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Expression and hypophysiotropic actions of corticotropin-releasing factor in *Xenopus laevis*

Graham C. Boorse[a] and Robert J. Denver[a,b,*]

[a] *Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109, USA*
[b] *Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA*

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Abstract

Members of the corticotropin-releasing factor (CRF) family of peptides play pivotal roles in the regulation of neuroendocrine, autonomic, and behavioral responses to physical and emotional stress. In amphibian tadpoles, CRF-like peptides stimulate both thyroid and interrenal (adrenal) hormone secretion, and can thereby modulate the rate of metamorphosis. To better understand the regulation of expression and actions of CRF in amphibians we developed a homologous radioimmunoassay (RIA) for *Xenopus laevis* CRF (xCRF). We validated this RIA and tissue extraction procedure for the measurement of brain CRF content in tadpoles and juveniles. We show that the CRF-binding protein, which is highly expressed in *X. laevis* brain, is largely removed by acid extraction and does not interfere in the RIA. We analyzed CRF peptide content in five microdissected brain regions in prometamorphic tadpoles and juveniles. CRF was detected throughout the brain, consistent with its role as both a hypophysiotropin and a neurotransmitter/neuromodulator. CRF content was highest in the region of the preoptic area (POa) and increased in all brain regions after metamorphosis. Exposure to 4 h of handling/shaking stress resulted in increased CRF peptide content in the POa in juvenile frogs. Injections of xCRF into prometamorphic tadpoles increased whole body corticosterone and thyroxine content, thus supporting findings in other anuran species that this peptide functions as both a corticotropin- and a thyrotropin (TSH)-releasing factor. Furthermore, treatment of cultured tadpole pituitaries with xCRF (100 nM for 24 h) resulted in increased medium content, but decreased pituitary content of TSHβ-immunoreactivity. Our results support the view that CRF functions as a stress neuropeptide in *X. laevis* as in other vertebrates. Furthermore, we provide evidence for a dual hypophysiotropic action of CRF on the thyroid and interrenal axes in *X. laevis* as has been shown previously in other amphibian species.

Keywords: Corticotropin-releasing factor; Metamorphosis; *Xenopus laevis*; Amphibian; Radioimmunoassay

1. Introduction

Corticotropin-releasing factor (CRF) and related peptides are known to play essential roles as hypophysiotropins and as neurotransmitters/neuromodulators in the regulation of autonomic and behavioral responses to physical and emotional stress. CRF is the primary neurohormone controlling adrenocorticotropin (ACTH) secretion by the mammalian pituitary (see Vale et al., 1997), and is also a potent secretagogue for ACTH in other vertebrates including amphibians (Fryer et al., 1983, 1985; Tonon et al., 1986; Tran et al., 1990). Recent findings suggest that a more primitive role for this peptide in vertebrates is as a dual hypophysiotropic factor for both the thyroid and the interrenal (adrenal) axes (Denver, 1999). CRF-like peptides have been shown to function as thyrotropin-(TSH) releasing factors (TRFs) in representatives of each class of non-mammalian vertebrate (reviewed by Denver, 1999).

In amphibian larvae, thyroid hormone is the primary morphogen controlling metamorphosis (see Shi, 2000). A role for CRF (and related peptides) as a TRF has been shown in larvae of several amphibian species, and CRF can accelerate metamorphosis (*Rana catesbeiana* and *Spea hammondii*, Denver, 1993; *Rana perezi*, Gancedo et al., 1992; *Bufo arenarum*, Miranda et al., 2000; and *Ambystoma tigrinum*; Boorse and Denver, 2002).

* Corresponding author. Fax: 1-734-647-0884.
E-mail address: rdenver@umich.edu (R.J. Denver).

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This effect of CRF on metamorphosis is driven by the upregulation of the thyroid and the interrenal axes (Denver, 1993, 1997a, 1998; Gancedo et al., 1992).

In addition to its role in tadpole metamorphosis, CRF functions in the control of locomotion and appetite in amphibians. Recent work of ours and others has shown that CRF can activate locomotion, and it functions as a potent anorectic agent in both tadpoles and juvenile frogs (*Bufo*: see Carr, 2002; *Xenopus laevis*: Crespi et al., 2004; *S. hammondii*: Crespi and Denver, 2004). We have mapped the neuroanatomical distribution of CRF perikarya and fibers in juvenile *X. laevis* and found specific CRF immunoreactivity (CRF-ir) in several hypothalamic, thalamic, limbic, brain stem and spinal cord nuclei, and in the median eminence (Westphal et al., 2002). We also found a strong increase in CRF-ir in the preoptic nucleus (homolog of the mammalian paraventricular and supraoptic nuclei) of juvenile frogs subjected to 4 h of handling/shaking stress (Westphal et al., 2002; stress paradigm as in Glennemeier and Denver, 2002). These and other findings support the view that the regulation of CRF expression by stress, and the actions of CRF in behavioral and autonomic responses to stress arose early in vertebrate evolution.

Towards understanding the factors that influence CRF biosynthesis and secretion we have developed a homologous radioimmunoassay (RIA) for *X. laevis* CRF. We validated the RIA for the measurement of CRF in brain extracts of tadpole and juvenile *X. laevis*, and we used the RIA to analyze CRF peptide content in several microdissected brain regions. We also analyzed stress-induced changes in CRF peptide content. While CRF is known to activate the interrenal and thyroid axes in other amphibian species, similar actions of CRF have not yet been analyzed in *X. laevis*. Thus, to determine if CRF plays a dual hypophysiotropic role in *X. laevis* we injected CRF into prometamorphic tadpoles and analyzed rapid changes in whole body corticosterone and thyroxine (T_4) content.

