (500 ng CRF: $t_6 = 4.55$, $P < 0.004$; 2000 ng CRF: $t_7 = 2.3$, $P < 0.05$) fewer retreats than icv CRF injected losers (Fig. 5B).

Ratio of Attacks to Retreats—Both the number of attacks and the number of retreats are reduced in icv CRF treated fish, and the relationship between these behaviors is unclear. Therefore, we qualitatively examined the ratio between attacks and retreats for all groups of fish (Fig. 5C). Non-injected and icv aCSF-treated fish show an attack/retreat ratio of less than one. Both groups of icv CRF treated fish show a ratio greater than one, indicating that these animals attack more than they retreat.

Cortisol

Non-injected controls and fish injected icv with aCSF show significantly increased plasma cortisol concentrations (one-way ANOVA, $F_{4,78} = 12.53$, $P < 0.001$) following aggressive social interaction when compared to resting control levels. The data presented here validate previous results that show both aggressive interactions (Summers and Winberg, 2006) and the icv injection procedure (Carpenter et al., 2007) are stressful (Fig. 6A). Furthermore, both winners and losers of aggressive interactions show an increase in plasma cortisol following a 15 minute bout (Fig. 6B; see statistics below). Fish treated icv with aCSF showed significantly higher levels of plasma cortisol compared to resting non-interacting controls, non-injected fight controls and icv CRF treated fish (Fig. 6A). In addition, both groups of icv CRF treated fish showed significantly increased plasma cortisol levels compared to resting non-interacting control fish, but significantly less plasma cortisol compared to icv saline treated fish (Fig. 6A).

For simplicity, when comparing treatment groups in Fig. 6B, Greek letter notations at the base of each bar are indications of appropriate comparisons, because fish treated with 500 ng CRF were never paired with other CRF treated conspecifics, but rather with aCSF treated opponents (for example: groups labeled with a β can be compared to β_1 and β_2 CRF treated groups). When the treatment groups are separated and compared to their fight outcome, non-injected winners and losers (both marked α) were not significantly different (Fig. 6B). Regardless of fight outcome, fish treated icv with 500 ng CRF had significantly less plasma cortisol (two way ANOVA CRF treatment effect; $F_{3,63} = 10.25$, $P < 0.0001$) compared to their aCSF treated opponent (compare β to β_1 , $t_{25} = 1.86$, $P < 0.075$; and compare γ to γ_1 , $t_{16} = 3.04$, $P < 0.008$). However, winning fish treated icv with 500 ng CRF showed significantly more plasma cortisol than losing fish also treated with the same dose of CRF (compare β_1 to γ_1 , $t_7 = 2.45$, $P < 0.044$; note that these fish were similarly treated but never opponents). Winning fish treated icv with 2000 ng CRF showed significantly less plasma cortisol than their aCSF treated opponents (compare β with β_2 ; $t_{25} = 2.42$, $P < 0.023$), but were not significantly different from winning fish treated with 500 ng CRF (compare β_1 with β_2 ; $t_{10} = 2.42$, $P > 0.49$). While losing fish treated with 2000 ng CRF were not significantly different from their aCSF treated opponents (compare γ with γ_2 ; $t_{17} = 1.44$, $P > 0.17$), they also were not significantly different in plasma cortisol concentrations from winning fish treated with 2000 ng CRF (compare β_2 with γ_2 ; $t_8 = 1.35$, $P > 0.21$). In contrast, losing fish treated with 2000 ng CRF had significantly greater plasma cortisol concentrations than losing fish treated with 500 ng CRF (compare γ_1 with γ_2 ; $t_5 = 5.25$, $P < 0.003$; Fig 6B). However, when considering treatment irrespective of fight outcome, icv CRF reduced plasma cortisol levels compared to icv aCSF treatment (Fig. 6A). Therefore, CRF treatment followed by an aggressive social interaction blunted plasma cortisol response when compared to fish subjected to social stress following icv injection of aCSF.

