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Ontogeny of corticotropin-releasing factor effects on locomotion and foraging in the Western spadefoot toad (*Spea hammondii*)

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Abstract

We investigated the effects of corticotropin-releasing factor (CRF) and corticosterone (CORT) on foraging and locomotion in Western spadefoot toad (*Spea hammondii*) tadpoles and juveniles to assess the behavioral functions of these hormones throughout development. We administered intracerebroventricular injections of ovine CRF or CRF receptor antagonist αhelical $CRF_{(9\text{-}41)}$ to tadpoles and juveniles, and observed behavior within 1.5 h after injection. In both premetamorphic (Gosner stage 33) and prometamorphic (Gosner stages 35–37) tadpoles, CRF injections increased locomotion and decreased foraging. Injections of αhelical $CRF_{(9\text{-}41)}$ reduced locomotion but did not affect foraging in premetamorphic tadpoles, but dramatically increased foraging in prometamorphic tadpoles compared to both placebo and uninjected controls. Similarly, αhelical $CRF_{(9\text{-}41)}$ injections stimulated food intake and prey-catching behavior in juveniles. These results suggest that in later-staged amphibians, endogenous CRF secretion modulates feeding by exerting a suppressive effect on appetite. By contrast to the inhibitory effect of CRF, 3-h exposure to CORT (500 nM added to the aquarium water) stimulated foraging in prometamorphic tadpoles. These tadpoles also exhibited a CORT-mediated increase in foraging 6 h after CRF injection, which was associated with elevated whole-body CORT content and blocked by glucocorticoid receptor (GR) antagonist (RU486) injections. Thus, exogenous CRF influences locomotion and foraging in both pre- and prometamorphic tadpoles, but endogenous CRF secretion in relatively unstressed animals does not affect foraging until prometamorphic stages. Furthermore, the opposing actions of CRF and CORT on foraging suggest that they are important regulators of energy balance and food intake in amphibians throughout development.

Keywords: Corticotropin-releasing factor; Corticosterone; Foraging; Locomotion; Tadpole; Amphibian

Introduction

Corticotropin-releasing factor (CRF) is the primary neurohormone regulating the vertebrate hypothalamo-pituitary-adrenal axis (Vale et al., 1981). In addition to its hypophysiotropic role, CRF also functions as a neurotransmitter/neuromodulator, influencing behaviors often affected by stress such as locomotion (Lowry and Moore, 1991; Takamatsu et al., 1991), appetite and foraging (Heinrichs and Richard, 1999), and sexual behavior (Lowry et al., 1990). Because of its multiple behavioral and physiological effects, CRF is considered to be a neurohormone that integrates behavioral and physiological responses to physical and emotional stress.

Corticotropin-releasing factor is known to influence locomotion in the context of the stress response in vertebrates. In adult mammals, fishes, and amphibians, exogenous CRF stimulates locomotory behavior, while CRF receptor antagonists block stress-induced increases in locomotion (Clements et al., 2002; Koob and Bloom, 1985; Lowry and Moore, 1991; Takamatsu et al., 1991). The stimulation of locomotor activity has been associated with CRF actions in the forebrain (Takamatsu et al., 1991), hypothalamus (Lowry et al., 2001), and the hindbrain (Lowry et al., 1996). Rapid behavioral effects of CRF have been linked to increased biosynthesis of dopamine, serotonin, and catecholamines in the brain (Koob, 1999; Lowry and Moore, 1991; Lowry et al., 2001; Price et al., 1998).

Corticotropin-releasing factor also is involved with the central regulation of energy balance and food intake (Hein-

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richs and Richard, 1999). Most studies investigating the role of CRF in the regulation of food intake focus on its involvement in stress-induced anorexia. Work primarily done with rodents has implicated stress-induced increases in CRF secretion by neurons in the paraventricular nucleus (PVN) with the inhibition of food intake (Krahn et al., 1986). Indeed, intracerebroventricular injection of CRF inhibits food intake in all vertebrate taxa tested (see review, Carr, 2002), and injection of the CRF receptor antagonist αhelical $CRF_{(9-41)}$ directly into the PVN attenuates the anorectic effects of stress (Heinrichs and Richard, 1999). Thus far, the mechanisms linking the up-regulation of CRF expression and peptide content in PVN neurons with appetite suppression have yet to be determined.

To better understand the development and evolution of the behavioral actions of CRF in vertebrates, we investigated the effects of CRF on foraging and locomotory behaviors in tadpoles and juveniles of the Western spadefoot toad (*Spea hammondii*). We and others have shown that exogenous CRF inhibits prey-catching behavior and reduces meal size (Carr et al., 2002; Crespi and Denver, 2004; Gancedo et al., 1992), and stimulates locomotion (Lowry and Moore, 1991; Lowry et al., 1996) in juvenile and adult amphibians. However, no studies of CRF action on feeding or locomotion have been conducted in the tadpole stage of any species, and it is possible that the behavioral actions of CRF differ between larvae and adults. Yet, studies have shown that CRF stimulates both the pituitary–interrenal and the pituitary–thyroid axes in tadpoles (reviewed by Denver et al., 2002), and tadpoles even at early developmental stages show an increase in whole-body corticosterone (CORT) content in response to environmental stressors such as forced physical activity, food deprivation, or high density (Glennemeier and Denver, 2002a,b). Although the hypophysiotropic function of CRF is present in the tadpole (Carr and Norris, 1990; Glennemeier and Denver, 2002b; Kloas et al., 1997), it is possible that the neural structures and networks needed for CRF to regulate behavior, such as locomotion or feeding, are not fully developed at this stage.