2. Methods

2.1. Animal care and husbandry

Spawning was induced in *X. laevis* by injection of a gonadotropin-releasing hormone agonist (1 μg [des-Gly^{10}, D-His(Bzl)^{6}]-LH-RH ethylamide: Sigma Chemical, St. Louis, MO). Tadpoles were maintained in 170 L holding tanks in dechlorinated tap water at a density of 500 tadpoles/tank. Tadpoles were fed pulverized rabbit chow (Purina) and after metamorphosis, juvenile frogs were fed fish meal pellets. Animals were maintained at a temperature of 22–23 °C on a 12:12 light:dark cycle. Animal care and husbandry was conducted in accordance with guidelines established by the University Committee on the Care and Use of Animals at the University of Michigan.

2.2. Boiling acetic acid tissue extraction

Individual brain regions isolated from tadpoles or juvenile frogs were acid extracted prior to radioimmunoassay as described by Mastorakos et al. (1995). Briefly, 10 volumes of boiling 2 M acetic acid were added to individual brain sections and incubated for 10 min at 95 °C. Tissues were placed on ice and homogenized using a handheld pestle followed by a brief sonication (20 s). The homogenates were then centrifuged (15,000*g*, 30 min, and 4 °C) and the supernatant was transferred to a fresh tube. One tenth of the supernatant was dried in a speedvac and resuspended in 0.02 M phosphate buffered saline (PBS) to determine the total protein content in the extract using the BCA method (Pierce Chemical). After addition of three volumes of acetone, the homogenate was mixed and centrifuged (15,000*g*, 30 min, and 4 °C). The supernatant was transferred to a fresh tube and dried in a speedvac. The sample was reconstituted in CRF assay buffer (see below). Recovery of CRF following tissue extraction was estimated in each sample by the addition of ^{125}I-xCRF (500 cpm/0.05 ml 0.02 M PBS/tube) to tissue homogenates following the sonication step. Samples were then incubated at 4 °C for 10 min prior to continuing with the extraction procedure. Recoveries ranged from 60 to 90%, and potency estimates from the RIA were normalized based on recoveries determined in individual samples.

2.3. Radioimmunoassay

The antiserum used in the RIA was produced in rabbits against synthetic *X. laevis* CRF (xCRF) conjugated to human α globulins following the methods of Vale and colleagues (1983; see Denver, 1997a). The xCRF was radioiodinated using the iodogen method (Pierce) and the tracer was purified by reversed-phase high performance liquid chromatography (HPLC) as described by Valverde et al. (2001).

The composition of the assay buffer was 0.1 M sodium phosphate, 0.05 M sodium chloride, 0.01% (w/v) sodium azide, 0.1% (w/v) BSA, and 0.1% (v/v) Triton X-100. All incubations were conducted at 4 °C. Tubes (12 × 75 mm, polypropylene) containing 0.1 ml standard (4.9–5000 pg) or sample and 0.1 ml anti-xCRF serum (diluted 1:6000 in assay buffer) and 0.2 ml assay buffer were incubated overnight. ^{125}I-labeled xCRF (approximately 10,000 cpm/0.1 ml assay buffer/tube) was then added and incubated overnight. Separation of bound from free tracer was achieved by the addition of 1 ml ice-cold polyethylene glycol (5%) containing goat-anti-rabbit IgG (1:200) and normal rabbit serum (1:750). Tubes were incubated at 4 °C for 30 min before centrifugation

at 2000*g* for 30 min at 4 °C. The supernatant was removed by decanting and the pellet was counted in an ICN gamma counter for 1 min/tube.

2.4. Validation of the RIA and tissue extraction procedure

To determine the specificity of the xCRF RIA, the cross-reactivities of the following neuropeptides were evaluated: ovine CRF, rat/human CRF, sauvagine (SV), teleost urotensin I (UI; Sigma Chemical, St. Louis, MO), rat urocortin (UCN; Sigma), and TRH (Sigma). The relative displacement of ^{125}I-xCRF with increasing concentration (4.9–5000 pg/tube) of each neuropeptide was determined.

Brain extracts (after acetic acid extraction) were serially diluted in assay buffer and assayed as described above. Parallelism of the displacement curves from serial dilutions of brain extracts with that of the xCRF standard was compared (see data analysis section for details).

CRF has a specific binding protein (CRF-BP) that is expressed at a high level in the *X. laevis* tadpole brain (Valverde et al., 2001). The following experiment was designed to test if CRF-BP is present in the tissue extract, and if present, whether it interferes with the RIA. Five mid/forebrains from juvenile *X. laevis* were pooled and homogenized using a polytron (homogenization buffer: 0.02 M PBS, 1% (v/v) Triton X-100, and 1× protease inhibitor cocktail; Sigma). One half of this homogenate was acid extracted following the method described above. Total protein content of the homogenate was determined using the BCA method (Pierce Chemical). A chemical crosslinking assay as described by Valverde et al. (2001) was used to test for the presence of CRF-BP in the extract. Conditioned medium from stably transfected AtT-20 cells that overexpress mouse CRF-BP (mCRF-BP; see Cortright et al., 1995) and a *X. laevis* brain homogenate (see Valverde et al., 2001) were used as positive controls in the crosslinking assay (without extraction).

To determine if residual CRF-BP in the extract interferes with the xCRF RIA, conditioned medium from AtT-20[mCRF-BP] cells (2.5 μl or 5 μl) was added to the standard curve and the parallelism of the displacementcurves with or without the added mCRF-BP were compared.

A series of recovery tests with added cold hormone were conducted. A known amount of radioinert xCRF (13 ng) was added to an aliquot of pooled brain homogenate, the homogenate was acid extracted and analyzed by RIA to estimate the recovery of xCRF. These values were compared to unextracted buffer controls to which the same amount of radioinert xCRF was added. We also analyzed whether the addition of β-mercapthoethanol (added after homogenization to a final concentration of 5%) to the extracts (to reduce intramolecular disulfide bonds in the CRF-BP and render it inactive) would alter the recovery of xCRF. Conditioned medium from AtT-20[mCRF-BP] cells (5 μl) was added to brain homogenates to determine the recovery of xCRF in the presence of added CRF-BP.