Monoamines

Hippocampus (dorsolateral pallium)—There was a significant increase (one-way ANOVA, $F_{4,79} = 3.47$, $P < 0.012$) in 5-HT in the dorsolateral pallium in treated behaviorally interacting fish when compared to non-interacting control fish (Fig. 7A). Since icv injections of aCSF or CRF had no effect on hippocampal 5-HT when compared to non-injected controls exposed to a conspecific, the results suggest that the increase was due to social interaction. There was also no significant effects of treatment or win/loss status (two-way ANOVA main effect, $F_{7,69} = 0.55$, $P > 0.79$) when 5-HT in the hippocampus was analyzed. Similarly, no significant difference (two-way ANOVA, $F_{7,69} = 1.02$, $P > 0.43$) in the serotonergic catabolite 5 hydroxyindoleacetic acid (5-HIAA) was found in the hippocampus when comparing winners and losers (Fig. 7B). However, there was a significant increase (one-way ANOVA, $F_{4,79} = 3.78$, $P < 0.007$) in 5-HIAA in fish that participated in an aggressive interaction when compared to non-interacting control fish.

Social interaction resulted in a significant decrease (one-way ANOVA, $F_{4,80} = 10.89$, $P < 0.001$) in dopamine (DA) in the hippocampus in all groups compared to non-interacting control fish (Fig. 8A). However, there was no significant effect (two-way ANOVA, $F_{7,69} = 0.57$, $P > 0.78$) on DA with respect to CRF treatment or win/loss status in this region. A significant decrease (two-way ANOVA, $F_{7,69} = 3.39$, $P < 0.004$) in the DA catabolite DOPAC was observed in CRF-treated fish (two-way ANOVA, CRF effect, $F_{3,69} = 6.95$, $P < 0.0001$) compared to non-injected controls and aCSF injected fish (Fig. 8B). Further, DOPAC levels in non-injected but aggressively interacting fish were significantly less than in non-injected non-interacting controls (One-way ANOVA, $F_{4,79} = 5.91$, $P < 0.001$). In addition, aCSF treated winners had significantly higher levels of DOPAC than winners treated with 500 ng (γ vs β_1 : $t_{20} = 2.42$, $P < 0.025$) or 2000 ng CRF (γ vs β_2 : $t_{47} = 2.61$, $P < 0.012$), but not when compared to their opponents (compare γ vs γ_1 and γ_2 ; data not shown). However, aCSF losers had significantly higher DOPAC concentrations in the hippocampus compared to CRF-treated winners at 500 (β vs β_1 : $t_{26} = 2.23$, $P < 0.035$) and, at the 2000 ng dose, increases in DOPAC approached significance (β vs β_2 : $t_{27} = 1.95$, $P < 0.062$).

Amygdala (dorsomedial pallium)—There were no significant changes in 5-HT in response to treatment or status (two-way ANOVA main effect: $F_{7,55} = 1.18$, $P > 0.33$) or 5-HIAA (two-way ANOVA main effect: $F_{7,53} = 1.42$, $P > 0.22$) in the dorsomedial pallium stimulated by CRF, aggression or fight outcome (data not shown). Similarly, no significant changes in DA (two-way ANOVA main effect: $F_{7,52} = 0.44$, $P > 0.87$) or NE (two-way ANOVA main effect: $F_{7,54} = 1.37$, $P > 0.24$) concentration were detected in this area (data not shown).

Amygdalar subpallium (ventral commissure)—In the amygdalar subpallium, aggressively interacting trout, irrespective of fight outcome, exhibited significantly elevated 5-HT (one-way ANOVA: $F_{4,83} = 45.96$, $P < 0.001$) and 5-HIAA (One-way ANOVA: $F_{4,78} = 7.06$, $P < 0.001$) compared to controls not subjected to social interaction (Fig. 7C,D).

There was a significant effect (two-way ANOVA aggression effect: $F_{1,66} = 10.06$, $P < 0.002$) of fight outcome on DA concentrations in the amygdalar subpallium observed in fish treated with CRF. This effect was significant (compare γ_2 with β_2 ; $t_9 = 2.27$, $P < 0.049$) only at the 2000 ng CRF dose (Fig. 9A).