Behavioral data suggest that environmental stress has inhibitory effects on foraging in tadpoles. Although foraging per se was not measured, Lawler (1989) showed that exposure to predators inhibited activity in several species of tadpoles. Reduction in aquarium water volume, which simulates pond drying (a common environmental stressor for tadpoles), causes Western spadefoot toad tadpoles to cease foraging (Denver et al., 1998; M. Phillips and R. Denver, unpublished data). Because the same pond drying treatment also increases hypothalamic CRF and whole-body CORT content, Denver (1997) hypothesized that these hormones also may be involved in the regulation of foraging in tadpoles.

Physiological responses to environmental stress depend on developmental stage in tadpoles. At stages before hindlimb growth, when growth and development is independent of thyroid hormone (premetamorphic, Etkin, 1968), tadpoles respond to deteriorating environmental conditions by reducing development rate and growth (Glennemeier and Denver, 2002b; Wilbur and Collins, 1973). By contrast, tadpoles at developmental stages after hindlimb growth, when thyroid hormone is secreted (prometamorphic), accelerate development in response to deteriorating environmental conditions (see review, Denver et al., 2002). Treatments with stress hormones also produce opposite responses in premetamorphic and prometamorphic tadpoles. Treatment with CORT or CRF slows development and growth in premetamorphic tadpoles (CORT: Glennemeier and Denver, 2002b; Hayes, 1997; CRF: Denver, unpublished data), while CORT or CRF accelerates development in prometamorphic tadpoles (reviewed by Denver et al., 2002). Therefore, it is possible that the behavioral effects of stress hormones could differ between premetamorphic and prometamorphic tadpoles, and likewise between tadpoles and post-metamorphic frogs (i.e., juvenile or adult).

To test the hypothesis that CRF influences foraging and locomotory behavior in tadpoles as it does in post-metamorphic amphibians, we conducted a series of experiments in which we injected CRF into the ventricular system of the tadpole brain to simulate acute increases in CRF secretion that would result from an environmental stressor. We also injected the CRF receptor antagonist αhelical-$CRF_{(9-41)}$ alone or in combination with CRF to (1) verify the specificity of the effects of exogenous CRF on behavior and (2) determine whether endogenous CRF secretion influences tadpole behavior. Because CORT secretion is induced by CRF, and CORT is also known to affect food intake (Crespi and Denver, 2004; Dallman et al., 1993; Tataranni et al., 1996) and locomotion (Bernier et al., 2004; Overli et al., 2002; Sandi et al., 1996) in other vertebrates, we incorporated experiments investigating the effects of CORT treatment on tadpole behavior. Furthermore, we used the glucocorticoid receptor (GR) antagonist RU486 to test whether early and late behavioral changes following CRF treatment result from a direct, central action of CRF, or the subsequent increase in plasma CORT.

Methods

Animals

Western spadefoot toad (*S. hammondii*) egg clutches were collected in Riverside County, CA, under California scientific collecting permit #802003-01 issued to R.J.D. Tadpoles were raised in the laboratory in aquaria at 23 °C on a 12L:12D photoperiod. Tadpoles were fed a mixture of rabbit chow, agar, and gelatin molded into cubes (approximately 3 × 3 × 3 cm; see Rugh, 1962). Tadpoles were randomly chosen from six clutches and dispersed evenly among experiments and treatments. Following Etkin (1968), tadpoles classified as premetamorphic were of a developmental stage before significant hindlimb development (Gos-

ner stage 33, 1.0–2.0 g); prometamorphic tadpoles were of developmental stages during hindlimb development (Gosner stages 35–37, 4.0–6.0 g). Animal husbandry and use was conducted in accordance with the guidelines set by the Animal Care and Use Committee at the University of Michigan.

Hormones and reagents

Ovine CRF (CRF) and αhelical-CRF $_{(9\text{-}41)}$ [αhelCRF$_{(9\text{-}41)}$] were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). We chose to use ovine CRF rather than *Xenopus laevis* CRF (which is identical in peptide sequence to *S. hammondii* CRF; GenBank accession numbers: *X. laevis*, S50096; *S. hammondii*, AY262255; Boorse and Denver, 2004), as the former binds to *X. laevis* CRF receptors with an affinity similar to that of *X. laevis* CRF (Dautenberg et al., 1997) but does not bind to the *X. laevis* CRF binding protein (CRF-BP, Valverde et al., 2001). Thus, the use of ovine CRF should remove any confounding effects of binding to endogenous CRF-BP (which is expressed at a high level in the amphibian brain; *X. laevis*: Valverde et al., 2001; *S. hammondii*: Boorse and Denver, 2004). Corticosterone was obtained from ICN Biomedicals (Aurora, OH) and RU486 (mifepristone) and metyrapone were from Sigma-Aldrich. Anti-corticosterone serum was purchased from Esoterix Endocrinology (formerly Endocrine Sciences, Calabasas, CA) and [^{3}H]-corticosterone (70 Ci/mmol) from Perkin Elmer Life Science Products, Inc. (Boston, MA).

Intracerebroventricular injections and food intake behavioral assay

Corticotropin-releasing factor or αhelCRF$_{(9\text{-}41)}$ were administered by intracerebroventricular injection into the third or fourth ventricle. A micropipette was inserted through the cartilaginous skull into either the third or fourth ventricle and a volume of 100–150 nl was pressure injected using a Drummond Nanoject apparatus (Drummond Scientific Co., Bromwell, PA). The positioning of the pipette for injection was determined in preliminary experiments using fast green dye or fluorescein [each at 1% in phosphate-buffered saline (PBS); 0.02 M sodium phosphate, 0.6% saline; pH 7.3]. After injection, the brain was dissected to identify the location and spread of the dye solution. For the average prometamorphic tadpole, the third ventricle injection site was on the midline approximately 3 mm posterior of the eyes, and the fourth ventricle injection site was on the midline approximately 6 mm posterior of the eyes (these locations varied proportionally depending on the size of the tadpole). In premetamorphic tadpoles, injections into either the third or fourth ventricle resulted in the spread of dye solution throughout the forebrain, midbrain, and hindbrain regions. However, in prometamorphic tadpoles, injections into the third ventricle resulted in spread of the dye solution throughout the fore- and midbrain, while injections into the fourth ventricle were restricted to the hindbrain. These marking solutions were not used during behavioral experiments.