2.5. HPLC fractionation of brain extracts

Microdissected brain tissue representing the preoptic area/diencephalon was isolated from ten *X. laevis* juveniles and acid-extracted as described above. The extract was dried in a speedvac and then resuspended in 0.02 M phosphate buffer (PB) before application to a Sep-Pack C18 column (Waters) previously activated with methanol. The column was washed with PB and eluted with 95% acetonitrile (ACN). The eluate was dried under a stream of nitrogen and the total protein in the extract was determined by BCA protein assay (Pierce Chemical). The partially purified brain extract (60 μg) was loaded onto a Supelcosil C18 reverse phase column (5 μm: 250 × 4.6 mm, Supelco) and eluted with the following linear gradient: 20–80% ACN in 0.1% trifluoroacetic acid (TFA) for 36 min, 80–95% ACN in 0.1% TFA for 2 min at 1.5 ml/min. Fractions were collected every 0.3 min, dried in a speedvac, and resuspended in assay buffer prior to RIA analysis. Sauvagine (SV), rat urocortin (UCN), xCRF, and teleost urotensin I (UI) were fractionated in a separate run to determine the elution profiles of CRF-like peptides under these conditions. The elution profiles of these peptides were determined by real-time spectrophotometry at 210 nm.

Previous studies have demonstrated the presence of oxidized forms of CRF in chromatographic profiles (Baigent and Lowry, 2000). To determine the elution profile of oxidized xCRF, we oxidized synthetic xCRF using the Iodogen reagent (Pierce) and fractionated the peptide by HPLC using the same elution gradient as above. The oxidized xCRF peak was detected by real-time spectrophotometry at 210 nm.

2.6. CRF peptide content in microdissected brain regions and effects of shaking/handling stress

To investigate the responsiveness of the frog CRF system to a stressor, prometamorphic tadpoles and juveniles were exposed to shaking stress. This stress paradigm has been shown to produce rapid elevations in whole body corticosterone content in tadpoles (Glennemeier and Denver, 2002) and plasma corticosterone concentration in juveniles (K.A. Glennemeier and R.J. Denver, unpublished data). We have also found that this stress paradigm dramatically increases CRF-ir in the preoptic nucleus of juvenile *X. laevis*, as analyzed by immunocytochemistry (Westphal et al., 2002). Briefly, tadpoles (Nieuwkoop and Faber, 1956 stage 58–59) and juveniles were shaken at 100 rpm on an orbital shaker for 4 h. Unstressed controls were maintained undisturbed in shielded holding tanks. Animals were rapidly

anesthetized by the addition of benzocaine to a final concentration of 0.01% to the holding tank water and five discrete brain regions [see Fig. 5; olfactory bulb, telencephalon, preoptic/diencephalon/pituitary (hereafter referred to as the POa), optic tectum, and rhombencephalon] were microdissected, snap frozen on dry ice, and stored at −80 °C until assay. CRF peptide content in each brain region was determined by RIA following acid extraction.

2.7. Effects of xCRF on whole body corticosterone and T_4 content in prometamorphic tadpoles

Two separate experiments were conducted to analyze the effects of xCRF injections on whole body hormone content. In the first experiment in which corticosterone was measured, tadpoles (NF stage 54–56; $n = 5$–6) were given intraperitoneal (i.p.) injections of saline vehicle or 1 μg xCRF (25 μl injection volume). Two hours later tadpoles were euthanized by immersion in 0.01% benzocaine, snap frozen, and stored at −20 °C until extraction and assay. Whole body corticosterone content was determined by RIA following extraction as described by Hayes and Wu (1995) with modifications described by Denver (1998). All samples were analyzed in a single RIA.

In the second experiment in which T_4 was measured, tadpoles (NF stage 57–59; $n = 6$) were injected i.p. with saline vehicle, 0.1 μg xCRF, 0.3 μg xCRF, or 1 μg xCRF (25 μl injection volume). Two hours later tadpoles were euthanized by immersion in 0.01% benzocaine, snap frozen, and stored at −20 °C until assay. Whole body T_4 content was determined by radioimmunoassay following extraction as described by Denver (1993, 1998). All samples were analyzed in a single RIA.

2.8. Effects of CRF in vitro on medium and pituitary TSHβ-ir

Pituitaries from NF stage 58 tadpoles were collected into wells (5/well) in a 24-well culture dish containing 1 ml ice-cold Dulbecco's modified Eagle's medium (DMEM: diluted 1:1.5 for amphibian tissues). Tissues were incubated in a humidified chamber under an atmosphere of 95% O_2, 5% CO_2 at room temperature with gentle shaking for 2 h. Medium was then changed and tissues were incubated in a volume of 250 μl DMEM with or without 100 nM xCRF for 24 h. At the end of this period medium was collected and pituitaries were harvested in 200 μl ice-cold PBS containing 0.1% Triton X-100. Pituitaries were sonicated on ice for 20 s, centrifuged and the supernatant was collected for Western blot analysis. Protein content in pituitary extracts was determined by the BCA method (Pierce Chemical).

For Western blot analysis, an equivalent amount of culture medium (50 μl/lane) or pituitary extract protein (2 μg/lane) were fractionated by 10% denaturing SDS–PAGE. Proteins were transferred to nitrocellulose and incubated overnight at 4 °C in TBS-BSA (10 mM Tris–HCl, pH 7.4; 0.9% saline; and 0.1% BSA) with anti-human TSHβ serum (1:1000; obtained from NIADDK; this antiserum has been previously shown to specifically stain thyrotrope cells in the anuran pituitary gland; Gracianavarro and Licht, 1987; Gracianavarro et al., 1988; Malagon et al., 1988). Following incubation with goat anti-rabbit-HRP secondary antiserum, immune complexes were detected by enhanced chemiluminescence (Amersham).

2.9. Data analysis

Radioimmunoassay data were analyzed using the computer program RIAMENU (P. Licht, Berkeley, CA). A logistic 3-parameter regression model ($y = a/(1 + (x/c)^b$; where x is the sample, y is CPM, a is total binding, b is slope, and c is ED_{50}) was used to determine the slope. Parallelism of the displacement curves from serial dilutions of brain extracts with that of the xCRF standard was tested by Student's t test of the slopes from the regression of data ($p < 0.05$).