Pre-Optic Area (POA)—There was a significant increase in 5-HT (one-way ANOVA: $F_{4,72} = 3.45$, $P < 0.012$) and DA (one-way ANOVA: $F_{4,72} = 4.96$, $P < 0.001$), but not in their catabolites 5-HIAA (One-way ANOVA: $F_{4,71} = 1.09$, $P > 0.367$) or DOPAC (one-way ANOVA: $F_{4,74} = 0.51$, $P > 0.728$) in the pre-optic area in response to aggressive social

interaction regardless of other treatments (Figs. 7E,F; DA and DOPAC data not shown). Furthermore, no significant change in NE (one-way ANOVA: $F_{4,70} = 0.975$, $P > 0.427$) was measured in the pre-optic area in response to aggressive social interaction or treatment.

When comparing winners and losers the interaction between CRF and aggressive social interaction is significant (two-way ANOVA interaction effect: $F_{3,46} = 3.56$, $P < 0.021$) there is a trend ($t_{18} = 1.77$, $P < 0.09$) toward increased DA in aCSF treated losers compared to winners that appears to be reversed ($t_9 = 1.9$, $P < 0.09$) when comparing CRF treated winners and losers (Fig. 9B). This trend is also apparent in the hypothalamus (see below).

Hypothalamus (NT)—A significant increase in hypothalamic 5-HT (one-way ANOVA: $F_{4,56} = 6.2$, $P < 0.001$), but not 5-HIAA (one-way ANOVA: $F_{4,54} = 0.99$, $P > 0.42$) was present in all groups exposed to social interaction when compared to non-treated, non-interacting control fish (data not shown). Further, both DA (one-way ANOVA: $F_{4,62} = 9.84$, $P < 0.001$) and DOPAC (one-way ANOVA: $F_{4,51} = 3.91$, $P < 0.008$) were also significantly increased by aggressive social interaction, but not in response to icv injection or CRF treatment (Fig. 8E, F). Like DA, NE also showed a significant (one-way ANOVA: $F_{4,60} = 8.71$, $P < 0.001$) increase in response to aggressive social interaction (data not shown). While all the groups involved in aggressive social interactions showed similar increases in hypothalamic monoamines, no changes in activity were apparent due to icv aCSF or CRF treatments.

However, when aggressive interaction outcome (winners vs losers) is considered, there was a significant interaction effect (Two-way ANOVA main effect: $F_{7,41} = 3.23$, $P < 0.008$) such that winners differed from losers in hypothalamic DOPAC (Fig. 9C). This interaction effect (two-way ANOVA CRF $\times$ outcome interaction effect: $F_{3,41} = 6.02$, $P < 0.002$) was significant when comparing aCSF treated winners and losers (β vs γ ; $t_{13} = 2.6$, $P < 0.022$) and also comparing winners and losers at the highest dose of CRF (β_2 vs γ_2 ; $t_9 = 2.4$, $P < 0.039$). Similar to DA in POA, DOPAC in the hypothalamus was higher in aCSF-treated losers, but reversed in CRF-treated trout such that winners had higher hypothalamic DOPAC concentrations compared with losers (Fig. 9C).

Discussion

Trout treated icv with either of two doses CRF tended to win aggressive interactions when paired with size-matched fish injected icv with saline (Fig. 2). However, the CRF treated trout did not win all such interactions. Nonetheless, CRF treatment did influence specific behaviors that control the fight outcome, such as total attacks, latency to attack, retreats and ratio of attacks to retreats (Figs. 3, 4, 5). Treatment with CRF suppressed plasma cortisol concentrations elevated by the icv injection procedure, but not below levels stimulated by aggression (Fig. 6). Further, aggression stimulates regionally specific monoaminergic activity, which can be influenced by treatment with CRF (Figs. 7, 8, 9).