Animals were anesthetized before injection by submersion in 0.001% benzocaine for premetamorphic tadpoles or 0.005% benzocaine for prometamorphic tadpoles and juveniles; the dose was adjusted according to the average body mass difference between developmental stages. After injection, tadpoles were placed in aquaria until recovery. Both pre- and prometamorphic tadpoles rapidly recovered from the anesthetic (within 10 min) and behavioral observations began approximately 1 h after injection; all tadpoles were observed within 2 h after injection.

Behavioral observations were made in a temperature- and light-controlled chamber between 1400 h and 1600 h. Tadpoles were placed in containers filled with 2 l well water, three per container (25 × 19 × 12.5 cm polystyrene cages) sorted by treatment. Two replicate containers per treatment were typically used in each experiment, resulting in a sample size of 6 tadpoles per treatment (or 12 per treatment if an experiment was conducted in two trials). Containers were placed in random order on a single shelf, and the observer was blind to the treatment during observations. Each tadpole was observed for 2 min, during which the amount of time spent swimming, resting, or foraging was recorded with the use of a computer program designed specifically for such observations (Glennemeier and Denver, 2002c). Foraging behavior was scored as the scraping of mouthparts on food or along the container wall. We statistically analyzed the number of seconds each tadpole spent displaying each of the three behaviors with analysis of variance (ANOVA), although we graphically represent data in percentage of time in our figures. If the ANOVA revealed a significant treatment effect ($\alpha = 0.05$), Duncan's multiple comparisons tests were used ($\alpha = 0.05$) to determine individual group differences.

Tissue extraction and corticosterone radioimmunoassay (RIA)

The tissue extraction procedure used is described by Hayes and Wu (1995) and was validated for *S. hammondii* tadpoles by Denver (1998). Briefly, tadpoles were weighed then homogenized in ethyl acetate. To estimate recoveries, 3500 cpm of [^{3}H]-corticosterone was added to each homogenate. The extracts were fractionated by thin layer chromatography (TLC) to separate CORT from other lipids. The silica was extracted with diethyl ether, dried under nitrogen, and resuspended in PBS plus 10% gelatin for recovery analysis and CORT RIA. The RIA was conducted as described by Licht et al. (1983). Intra- and inter-assay coefficients of variation were 5% and 16%, respectively. The mean minimum detectable limit of the assay (−2 standard deviations of B_0) was 23 pg per tube. Dilutions

of tadpole extracts exhibited parallelism to the standard curve in the RIA. Whole-body CORT content was measured in ng/g body weight (g BW).

Experiment 1

CRF dose-response

Prometamorphic tadpoles were weighed and assigned to treatments (n = 6) such that there were no differences in body weight between groups. Tadpoles received intracerebroventricular injections (100 nl volume) into the region of the third ventricle of vehicle (PBS) or CRF at 0.2, 2.0, 20.0 ng/g body weight. After injection, tadpoles were placed in 4 l aquaria by treatment, three per aquarium (two aquaria per treatment) for recovery from anesthesia. Behavioral observations were conducted as described above.

Experiment 2

Effect of CRF on swimming speed

Prometamorphic tadpoles received intracerebroventricular injections (100 nl volume) into the region of the third ventricle of vehicle (PBS 100 nl) or 2 ng/g BW CRF (n = 8 per treatment) and allowed to recover for 1 h before the behavioral assay. Tadpoles were tested for how fast they could swim a 150-cm distance in a linear racetrack filled with water. The racetrack was fashioned from aluminum sheet folded into a v-shape, with a removable door at one end and markings every 1 cm. Each tadpole was tested twice, and the average time was used in the analysis. Times were converted into swimming speed (cm/s) and analyzed with a two-sample t test.

Experiment 3

Effects of CRF or αhelCRF$_{(9\text{-}41)}$ on locomotion and foraging behavior

To assess specificity of the CRF injections and the role of endogenous CRF on tadpole behavior, we administered intracerebroventricular injections of αhelCRF$_{(9\text{-}41)}$ to block CRF receptors. Both premetamorphic and prometamorphic tadpoles were tested in separate experiments. Premetamorphic tadpoles were assigned to one of five treatments: no injection (handling control), and intracerebroventricular injections of PBS (100 nl), 20 ng/g BW CRF, 200 ng/tadpole αhelCRF$_{(9\text{-}41)}$, or co-injection of CRF and αhelCRF$_{(9\text{-}41)}$ at the above doses. Tadpoles received injections into the third ventricle (but note that fluorescein analysis, described earlier, revealed that solution spread throughout the brain ventricular system in premetamorphic tadpoles). This experiment was conducted in two trials, six tadpoles per treatment in each trial (n = 12 per treatment). Behavioral scores from both trials were combined for analysis.

The same general experimental procedure was applied to prometamorphic tadpoles (n = 12 per treatment), except that intracerebroventricular injections were given in two locations to determine if the behavioral effects of CRF or αhelCRF$_{(9\text{-}41)}$ differ when administered to different brain regions. One group received injections of 20 ng/g BW CRF or 1 μg per tadpole αhelCRF$_{(9\text{-}41)}$ into the third ventricle (these injections spread to the fore- and midbrain regions—see earlier), and vehicle injection into the fourth ventricle. Another group received injections of CRF or αhelCRF$_{(9\text{-}41)}$ at the same doses into the fourth ventricle (these injections were restricted to the hindbrain region—see earlier), and vehicle injection into the third ventricle. There were two control groups in this experiment: (1) tadpoles that were not injected before behavioral observations (handling controls), and (2) tadpoles that received vehicle (PBS) injections into both the third and fourth ventricle regions to serve as placebo controls. Because the availability of animals was limited, the combination CRF and αhelCRF$_{(9\text{-}41)}$ treatment was not included in this experiment.