For each brain region, CRF peptide content between stressed and unstressed animals was compared using the non-parametric Mann–Whitney rank sum test. Student's unpaired t test was used to compare whole body corticosterone content in CRF-injected and control tadpoles. Whole body T_4 content after injection with various doses of CRF was analyzed using one-way ANOVA with T_4 content as the response variable and CRF dose as the treatment variable. Fisher's least squares difference test of pairwise comparisons was used to compare each CRF dose to the saline-injected group. p values less than 0.05 were accepted as significant.

3. Results

3.1. Development and validation of the xCRF radioimmunoassay

We determined the cross-reactivities of ovine CRF, rat/human CRF, SV, UI, UCN, or TRH in the xCRF RIA (Fig. 1). Both mammalian CRF peptides tested exhibited similar cross-reactivities with slope and potency similar to the xCRF displacement curve. Other members of the CRF peptide family (SV, UI, and UCN) exhibited minor cross-reactivity with the xCRF antisera. For example, cross-reactivity with SV and UI was ~10%, while cross-reactivity with UCN was only ~1%. The unrelated peptide TRH did not cause displacement of the bound xCRF label.

Serial dilutions of a brain pool extract from *X. laevis* juveniles produced displacement curves similar to those

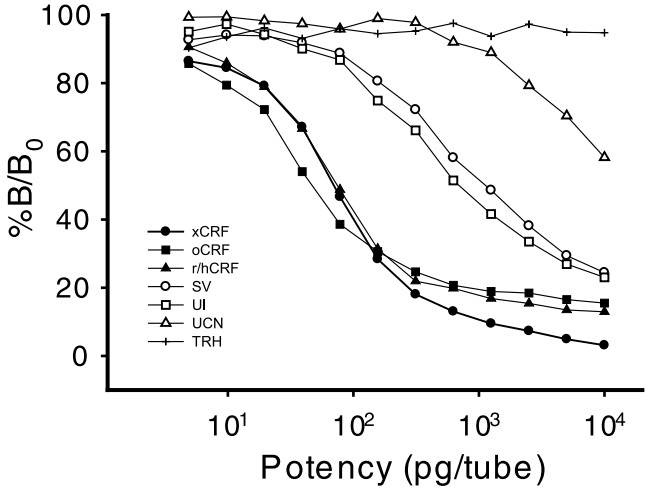


Fig. 1. Relative displacement of ^{125}I-labeled *X. laevis* CRF by increasing concentrations of the following radioinert peptides: *X. laevis* CRF (xCRF), ovine CRF (oCRF), rat/human CRF (r/hCRF), sauvagine (SV), fish urotensin I (UI), and rat urocortin (UCN). All points represent the means of two determinations.

of the xCRF standard (Fig. 2; $T = -0.249$; $p = 0.8115$). The range (average ± SEM) of the xCRF RIA ($n = 5$) was from 9.5 ± 1.0 pg/tube to 6.5 ± 0.5 ng/tube, and the intra- and interassay coefficients of variation were 2.1 and 11.3%, respectively.

Acetic acid extraction decreased the amount, but did not completely eliminate CRF-BP from the tissue extract (Fig. 3A). However, we determined that the residual CRF-BP in the extract did not interfere with the RIA. For example, the addition of rmCRF-BP to the standard curve did not alter the slope of the curve (Fig. 3B). The highest dose of rmCRF-BP tested (5 μl) represented

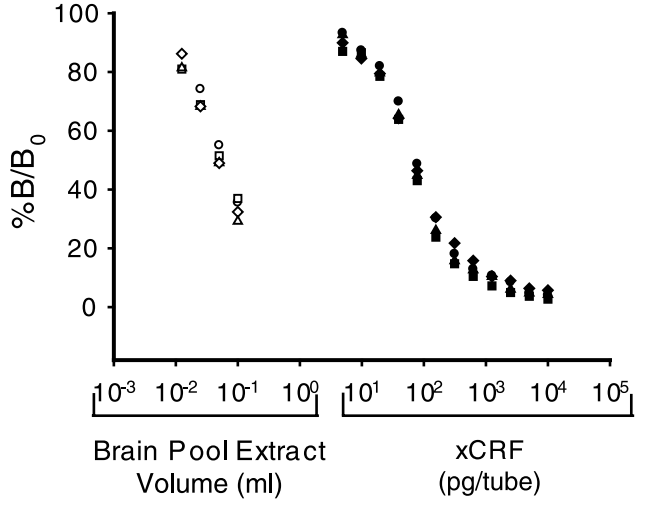


Fig. 2. Displacement of ^{125}I-labeled *X. laevis* CRF by radioinert xCRF (closed symbols) or dilutions of juvenile *X. laevis* brain extract (open symbols). The slopes of the displacement curves (determined using regression analysis; 3-parameter logistic model) were not significantly different.

roughly two times the amount of residual CRF-BP in the extract as determined by crosslinking assay (see Fig. 3A). Furthermore, we recovered greater than 95% of unlabeled ('cold') xCRF added to tissue homogenates prior to extraction (data not shown), thus arguing against interference by residual CRF-BP. Finally, addition of rmCRF-BP (5 μl) to tissue homogenates prior to extraction did not alter the recovery of added cold xCRF (data not shown). Addition of β-mercapthoethanol to the extracts to a final concentration of 5% did not cause interference in the RIA (data not shown) nor did it alter the potency estimates of tissue extracts and thus was not used in subsequent analyses.

3.2. Analysis of CRF content in brain extracts by reversed-phase HPLC

Fractionation of *X. laevis* brain extracts by reversed-phase HPLC produced two prominent CRF-ir peaks (Figs. 4A and B). A clear peak of CRF-ir coeluted with the xCRF standard; an earlier peak had a retention time similar to UI. Others have reported the presence of an oxidized form of CRF that elutes earlier than the xCRF standard (Baigent and Lowry, 2000). We found that oxidized xCRF generated by exposure to the Iodogen reagent had a retention time similar to the early CRF-ir peak (Fig. 4C).