Aggressive behavior is modified by CRF

Treatment effects—Rainbow trout treated icv with both 500 and 2000 ng doses of CRF showed a propensity to defeat aCSF-treated trout in aggressive interactions. These CRF-treated fish adopted different fight strategies compared to non-treated interacting fish. Surprisingly, fish treated with CRF showed a significant decrease in the total number of attacks compared to non-injected controls (Fig. 3A). However, even though the number of attacks was decreased in CRF-treated fish, the latency to first attack in a given interaction was significantly reduced. Previous work in salmonids suggests that latency to first attack determines dominant social status (Höglund et al., 2001). In addition, all groups of treated fish had fewer retreats when compared to non-injected control fish (Fig. 5A). We also considered that the relationship between attack and retreat behavior may play a role in determining fight outcome, and

qualitatively compared the ratio of average attacks to retreats (Fig. 5C). Untreated and aCSF-treated animals showed slightly fewer attacks than retreats, whereas CRF-treated fish attacked more often than they retreated (Fig. 5C). While retreats and attacks require additional locomotion, which icv CRF stimulates (Carpenter et al., 2007; Clements et al., 2002), we measured reduced retreat and attack behavior in CRF-treated fish. Therefore, it appears that while CRF influences both locomotion and social interaction, the CRF effects on aggressive behavior appear to be independent of those associated with increased locomotion. In addition, icv CRF stimulated a head-shaking behavior in trout that may be related to anxiogenic effects of CRF (Carpenter et al., 2007). The CRF treatments decreased chasing, ramming, and biting, suggesting at first a generalized behavioral inhibition as has been proposed for subordinates (Fuchs and Flugge, 2002; Øverli et al., 2004a). This also suggests an increase in anxiety-like behavior reflected by reduced overall levels of social interaction. However, CRF treatment reduced retreats and also made first attacks come more quickly. Together these CRF effects significantly increased the likelihood of winning a social interaction (see below), but not via the expected route of overall increased attack behavior.

Social outcome effects—In untreated and aCSF-injected fish, winners attacked more frequently than similarly treated losers, but CRF treatment abolished that difference. A significant decrease in latency to first attack was seen in winning fish treated with both doses of CRF compared to aCSF-treated winners. Further, at the lower dose of CRF, winners had a significantly shorter latency to attack than similarly treated losers. In un-treated control fish, when fight outcome is considered, winners retreated significantly fewer times than losers (Fig. 5B). In both groups of CRF-treated fish, winners retreated significantly less than losers. In addition, at the lower CRF dose, winners also retreated significantly less than their aCSF treated opponents (Fig. 5B). It is not surprising that CRF might have different effects on different individuals in paired encounters, for the following reasons: 1) Only one fish in each dyad received icv CRF, the other was treated with aCSF, 2) Each fish has a propensity for aggressive interaction that is influenced by genetic and environmental factors 3) Each opponent also has a unique predisposition for aggression, and 4) Each fish and its opponent also have propensities for anxiety, which will also influence the motivation and actualization of aggressive behavior. Conflicting motivations, such as reducing attacks while reducing retreats, or reducing total attacks while speeding the onset to first attack, suggests that CRF influences anxiety among treated fish, and that anxiety modified the strategy used during social aggression. This anxiety may be expressed through a general reduction of aggression, or by potentiating focally induced aggressive responsiveness due to forced close proximity. While the effects of CRF may include increased anxiety, the social behaviors stimulated by icv CRF treatment significantly influence the outcome of social interaction.

Aggression and CRF Modify Plasma Cortisol Response

Treatment effects—Aggressive social interaction stimulated a significant increase in plasma cortisol compared to non-interacting control fish (Fig. 6). What is more, the icv injection procedure (Clements et al., 2002) also caused an additional significant increase in plasma cortisol (Carpenter et al., 2007). However, treatment with both doses of CRF suppressed the injection-induced increase in plasma cortisol (Fig. 6). In our previous work with socially isolated fish, icv injections of either aCSF, 500 ng CRF or 2000 ng CRF markedly increased plasma cortisol to similar levels when compared to un-injected controls, but only CRF treated fish showed increases in locomotion (Carpenter et al., 2007). In the current study, aggressive social interaction modified the effect of CRF on stress responsiveness in an unexpected manner. In socially interacting fish, both doses of icv CRF resulted in a significant decrease in plasma cortisol compared to aCSF injected conspecifics (Fig. 6A). In CRF treated fish, cortisol secretion remained elevated equivalent to uninjected, aggressively interacting controls, suggesting that CRF blunted the stressful effects of icv injection, but not those associated with