Experiment 4

Effects of CRF or αhelCRF$_{(9\text{-}41)}$ on food intake

To determine if our foraging behavioral assay represented a true measure of food intake, we used food blocks containing the same ingredients as described above with the addition of approximately 200-μm lead beads (1% of the total volume of food). These beads have been used in fish to monitor food intake (Silverstein and Plisetskaya, 2000). X-ray analysis can be used after lead beads are eaten to compare the relative amount of food ingested by each tadpole. Tadpoles received third ventricle intracerebroventricular injections (100 nl/injection) of vehicle, 20 ng/g BW CRF, or 1 μg per tadpole αhelCRF$_{(9\text{-}41)}$ (n = 8 per treatment). Thirty minutes after injection, tadpoles were allowed to feed on the lead bead-impregnated food blocks for 1 h. During this foraging period, 2-min behavioral observations were made on each tadpole to confirm that their behavior was similar to those in previous experiments. At the end of the hour, animals were anesthetized with 0.005% benzocaine and X-ray images were taken with a Specimen Radiograph System (Model No. MX-20, Faxitron X-ray Corp, Buffalo Grove, IL), which produces low-energy, long-exposure, magnified radiographs. The area of lead beads was measured in each tadpole from digitized images of the radiographs using MetaMorph software (v. 6.1; Universal Imaging Corp., Downingtown, PA).

Experiment 5

Effects of CORT or metyrapone on tadpole behavior

To determine if CORT plays a role in appetite control in tadpoles, prometamorphic animals were placed in aquaria containing 2 l of water (three tadpoles per aquarium, two aquaria per treatment) and treated by addition to the aquarium water with one of the following: vehicle (etha-

nol, 0.0048%), CORT (500 nM), metyrapone (a corticoid synthesis inhibitor, 0.11 μM), or 500 nM CORT + 0.11 μM metyrapone. Tadpoles were exposed to the treatments for 3 h before behavioral observations were conducted (n = 6 per treatment). A separate set of six tadpoles from each treatment were anesthetized in 0.005% benzocaine and snap frozen for later whole-body CORT analysis.

Experiment 6

Effects of CRF with or without RU486 on tadpole behavior

To determine if the behavioral effects of intracerebroventricular CRF injection resulted from a direct action of CRF on central nervous system feeding centers, or from the effects of the subsequent rise in circulating CORT (which also can have rapid behavioral effects; Orchinik, 1998), we administered CRF with or without the GR antagonist RU486. Prometamorphic tadpoles received intraperitoneal injections of RU486 (1 μg/50 μl vegetable oil) or oil vehicle (50 μl) in combination with intracerebroventricular injection into the third ventricle of CRF (20 ng/g BW) or vehicle. Behavioral observations were made as described above on the same individuals at 1 and 6 h after injection (n = 6 per treatment). At each time point, separate samples (n = 6 per treatment) were anesthetized in 0.005% benzocaine and snap frozen for whole-body CORT analysis.

Experiment 7

Effect of αhelCRF$_{(9\text{-}41)}$ on prey-catching behavior in juvenile toads

To determine if endogenous CRF modulates foraging in juvenile toads (2–3 weeks post-metamorphosis), we administered intracerebroventricular injections (100 nl/injection) of vehicle (PBS), 200 ng αhelCRF$_{(9\text{-}41)}$, or 20 ng/g BW CRF + 500 ng/frog αhelCRF$_{(9\text{-}41)}$, and assayed prey-catching behavior. All toads were fed one pinhead cricket (approximately 1 cm length) at 0900 h on the day of the experiment in an effort to reduce and normalize hunger levels among animals. Behavioral tests (n = 7 per treatment) were conducted 1 h after injection at the same time of day (1400 h) used in the tadpole experiments.

Prey-catching behavior was assayed as follows. Juvenile toads were placed in the middle of a 29 × 19 × 12.5 cm plastic container that had live crickets along all four walls. Within the first 3 min in the tank, we recorded time until first strike, time until first cricket eaten, total number of strikes, and number of crickets eaten. After the 3-min observation period, toads were individually placed into enclosures containing six crickets and were allowed to forage undisturbed for an additional 30 min. The time until first strike and first cricket eaten was analyzed by ANOVA; the number of strikes and crickets eaten in 3 and 33 min were analyzed using Kruskal–Wallis tests.

Results

Experiment 1

CRF dose-response

All three doses of CRF significantly increased the time spent swimming [ANOVA: $F(3,20) = 13.17$, $P < 0.0001$] and decreased the time spent foraging [ANOVA: $F(3,20) = 39.77$, $P < 0.0001$; Fig. 1]. There were no statistically significant differences among the three doses of CRF, although the highest dose (20 ng/g BW) tended to have the largest effect on locomotion and feeding. There were no differences in the time spent resting among treatments [ANOVA: $F(3,20) = 1.03$, $P = 0.41$; data not shown].

Experiment 2

CRF effect on swimming speed

Tadpoles injected with CRF swam significantly faster (12.4 ± 0.5 cm/s) than saline-injected tadpoles (10.1 ± 0.3 cm/s; t test: $df = 14$, $t = 2.53$, $P = 0.024$).