3.3. CRF peptide content in microdissected brain regions and the effects of shaking/handling stress

Brain CRF peptide content increased after metamorphosis as juveniles had significantly higher values in each brain region compared with tadpoles (Fig. 5A). While we found CRF-ir widely distributed in *X. laevis* brain, the preoptic area (POa) and rhombencephalon contained the highest amount of CRF peptide in both tadpole and juvenile *X. laevis* (Fig. 5A). Shaking stress significantly increased CRF peptide content in the juvenile POa (Fig. 5B; $p = 0.016$; Mann–Whitney rank sum test). The mean POa CRF content was greater in prometamorphic tadpoles exposed to shaking stress but this difference was not statistically significant (data not shown). Shaking stress did not alter CRF peptide content in other brain regions of juveniles (Fig. 5B) or tadpoles (data not shown).

3.4. Effects of xCRF injections on whole body corticosterone and T_4 content in prometamorphic X. laevis tadpoles

Injection of xCRF (1 μg) produced a rapid (by 2 h) elevation in whole body corticosterone content compared with saline injected controls (Fig. 6A; $T = -2.075$; $p = 0.0339$). In a separate experiment, injection of xCRF significantly increased whole body T_4 content by 2 h

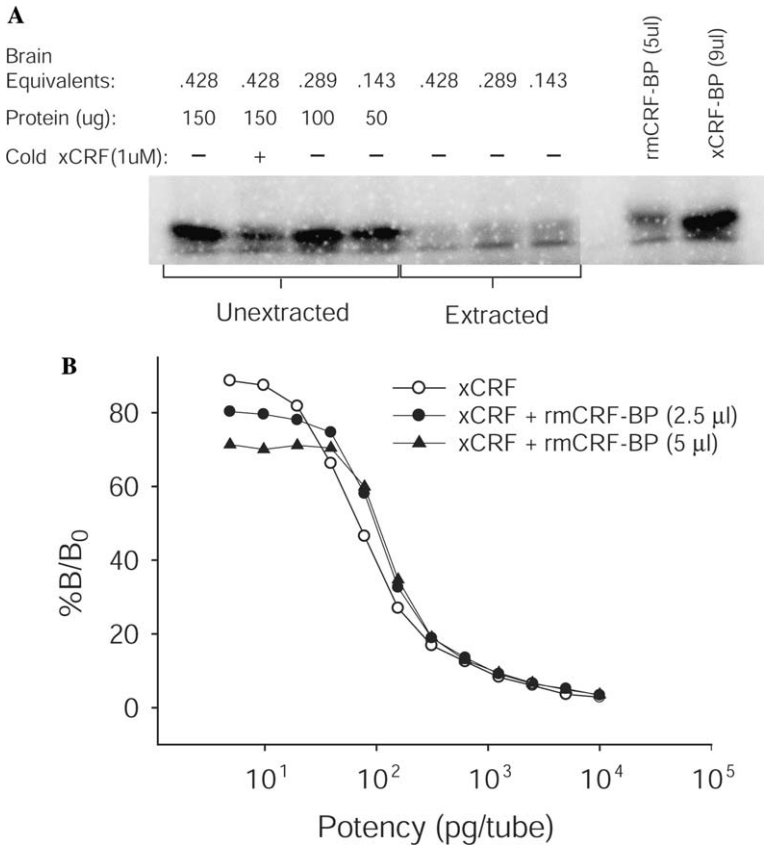


Fig. 3. (A) Crosslinking assay of boiling acid-extracted brain homogenates from juvenile *X. laevis* (see Section 2 for details). Specific binding is demonstrated by displacement of ^{125}I-labeled xCRF by the addition of radioinert xCRF (1μM). Recombinant mouse CRF-BP (rmCRF-BP) and *X. laevis* brain extract (xCRF-BP) were included as positive controls for the crosslinking assay. (B) Effects of addition of rmCRF-BP to the standard curve in the xCRF RIA.

(Fig. 6B; $F_{(3,20)} = 10.237$; $p = 0.0003$) at doses of 0.3 and 1 μg. The lowest xCRF dose tested (0.1μg) did not alter whole body T_4 content (Fig. 6B).

3.5. Effects of xCRF in vitro on medium and pituitary TSHβ-ir

The amount of TSHβ-ir present in the medium of cultured tadpole pituitaries was increased by treatment with xCRF (100 nM) as determined by Western blotting (Fig. 7). By contrast, xCRF treatment reduced pituitary TSHβ content.

4. Discussion

We have developed and validated a homologous radioimmunoassay for *X. laevis* CRF, and we used this RIA to measure CRF content in discrete brain regions of tadpoles and juvenile frogs. The sensitivity of the xCRF RIA, which was in the low picogram range, was comparable to that of previously published CRF RIAs (Calogero et al., 1989; Chappell et al., 1986; Owens et al., 1990; Pepels et al., 2002b; Skofitsch and Jacobowitz, 1985). We found that CRF peptide was distributed throughout the brain of *X. laevis* tadpoles and juveniles, thus supporting a role for CRF as both a hypophysiotropin and a neurotransmitter/neuromodulator in frogs as has been shown in mammals. We also found that the POa CRF neurons in frogs are responsive to stress as has also been shown in mammals.