aggressive social interaction. Additionally, the results suggest that CRF action can be modified by either a prior stressor (Ronan et al., 1999) or a concurrent stressor, in this case aggression, and modifies behavioral and endocrine stress responses independently. While negative correlations between cortisol and aggression have been demonstrated convincingly (Øverli et al., 2004a), our results suggest that effects of CRF on specific social behaviors are independent of plasma cortisol concentrations.

Social outcome effects—Within treatments, winners and losers showed equivalent increases in plasma cortisol, with the exception of fish treated with 500 ng CRF. In this group, winners had significantly higher plasma cortisol levels than losers. When taking into consideration outcome of social interaction, CRF- injected (both doses) winners had significantly less cortisol than their aCSF injected opponents (Fig. 6B). Similarly, CRF-treated losers of social interactions show significantly less plasma cortisol when compared to their aCSF injected opponents at the lowest dosage of CRF. The ameliorative effects of CRF on plasma cortisol levels leaves us with the question of why administration of this stress related peptide resulted in a reduction of overall stress responsiveness. This effect may be due to increased negative feedback, or to activity of monoamine systems. As monoaminergic activity can be altered by social stress, corticosteroids and CRF (Carpenter et al., 2007; Summers and Winberg, 2006; Summers et al., 2003b), lower cortisol levels in CRF-treated fish may be due to modulation by monoaminergic activity (Lepage et al., 2002).

CRF and Aggression Modify Monoaminergic Response

Serotonergic activity—As with cortisol, aggression stimulated altered (mostly increased) serotonergic responses in all brain regions sampled, with the exception of the dorsomedial pallium. Social aggression has been shown to stimulate monoaminergic activity in the telencephalon of salmonids (Winberg et al., 1991; Winberg et al., 1992; Winberg and Nilsson, 1993). Our data suggest that serotonergic changes are expressed in specific neuroanatomical regions of the telencephalon including the hippocampus (DL), the striato-amygdalar complex (Vc), the preoptic area (POA), and the rest of the hypothalamus (NT); all part of stress and aggression neurocircuitry (Summers and Winberg, 2006). Low levels of serotonergic activity have been associated with aggression in vertebrate systems (Chiavegatto et al., 2001; Ferris et al., 1997; Summers et al., 2005). The effect of elevated serotonergic activity on aggression appears to be manifest through diminished excitability of aggression circuitry neurons through activation of 5-HT_{1A} and 5-HT_{1B} receptors (Ferris et al., 1999). However, during aggressive interactions social stress is high and serotonergic activity increases in specific brain regions (Summers and Winberg, 2006). These specific regional increases in serotonergic activity are similar to rapid, aggression-induced serotonergic increases in rodent and reptilian models (Blanchard et al., 1991; Summers et al., 2003a; Summers and Winberg, 2006). While serotonergic increases in the whole telencephalon of aggressively interacting fish has been previously reported (Höglund et al., 2001; Øverli et al., 1999), this demonstration of specific regional increases suggests an evolutionary conservation of function for brain regions associated with stress and aggression.