Experiment 3

Effects of CRF or αhelCRF$_{(9\text{-}41)}$ on locomotion and foraging

In premetamorphic tadpoles, CRF significantly decreased time spent resting, while blockade of CRF receptors with αhelCRF$_{(9\text{-}41)}$ increased resting time [ANOVA: $F(4,51) = 9.20$, $P < 0.0001$; Fig. 2]. Conversely, CRF injections increased swimming while αhelCRF$_{(9\text{-}41)}$ decreased swimming compared with both uninjected and placebo controls [ANOVA: $F(4,51) = 18.60$, $P < 0.0001$]. Only CRF affected foraging, as tadpoles spent significantly less time foraging

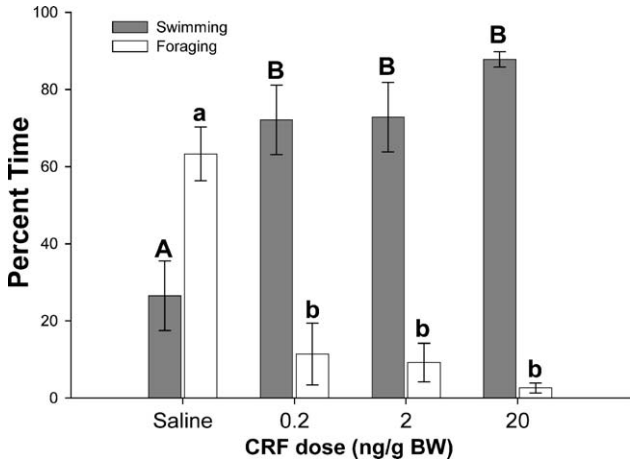

Fig. 1. Dose-dependent effects of intracerebroventricular injection of CRF on time spent swimming and foraging in *S. hammondii* prometamorphic tadpoles during a 2-min observation period. Bars indicate means ± SEM (n = 6); letters indicate significantly different treatment means for each behavior (capital: swimming, lowercase: foraging) per Duncan's multiple comparisons tests ($P < 0.05$).

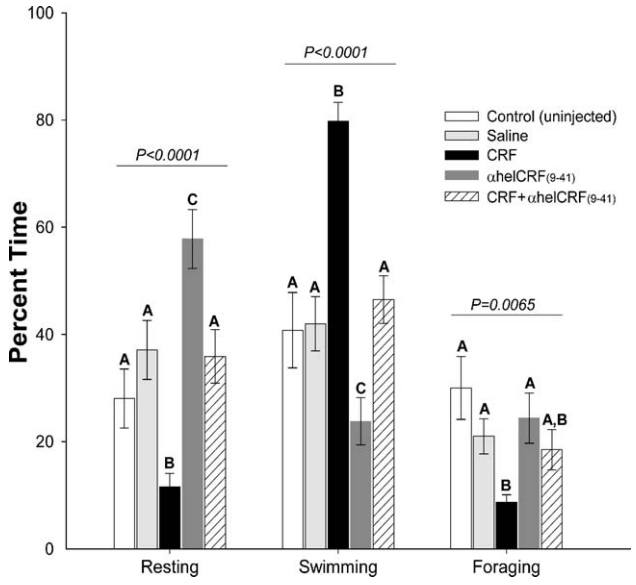

Fig. 2. Effects of CRF or αhelical $CRF_{(9-41)}$ on behavior of premetamorphic *S. hammondii* tadpoles. Bars indicate mean differences (±SEM) in the percentage of time tadpoles spent exhibiting a behavior in a 2-min observation trial (n = 12). Tadpoles were observed 1 h after intracerebroventricular injections of CRF (20 ng/g BW), 200 ng per tadpole $\alpha helCRF_{(9-41)}$, or co-injection of CRF and $\alpha helCRF_{(9-41)}$. Statistical analyses compared treatments means for each behavior; ANOVA significance values are indicated above the set of bars for each behavior, and letters indicate significantly different treatment means (Duncan's multiple comparisons tests, $P < 0.05$).

compared with uninjected and placebo controls [ANOVA: $F(4,51) = 4.03$, $P = 0.0065$]. Simultaneous treatment with $\alpha helCRF_{(9-41)}$ blocked the effects of CRF for each of the behaviors, that is, the CRF + $\alpha helCRF_{(9-41)}$ treatment did not differ from controls (Fig. 2).

In prometamorphic tadpoles, the effects of CRF on tadpole behavior varied with the site of injection (recall that intracerebroventricular injections in premetamorphic tadpoles resulted in diffusion of the solution throughout the brain ventricular system regardless of where the injection was placed, while in prometamorphic animals, third or fourth ventricle injections were restricted in their spread—see Methods). Tadpoles receiving third ventricle injections of CRF spent significantly less time foraging and tended to increase swimming time, although the effect was not statistically significant in this experiment (Fig. 3). However, fourth ventricle injections of CRF did not affect any behavior. Both third and fourth ventricle injections of $\alpha helCRF_{(9-41)}$ significantly increased foraging and decreased swimming relative to uninjected and placebo controls, and this effect was greater in animals receiving injections into the fourth ventricle (Fig. 3). Analysis of variance showed significant treatment effects for all behaviors measured [resting ANOVA: $F(5,64) = 8.66$, $P < 0.0001$; swimming ANOVA: $F(5,64) = 8.66$, $P < 0.0001$; foraging ANOVA: $F(5,64) = 24.51$, $P < 0.0001$].

Experiment 4

Effects of CRF or $\alpha helCRF_{(9-41)}$ on food intake

The purpose of this experiment was to determine if our analyses of foraging behavior provided an accurate measure of the rate of food intake in prometamorphic *S. hammondii* tadpoles. The results of 2-min behavioral observations of these tadpoles were similar to those described in Experiments 1 and 3: CRF-injected tadpoles spent less time foraging than placebo, while $\alpha helCRF_{(9-41)}$-injected tadpoles spent significantly more time foraging (data not shown). Analysis of the area of lead beads in each tadpole (as measured from radiographs) showed that CRF-injected