While the xCRF RIA exhibited strong cross-reactivity with CRFs (e.g., ovine CRF, rat/human CRF) we found low cross-reactivity with CRF-like peptides (~10% for UI and SV, ~1% for UCN). Thus, the contribution of UI/SV/UCN-like peptides to the potency estimates in the xCRF RIA is likely to be low (note that no UI/SV/UCN-like peptide has yet been identified in *X. laevis*). This conclusion has a bearing on the interpretation of the two CRF-ir peaks that we observed following fractionation of *X. laevis* brain by reversed-phase HPLC. While the late peak clearly corresponds to xCRF, the earlier peak eluted with a retention time similar to UI. This early peak produced a displacement curve in the RIA that was parallel to the xCRF standard, but curves generated with synthetic UI were not parallel. Given the low cross-reactivity of UI/SV/UCN-like peptides in the xCRF RIA, and the fact that the synthetic peptides produce displacement curves that are not parallel to xCRF, we conclude that these CRF-like peptides, if

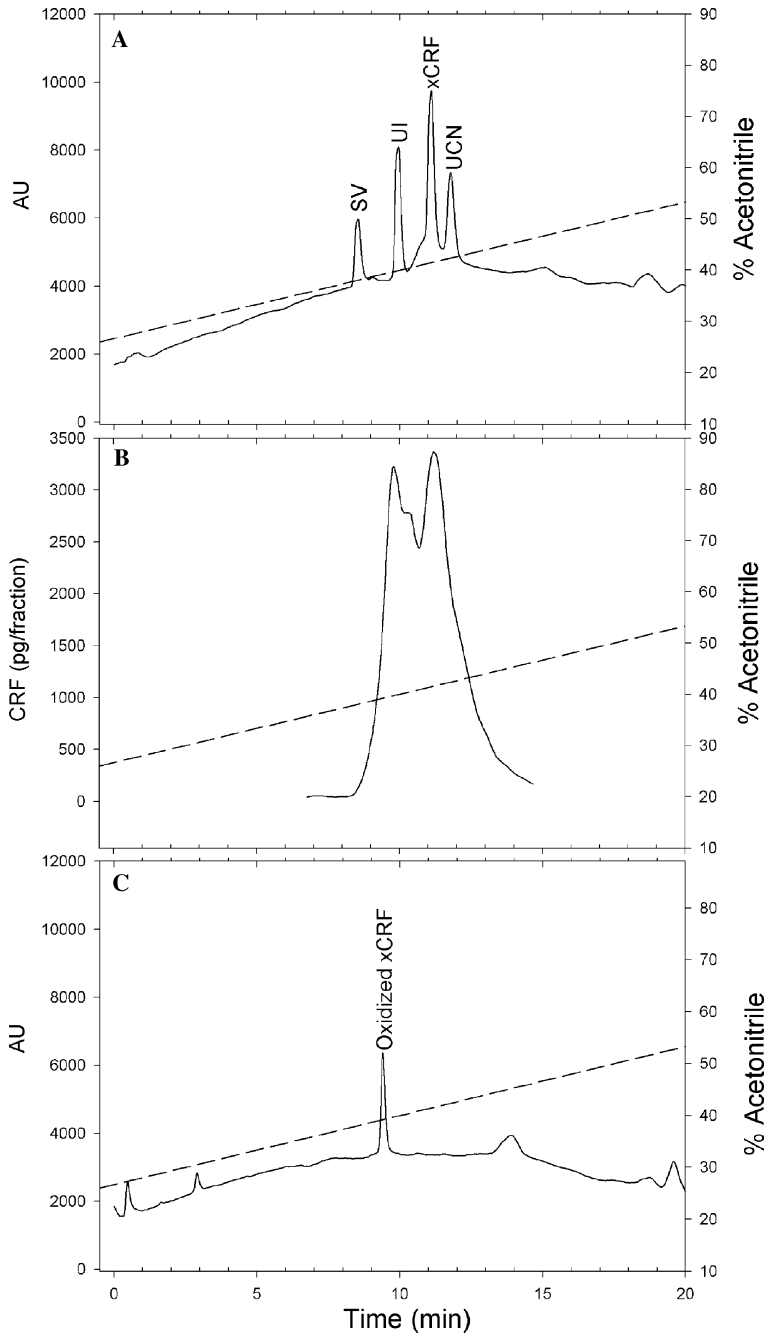
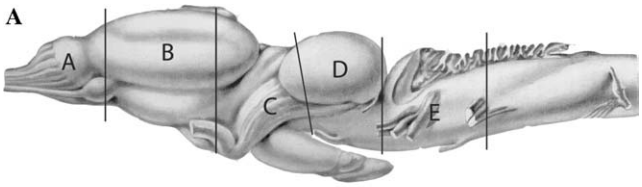


Fig. 4. Reversed-phase HPLC fractionation of juvenile *X. laevis* hypothalamic extract. (A) Elution profile of synthetic CRF-like peptides sauvagine (SV), teleost urotensin I (UI), *X. laevis* CRF (xCRF), and rat urocortin (UCN). (B) Elution profile of CRF-ir in juvenile *X. laevis* hypothalamic extract. CRF-ir in the fractions was determined by RIA. (C) Elution profile of oxidized xCRF. In all panels the dashed line represents the acetonitrile gradient.

present in *X. laevis* brain extracts, will contribute little if any to the potency estimates in the xCRF RIA.

We hypothesized that the early CRF-ir peak on HPLC represents oxidized xCRF. Like the rat/human CRF, xCRF has two methionine residues (residue 21 and 38) that can become oxidized to form methionine-sulphoxy CRF. The methionine-sulphoxy CRF exhibits a shorter retention time than CRF on reversed-phase HPLC (Fischman and Moldow, 1982; McMaster and Lederis, 1988; Rivier et al., 1983; Vale et al., 1981). Rat or ovine hypothalamic extracts obtained by boiling acetic acid extraction contained roughly equal amounts of CRF and methionine-sulfoxy CRF (Fischman and Moldow, 1982; Rivier et al., 1983) or only methionine-sulfoxy CRF (Vale et al., 1981). We found that oxidized xCRF exhibited a similar retention time to the early CRF-ir peak found in frog brain extract. Taken together with the low cross-reactivity and antiparallelism of



Brain Region	xCRF (pg/μg protein) Tadpole	Juvenile
A. Olfactory bulb	1.66 + 0.43	4.82 + 0.83
B. Telencephalon	2.38 + 0.60	9.61 + 1.70
C. Preoptic area, diencephalon & pituitary	4.84 + 0.82	17.45 + 1.28
D. Optic tectum	1.95 + 0.53	14.96 + 2.95
E. Rhombencephalon	4.01 + 0.37	17.96 + 1.07