Dopaminergic activity—High levels of dopaminergic activity have been shown to increase aggression intensity in mammals and fish (Ferrari et al., 2003; Siegel et al., 1999; Winberg and Nilsson, 1992), but have also been shown to suppress aggression in the lizard *Anolis carolinensis* (Höglund et al., 2005). Changes in dopaminergic activity during the early stages of social aggression appear to be important for motivation and learning, rather than aggression itself (Korzan et al., 2006; Korzan and Summers, 2007; Summers and Winberg, 2006). In our study, dopaminergic function appeared to be differentially influenced by aggression. Measures of dopaminergic activity were increased in the striato-amygdalar complex, POA and hypothalamus, but decreased in the hippocampus of aggressively interacting trout. Diminished

hippocampal dopaminergic activity in response to an aggressive interaction has also been reported in a reptilian model (Korzan et al., 2006; Ling et al., 2008). In this reptilian model, and also in our trout, after aggression has been initiated, hippocampal and amygdalar dopaminergic activity seem to have opposing effects, and appear to be regulating stress responsiveness, rather than modulating aggressive behavior directly. High and low dopaminergic activity in mammalian striatum, similar to what we see in trout amygdalar subpallium and hippocampus, differentially regulate neuronal excitability and Hebbian plasticity (Shen et al., 2008). When behaviorally important stimuli are present, aggression and CRF in our study, these mechanisms of may be available to promote associative learning.

Outcome and monoaminergic activity

When trout were grouped according to the outcome of the aggressive encounter (winners and losers), there were relatively few significant effects on monoamine concentrations and activity. However, pre-treatment with CRF did influence some regionally specific changes in dopaminergic activity and behavior during aggressive interaction. When considering outcome of the behavioral interaction, CRF treatment primarily affected losing fish in three brain regions (subpallium, POA and hypothalamus) and these changes appear to be associated with social behavior and anxiety. Losers treated with CRF had increased DA levels in the subpallium compared to similarly treated winners. However, these same CRF-treated losers had decreased DA levels in the POA and decreased DOPAC levels in the hypothalamus when compared with both CRF-treated winners and aCSF-injected controls. Results from a previous study suggested that increased dopaminergic activity in the striato-amygdalar pallium was related to increased locomotion (Carpenter et al., 2007). The finding that dopaminergic activity was elevated in CRF treated trout could explain the current behavioral results, such as reduced latency to first attack. However, changes in aggressive behavior that could possibly be linked to locomotion were only measured in winning trout; but importantly, there was no difference in overall locomotion between winners and losers. Thus, the change in striato-amygdalar pallium DA may be associated with a change in motivation, as the ratio of attacks to retreats also increased in CRF treated fish. Treatment with CRF also affected fight outcome, and the changes in dopaminergic parameters in POA, hypothalamus and striato-amygdalar complex may be related. In mammals, connections between POA, other hypothalamic nuclei and amygdala are influenced by dopaminergic activity (Perkins et al., 1977). In birds and mammals, dopaminergic activity in the POA is often associated with motivated sexual behavior (Cornil et al., 2002; Kato and Sakuma, 2000). Since establishment of social rank often determines the primary mating hierarchy, perhaps changes in POA DA are important in association with aggressive territorial encounters.

Possible Mechanisms of CRF Action

In mice, CRF decreases maternal aggression (Gammie et al., 2004). In addition, CRF₂ receptor-deficient females also show decreased maternal aggression due to hyper-secretion of CRF (Gammie et al., 2005). However, inter-male aggression was unaffected by CRF₂ receptor knock-down (Gammie et al., 2005). In hamsters, a CRF₁ receptor antagonist (SSR125543A) that produced an anxiolytic-like effect also produced a higher latency to bite and lower lateral attack frequencies and chase durations without affecting non-aggressive social activity (Farrokhi et al., 2004). In trout, the effect of icv CRF treatment was a higher ratio of attacks to retreats with the result that more CRF-treated trout were winners. It has been suggested that selective inhibition of defensive-escape tendency in agonistic interactions can be a good predictor of anxiolytic activity of drugs (Krsiak et al., 1984). It may be that in specific populations, such as maternal mice (Gammie et al., 2004) or winning trout, sensitivity to CRF may influence the outcome of aggressive interaction by modulating motivation through monoaminergic neurotransmission and by influencing anxiety.