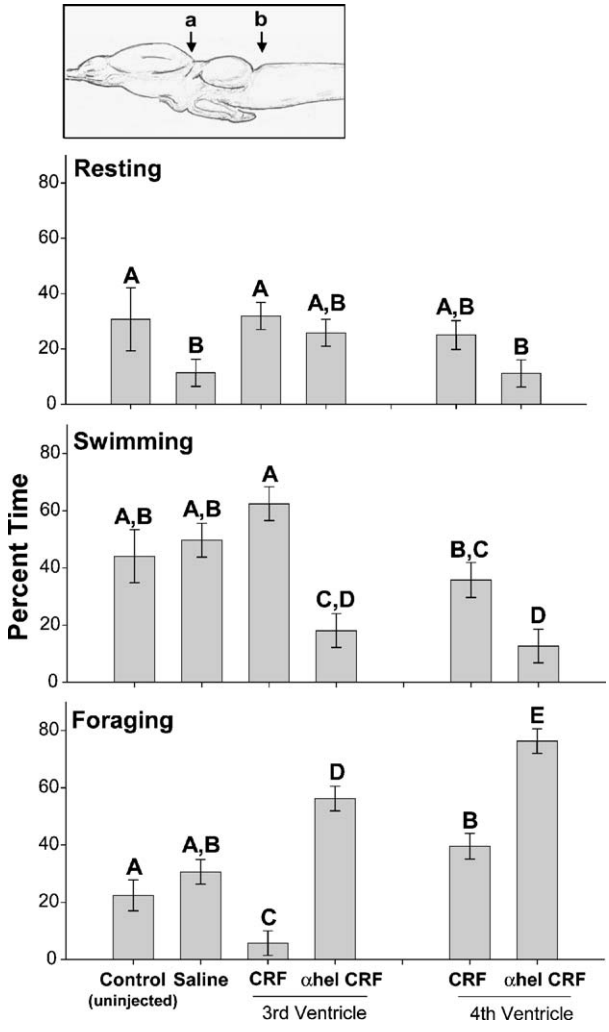

Fig. 3. Effects of CRF or $\alpha helCRF_{(9-41)}$ on behavior of prometamorphic *S. hammondii* tadpoles. Location of intracerebroventricular injection sites (a: third ventricle or b: fourth ventricle) is indicated in the top panel. Bars indicate mean differences (±SEM) in the percentage of time tadpoles spent exhibiting a behavior in a 2-min observation trial (n = 12). Tadpoles were observed 1 h after intracerebroventricular injections of 20 ng/g BW CRF, 1 μg per tadpole $\alpha helCRF_{(9-41)}$, or co-injection of CRF and $\alpha helCRF_{(9-41)}$. An ANOVA was conducted to compare treatment means for each behavior, and letters indicate significantly different treatment means for each behavior (Duncan's multiple comparisons tests, $P < 0.05$).

tadpoles ingested significantly fewer lead beads than controls, while αhelCRF$_{(9-41)}$ tadpoles ingested significantly more lead beads [ANOVA: $F(2,21) = 7.04$, $P = 0.004$; Fig. 4].

Experiment 5

Effects of CORT or metyrapone on tadpole behavior

Treatment of water with corticosterone, metyrapone, or both significantly affected all behaviors observed in prometamorphic tadpoles [swimming ANOVA: $F(3,20) = 4.08$, $P = 0.0206$; foraging ANOVA: $F(3,20) = 25.24$, $P < 0.0001$; and resting ANOVA: $F(3,20) = 12.47$, $P < 0.0001$]. Three-hour treatment with CORT or metyrapone and CORT caused tadpoles to spend less time swimming and more time foraging compared with vehicle-treated controls (Fig. 5). Tadpoles treated with metyrapone for 3 h (to block corticoid synthesis) spent significantly more time resting and less time foraging compared with vehicle controls (Fig. 5). Radioimmunoassay confirmed that treatments affected whole-body corticosterone content as expected [ANOVA: $F(3,20) = 4.32$, $P = 0.017$]. Relative to the whole-body corticosterone content of vehicle controls (2.1 ± 0.86 ng/g BW), corticosterone treatment significantly increased content (4.8 ± 0.98 ng/g BW) and metyrapone treatment decreased content (1.1 ± 0.46 ng/g BW), although this decrease was not statistically significant. Tadpoles treated with both corticosterone and metyrapone did not differ from vehicle controls (3.5 ± 0.26 ng/g BW).

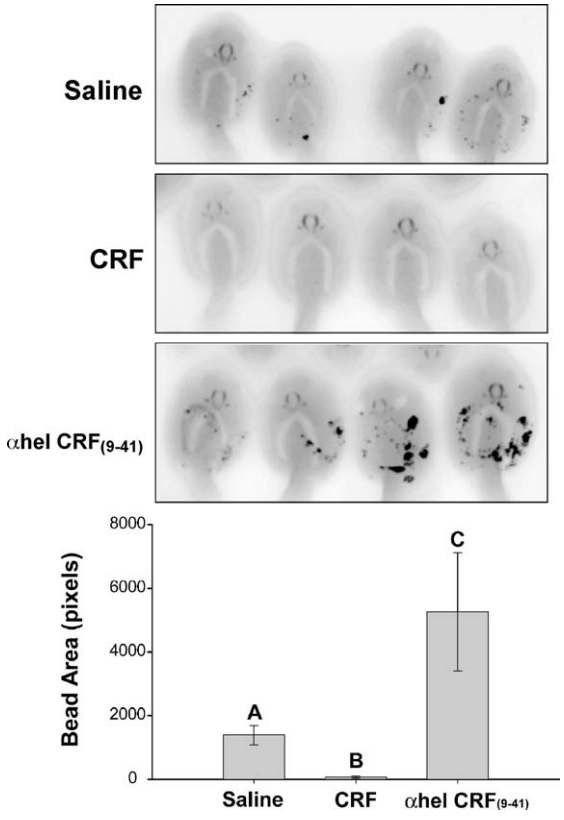

Fig. 4. Representative radiographs of prometamorphic *S. hammondii* tadpoles that received intracerebroventricular injections of saline vehicle, 20 ng/g BW CRF, or 1 μg per tadpole αhelCRF$_{(9-41)}$ and were allowed to forage on food containing lead beads for 1 h. Black spots indicate the presence of lead beads in the digestive tract of the tadpole and are shown in the top panel. Quantitation of the mean area of lead beads (±SEM) found in tadpoles of each treatment is shown in the bottom panel; letters indicate significantly different treatment means (Duncan's multiple comparisons tests, $P < 0.05$).