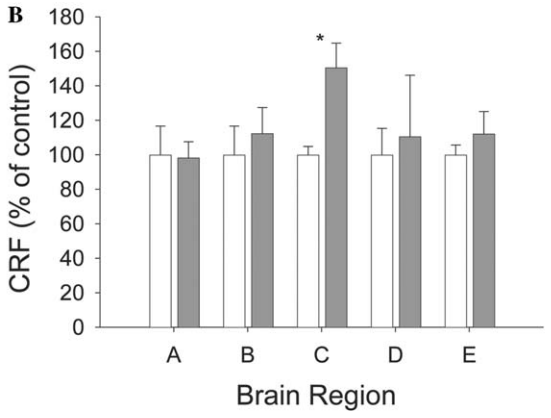


Fig. 5. (A) Brain CRF peptide content in *X. laevis* tadpoles and juveniles. Lateral view of the anuran brain indicating brain regions collected for CRF RIA (drawing modified from Ten Donkelaar, 1998). Brain tissue was extracted and CRF peptide content determined by RIA (mean ± SEM; $n = 6$ for tadpoles; $n = 3$ for juveniles). (B) Brain CRF peptide content in juvenile *X. laevis* following 4 h shaking stress. Open bars designate unstressed and filled bars designate 4 h shaking stress. Data are expressed as the mean percentage of control ± SEM ($n = 6$). Asterisk indicates significant difference from unstressed group ($p < 0.05$).

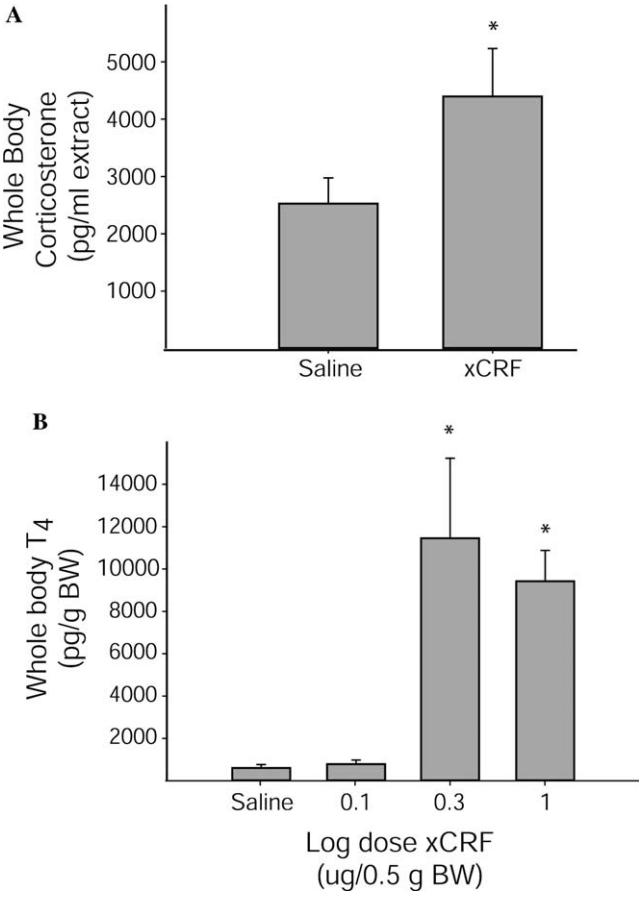


Fig. 6. Effects of xCRF injections on whole body corticosterone and thyroid hormone content in prometamorphic *X. laevis* tadpoles. (A) Whole body corticosterone content in NF stage tadpoles injected with xCRF (1μg/tadpole; $n = 5$) or saline vehicle ($n = 6$). (B) Whole body thyroxine (T_4) content in NF stage 57–59 *X. laevis* tadpoles injected with different doses of xCRF or saline vehicle ($n = 6$/treatment). In both experiments tadpoles were sacrificed 2 h after injection and analyzed for whole body hormone content by RIA following extraction (see Section 2). Bars represent means ± SEM and asterisks indicate significantly different from saline injected controls ($p < 0.05$).

UI/SV/UCN-like peptides, it is most likely that the early CRF-ir peak observed in brain extracts represents oxidized xCRF. The oxidized xCRF exhibits equal potency to xCRF standard in the xCRF RIA; thus, oxidation does not affect the potency estimates in the RIA (data not shown).

We evaluated the presence of CRF-BP in frog brain extracts since residual binding protein could theoretically lead to an overestimate of potencies by displacing radiolabeled CRF from the antibody (Orth and Mount, 1987). We found that the boiling acetic acid extraction procedure greatly reduced the amount of biologically active CRF-BP as determined by crosslinking assay. Furthermore, we provide evidence that the residual CRF-BP following extraction is unlikely to interfere with the RIA.

We then used our validated xCRF RIA and extraction procedure to analyze CRF peptide content in microdissected brain regions from *X. laevis* tadpoles and juveniles. The wide central distribution of CRF as determined by RIA in the present study is consistent with immunohistochemical studies that we have conducted in *X. laevis* (Westphal et al., 2002). Our finding of greatest CRF peptide content in the POa of *X. laevis* supports previous studies in other anurans in which immunohistochemistry was used (Carr and Norris, 1990; Miranda and Dezi, 1997; Tonon et al., 1985; Verhaert et al., 1984).

In each brain region, juvenile *X. laevis* had markedly higher CRF peptide content than did tadpoles, supporting previous studies showing that POa/hypothalamic neurosecretory neurons develop during metamorphosis (see Denver, 1996). We have also found that POa/hypothalamic CRF mRNA levels increase after metamorphosis in *X. laevis* (R.J. Denver, unpublished data). The maturation of the hypothalamic neurosecretory neurons and the median eminence is dependent upon increasing thyroid hormone concentrations during metamorphic climax (Etkin, 1968; Norris and Gern, 1976).