Conclusion

While icv CRF treatment did influence aggressive interactions, the results may be more easily understood as an effect on anxious and motivated behaviors. The outcome of an aggressive interaction is complex. Central CRF influenced overall attacks, latency to first attack, and retreat behavior, but only in winners. The icv injection procedure is stressful, and stimulated an increase in plasma cortisol, an effect that was suppressed by treatment with icv CRF. Aggressive social interaction had a significant effect on monoamine concentrations and activity, and pre-treatment with CRF influenced regionally specific changes in dopaminergic and serotonergic activity and behavior during aggressive interaction. Taken together these data suggest that CRF influences anxiety, which not surprisingly also influences the nature and outcome of aggressive social interactions.

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Fig. 1. Regional punch microdissection illustrated by black circles to indicate 500 μ m diameter samples taken for HPLC analysis. Top: Sagittal section oriented with rostral telencephalon (TEL) to the left, moving caudally to optic tectum (OT), hypothalamus (HYP), cerebellum (CER) and finally medulla (MED). Coronal sections are taken from **A**) telencephalon (TEL), **B**) rostral diencephalon and **C**) caudal diencephalon. In coronal slices, each circle is numbered to indicate where the punch removed tissue from: **A**) ❶ hippocampus (dorsolateral pallium), ❷ pallial amygdala (dorsomedial pallium) ❸ striato-amygdalar complex (subpallium), **B**) ❹ preoptic area (POA) and **C**) ❺ hypothalamus (NT).

Fig. 2.

Percentage of winners of aggressive interactions between size-matched pairs of rainbow trout. In each pair one fish was injected icv with aCSF (gray bars) and the other fish was injected icv with CRF (hatched bars). Trout treated with CRF were winners of over 60% of dyadic interactions (Fisher exact, $P < 0.022$); whether the dose of CRF was lower (500 ng; narrow hatching; leftmost bars) or higher (2000 ng; wide hatching; right bars).

Fig. 3.

Mean number of attacks ($\pm$ SEM) during social aggression between size-matched pairs of rainbow trout. **A** Attack number by treatment irrespective of fight outcome, in uninjected control fish (open bars) and icv treated fish (all gray bars) injected with aCSF (plain gray), 500 ng CRF (gray with forward hatching) and 2000 ng CRF (gray and backwards hatching). Fish treated with 500 ng CRF displayed significantly (*) fewer attacks than uninjected and aCSF injected fish. **B** Losing fish (open bars) had significantly (*) fewer attacks than winning fish (hatched bars) when uninjected (leftmost bars) and aCSF injected, but not when CRF injected.

Fig. 4.

Latency to first attack in seconds ($\pm$ SEM) during social interaction between size-matched pairs of rainbow trout. **A** Irrespective of fight outcome, apparently lower mean latency to attack among fish treated with 500 ng CRF (gray with forward hatching) and 2000 ng CRF (gray and backwards hatching) was not significantly reduced compared to uninjected control fish (open bars) and aCSF treated fish (plain gray bars). **B** Winners treated with 500 ng CRF exhibited significantly (*) shorter attack latency compared to similarly treated losers. In addition, winners treated with both doses of CRF were significantly (§) faster to attack than aCSF treated winners.

Fig. 5.

Mean number of retreats ($\pm$ SEM) during aggressive social interaction among size matched pairs of rainbow trout. **A** Irrespective of fight outcome, all icv injected fish (gray bars) showed a significant (*) decrease in retreats compared to uninjected controls (open bars). **B** While winning fish (hatched bars) did not show significantly fewer retreats than losing fish among uninjected controls, CRF treatment at both doses stimulated a significant (*) decrease in the number of retreats in winners compared to losers. **C** A qualitative comparison of the ratio of attacks to retreats, where uninjected (open bars) and aCSF injected (gray bars) fish showed more retreats than attacks, and treatment with both 500 ng CRF (gray forward hatching) and 2000 ng CRF (gray backwards hatching) treated fish performed more attacks than retreats.

Fig. 6.