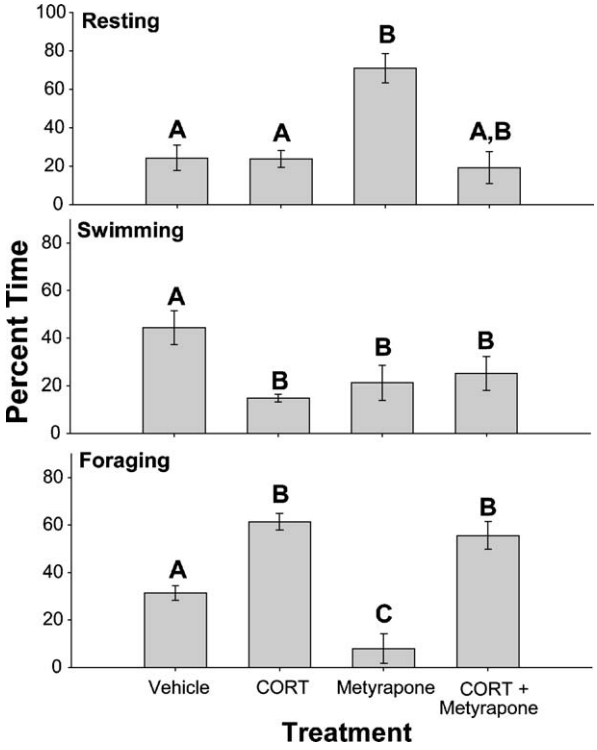

Fig. 5. Effects of exposure to vehicle (0.0048% ethanol), corticosterone (CORT, 500 nM), or metyrapone (MET, 0.11 μM) on the behavior of prometamorphic tadpoles. Tadpoles ($n = 6$ per treatment) were exposed to the hormone or drug for 3 h before behavioral observations were conducted for a 2-min period. Letters indicate significantly different treatment means (Duncan's multiple comparisons tests, $P < 0.05$).

Experiment 6

Effects of CRF with or without RU486 on tadpole behavior

At 1 h after intracerebroventricular injection of CRF (with intraperitoneal injection of oil or RU486), tadpoles significantly increased swimming [ANOVA $F(3,20) = 11.87$, $P = 0.0001$] and decreased foraging [ANOVA $F(3,20) = 29.63$, $P < 0.0001$] compared with vehicle-injected controls (Fig. 6), as shown in previous experiments. The time spent resting was not affected by any treatment [ANOVA $F(3,20) = 1.79$, $P = 0.34$]. Simultaneous injection of RU486 (i.p.) did not affect the immediate behavioral response to CRF, nor did it alter behavior relative to the saline + oil controls (Fig. 6).

By 6 h after injection, the effects of CRF on swimming and foraging continued to be statistically significant [ANOVA: $F(3,20) = 5.60$, $P = 0.0059$, and $F(3,20) = 17.55$,

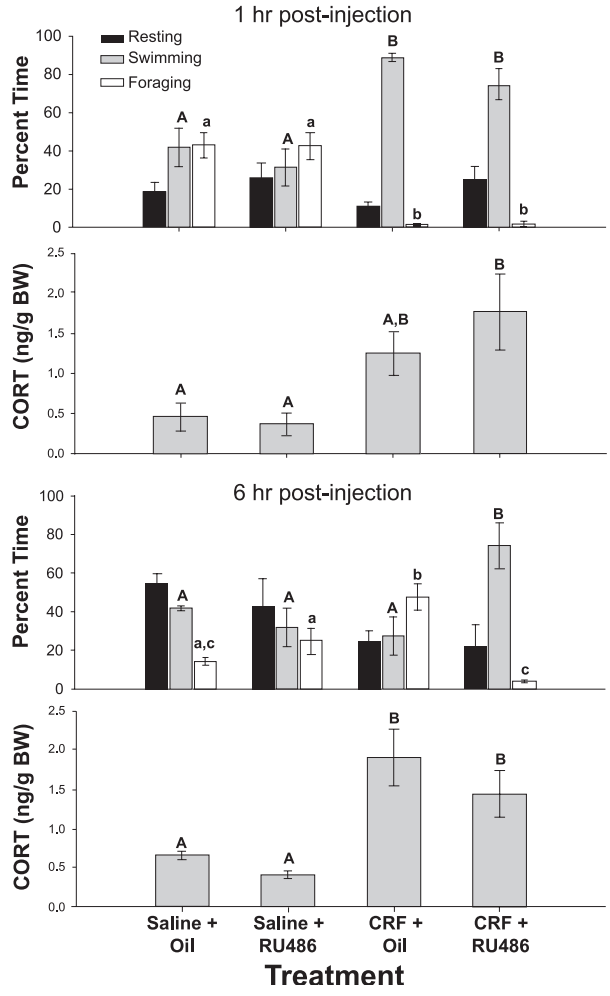

Fig. 6. Behavioral profiles and whole-body corticosterone content of prometamorphic *S. hammondii* tadpoles given intracerebroventricular injections of CRF into the third ventricle with or without intraperitoneal injection of the glucocorticoid receptor antagonist RU486. Behavioral assays were conducted at 1 and 6 h after injections. Behaviors were observed in the same individuals at each time point ($n = 6$ per treatment), but separate sets of tadpoles were analyzed for corticosterone ($n = 5–7$ per treatment). Letters indicate significantly different treatment means (Duncan's multiple comparisons tests, $P < 0.05$). For behavioral graphs: capital letters indicate significantly different treatment means for swimming; lowercase letters indicate significantly different treatment means for foraging.