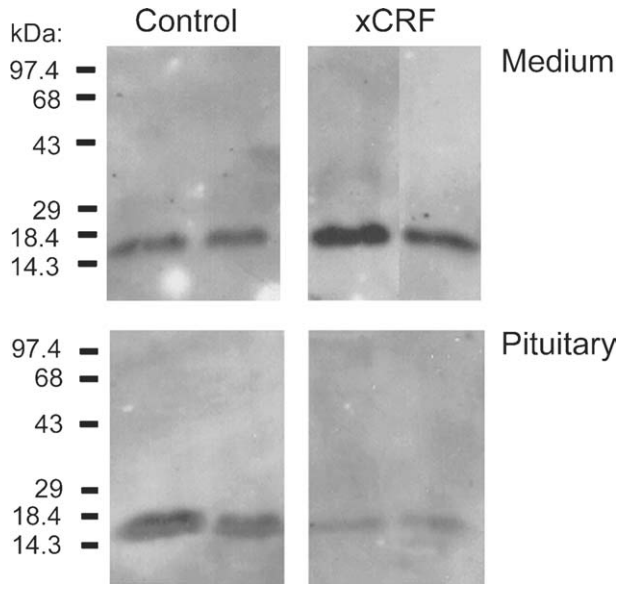


Fig. 7. Effects of xCRF in vitro on medium and pituitary TSHβ-ir. Pituitaries from prometamorphic *X. laevis* tadpoles (NF stage 58) were collected into wells (5/well) and cultured with or without 100 nM xCRF for 24 h. Culture medium and pituitaries were collected at the end of the culture period and analyzed for TSHβ-ir by Western blotting.

Developmental differences in the CRF system are also seen in the responsiveness of tadpoles and juveniles to a physical stressor. Juvenile *X. laevis* exhibited increased CRF peptide content in the POa following a 4 h shaking stress, but tadpoles exposed to this same stress paradigm showed modest increases in CRF content that were not statistically significant. Both tadpoles and juveniles are capable of responding to stressors as evidenced by increases in whole body (tadpoles) or plasma (juveniles) corticosterone (Glennemeier and Denver, 2002; K.A. Glennemeier and R.J. Denver, unpublished data). However, it is impossible to compare the relative responsiveness of tadpoles and juveniles using these measures, as one involves whole body hormone content while the other involves plasma hormone concentration.

Interestingly, we found that CRF peptide content in the hindbrain (rhombencephalon) of both tadpoles and juveniles was nearly comparable to that of the POa. We have identified well organized groups of CRF-positive neurons in the locus coeruleus, cerebellum, and lateral motor column of juvenile *X. laevis* using immunocytochemistry (Westphal et al., 2002). Studies in mammals (Sawchenko et al., 1993a,b; Skofitsch and Jacobowitz, 1985; Yasuda and Yasuda, 1985) and in two neoteleosts (*Poecilia latipinna*—Batten et al., 1990; and *Oreochromis mossambicus*—Pepels et al., 2002a,b) also identified CRF neurons in the rhombencephalon. The physiological significance of hindbrain CRF remains poorly understood, but studies in mammals have implicated these neurons in behavioral responses to stress (Lehnert et al., 1998; Page and Valentino, 1994). We found that simulated pond drying caused a robust increase in hindbrain CRF peptide content in *S. hammondii* tadpoles (Boorse and Denver, 2004), suggesting that these neurons play a role in responses to environmental stress in amphibians. Also, we have evidence that hindbrain CRF neurons play a role in foraging and locomotory behavior in *S. hammondii* (Crespi and Denver, 2004).

CRF was first isolated for its ability to stimulate ACTH secretion in mammals (Turnbull and Rivier, 1997; Vale et al., 1981) and regulation of ACTH by CRF is considered to be the primary hypophysiotropic role for this peptide (Vale et al., 1997). Similar to mammals, CRF stimulates the amphibian pituitary-interrenal axis (Denver, 1997b; Tonon et al., 1986). CRF has also been shown to be a potent TSH releasing factor in representatives of each class of non-mammalian vertebrate and can accelerate tadpole metamorphosis (reviewed by Denver et al., 2002). The hypophysiotropic actions of CRF have not previously been examined in *X. laevis*. We found that injections of xCRF resulted in rapid (by 2 h) increases in whole body corticosterone and T_4 content. Thus, CRF stimulates both the interrenal and the thyroid axes of *X. laevis* tadpoles as it does in other species (Denver, 1993, 1997a; Gancedo et al., 1992).

We also provide evidence that CRF can act directly on the *X. laevis* tadpole pituitary gland to stimulate the secretion of TSH. Media collected from pituitaries cultured in the presence of xCRF exhibited increased TSHβ-ir compared with controls. Previously, we and others showed that CRF can act directly on the amphibian pituitary to stimulate the secretion of thyrotropic bioactivity (Denver, 1988; Denver and Licht, 1989a; Jacobs and Kuhn, 1992). Recently, Okada et al. (2004) showed, using a homologous RIA for bullfrog TSH, that CRF is a potent secretagogue for TSH by cultured bullfrog tadpole and adult pituitaries. Also, specific TSH radioimmunoassays have been used to show that CRF directly stimulates TSH release in a reptile (turtle; Denver and Licht, 1989a,b, 1991) and a fish (salmon: Larsen et al., 1998; Moriyama et al., 1997). Berghman et al. (1993) used a subtractive RIA strategy to show that CRF is also a potent TSH secretagogue in the chick (see also Geris et al., 1996, 2003).

In summary, CRF is widely distributed throughout the brains of *X. laevis* tadpoles and juveniles, and exhibits a marked elevation in content after metamorphosis. The frog POa CRF neurons respond to physical stress similarly to mammals, and there may be developmental differences in the magnitude of the response. CRF is a potent secretagogue for corticosterone and thyroid hormone, and can act directly on the tadpole pituitary to stimulate the secretion of TSHβ. Our results support the hypothesis that, as in other amphibian species, CRF may mediate developmental responses to environmental stress in *X. laevis* tadpoles through its activation of both the interrenal and thyroid axes.

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