Mean plasma cortisol concentrations ($\pm$ SEM) following aggressive social interaction among size matched pairs of rainbow trout. **A** All groups of aggressively interacting fish, including uninjected (bars with fine forward hatching), aCSF (coarse hatching) and CRF injected (500 ng fine crosshatching; 2000 ng coarse crosshatching) fish had significantly (*) more cortisol than socially isolated animals that did not receive any injection treatment (clear bar). Fish injected icv with aCSF had significantly (§) greater plasma cortisol concentrations, than other aggressively interacting fish, including those injected with CRF. **B** Winning (hatched bars) and losing fish (clear bars) injected icv with aCSF (β for aCSF losers, γ for winners) had greater (§) plasma cortisol concentrations than all other fish except losing fish that were uninjected (α , clear bar) or injected icv with 2000 ng CRF (γ_1 , clear bar). Winning fish injected with 500 ng CRF (β_1 , hatched bar) had significantly (*) greater cortisol than similarly treated losing fish

(γ_1 , clear bar), but significantly (§) less cortisol than their aCSF opponents (β , clear bar). In dyads where one opponent was given CRF (β v β_1 or β_2 and γ v γ_1 or γ_2), winners given both the 500 ng (β_1) and the 200 ng (β_2) treatment resulted in reduced cortisol compared with aCSF treated losing opponents (β , clear bar), which was still elevated compared with non-interacting, uninjected control fish (gray bar). Similarly, CRF treated losers had less plasma cortisol than the winning aCSF opponent (γ , hatched bar), but only at the 500 ng dose (γ_1).

Fig. 7.

Mean serotonin (5-HT; **A, C, E**) and 5-hydroxyindoleacetic acid (5-HIAA; **B, D, F**) concentrations ($\pm$ SEM) following aggressive social interaction among size matched pairs of rainbow trout. **A, B, C, D, E** Socially isolated animals that did not receive any injection treatment (gray bar) had significantly (*) lower 5-HT and 5-HIAA in hippocampus (**A, B**), striato-amygdalar complex (**C, D**) and POA (**E**) than socially active fish that were uninjected (fine forward hatching), aCSF injected (coarse forward hatching), 500 ng (fine crosshatching) and 2000 ng CRF (coarse crosshatching) icv injected. **C, D** In striato-amygdalar subpallium 5-HT and 5-HIAA concentration were significantly (§) reduced compared to aCSF injected socially interacting fish.

Fig. 8.

Mean dopamine (DA; **A, C, E**) and dihydroxyphenylacetic acid (DOPAC; **B, D, F**) concentrations ($\pm$ SEM) following aggressive social interaction among size matched pairs of rainbow trout. **A, B** Socially isolated animals that did not receive any injection treatment (gray bar) had significantly (*) greater DA and DOPAC in hippocampus than socially active fish that were uninjected (fine forward hatching), aCSF injected (coarse forward hatching), 500 ng (fine crosshatching) and 2000 ng CRF (coarse crosshatching) icv injected. **C, D, E, F** In striato-amygdalar subpallium (**C, D**) and POA (**E, F**) DA and DOPAC concentrations were elevated by aggressive interaction, compared to uninjected isolated controls. **D** In striato-amygdalar complex icv injection of aCSF or CRF in socially interacting fish significantly (§) further increased the concentration of DOPAC.

Fig. 9.

Comparisons of mean ($\pm$ SEM) dopamine (DA; **A, B**) and dihydroxyphenylacetic acid (DOPAC; **C**) concentrations in striato-amygdalar subpallium (**A**), preoptic area (POA; **B**), and hypothalamus (NT; **C**) of isolated uninjected controls (gray bars), winners (hatched bars) and losers (open bars) treated with no injection, aCSF icv injection, and 500 or 2000 ng CRF injection (note X axis subheadings). **A** Fish that lost an aggressive encounter injected with 2000 ng CRF had significantly (*) higher subpallial DA than CRF injected winning fish. **B, C** Also in fish treated with 2000 ng CRF there was a trend toward increased DA in POA (**B**, $P < 0.09$) and significantly (*) increased hypothalamic DOPAC (**C**) in winners compared with

losers. C Hypothalamic DOPAC was also significantly (*) greater in losing fish treated icv with aCSF compared with winners.