$P < 0.0001$, respectively], but were opposite to the effects seen at 1 h. Injection of CRF + oil caused tadpoles to spend significantly more time foraging than controls (Fig. 6). By contrast, co-injection of CRF with the GR antagonist RU486 blocked the CRF-dependent increase in foraging at 6 h. CRF + RU486-treated tadpoles displayed the same behavioral profile at 6 h as they did at 1 h, that is, they swam for more time and foraged for less time than control tadpoles (Fig. 6). There were no significant differences in time spent resting [ANOVA: $F(3,20) = 2.57$, $P = 0.083$].

Radioimmunoassay analysis showed that CRF-injected tadpoles tended to have higher whole-body CORT content relative to vehicle-injected animals when measured at both 1 h and 6 h after the injection [ANOVA 1 h: $F(3,18) = 3.83$, $P = 0.028$; 6 h: $F(3,17) = 9.65$, $P < 0.001$]. The increase was statistically significant for the CRF/RU486 treatment in the 1-h sample, and for both CRF-injected treatments in the 6-h sample (Fig. 6).

Experiment 7

Effect of αhelCRF$_{(9-41)}$ on prey-catching behavior in juvenile toads

Toads receiving intracerebroventricular injections of αhelCRF$_{(9-41)}$ displayed increased prey-catching behavior (Table 1). Animals injected with αhelCRF$_{(9-41)}$ displayed a significantly shorter time until their first strike at a cricket [ANOVA: $F(2,18) = 7.04$, $P = 0.006$] and made significantly more strikes (Kruskal–Wallis $\chi^2 = 11.078$; $df = 2$; $P = 0.004$) than saline-injected controls. Toads treated with CRF + αhelCRF$_{(9-41)}$ showed intermediate values for these three variables. In addition, toads injected with αhelCRF$_{(9-41)}$ or CRF and αhelCRF$_{(9-41)}$ ate more crickets within both the 3- and 33-min time intervals compared with saline-injected controls (Kruskal–Wallis 3 min: $\chi^2 = 6.99$; $df = 2$; $P = 0.030$; 33 min: $\chi^2 = 7.67$; $df = 2$; $P = 0.021$).

Discussion

Our findings show that CRF exerts similar inhibitory effects on food intake in early, postembryonic life stages (i.e., the amphibian tadpole) to those that have been observed in adult vertebrates (Carr, 2002). We also found that CRF stimulated locomotory behavior in tadpoles as it does in adult amphibians and adults of other vertebrate species. These behavioral responses to exogenous CRF essentially mirror those that occur during an acute, stress-induced secretion of CRF (i.e., during the "fight or flight" re-

Table 1
Effect of intracerebroventricular injections of αhelCRF$_{(9-41)}$ on prey-catching behavior in juvenile spadefoot toads

Treatment	Time to first strike (s)**	Time to first eaten (s)	Number of strikes**	Number eaten in 3 min*	Number eaten in 33 min*
Saline	148 ± 14	154 ± 26	0.6 ± 0.3	0.0 ± 0.0	0.6 ± 0.3
αhelCRF$_{(9-41)}$	39 ± 9	83 ± 26	3.6 ± 0.6	1.4 ± 0.5	2.7 ± 0.7
αhelCRF$_{(9-41)}$ + CRF	85 ± 32	102 ± 34	1.4 ± 0.5	1.3 ± 0.5	3.3 ± 0.7

The mean ± SEM is given for each behavioral variable measured (refer to Methods for an explanation of the variables and statistical analyses).
* $P < 0.05$.
** $P < 0.01$.

sponse). Co-injection with the CRF receptor antagonist αhelCRF$_{(9\text{-}41)}$ reversed the effects of CRF, indicating that these behaviors are specifically mediated by CRF receptors. Furthermore, we confirmed that the immediate behavioral effects of CRF injection were not due to the subsequent increase in circulating corticosterone by showing that (1) treatment with the GR antagonist RU486 did not reverse the immediate behavioral effects of CRF injection, and (2) exogenous corticosterone caused the opposite behavioral effects to those of CRF injections (i.e., stimulated food intake).

Effects of CRF on locomotory and foraging behavior

In both premetamorphic and prometamorphic tadpoles, CRF injections increased swimming speed and the amount of time spent swimming. Similar increases in locomotory behaviors have been shown in adult amphibians and mammals (Clements et al., 2002; Koob and Bloom, 1985; Lowry and Moore, 1991; Takamatsu et al., 1991). In previous studies, treatment with a CRF receptor antagonist (αhelCRF$_{(9\text{-}41)}$) blocked stress-induced increases in locomotion (e.g., Lowry and Moore, 1991). We also found that αhelCRF$_{(9\text{-}41)}$ injection prevented CRF-induced increases in locomotion in premetamorphic tadpoles, and treatment with αhelCRF$_{(9\text{-}41)}$ alone reduced locomotion relative to uninjected animals. These data suggest that endogenous CRF secretion in premetamorphic tadpoles modulates locomotory behavior, and an acute increase in CRF secretion, as simulated by our injection, further stimulates locomotion.

Exogenous CRF inhibited foraging in both premetamorphic and prometamorphic tadpoles, but αhelCRF$_{(9\text{-}41)}$ dramatically stimulated foraging and food intake only in prometamorphic tadpoles and juveniles. In the prometamorphic tadpole experiment, αhelCRF$_{(9\text{-}41)}$ caused an increase in foraging compared with that observed in placebo and uninjected controls. Because all animals were anesthetized before injection and the unhandled control animals exhibited the same behavioral profiles and whole-body CORT content as the saline-injected controls, we conclude that the intracerebroventricular injection did not cause a stress response. Therefore, the stimulation in foraging caused by αhelCRF$_{(9\text{-}41)}$ injection suggests that endogenous CRF secretion plays a significant role in the regulation of food intake in unstressed animals at later developmental stages. In other vertebrates, αhelCRF$_{(9\text{-}41)}$ attenuated CRF- or stress-induced inhibition of food intake, but did not affect food intake in the absence of stress (fish: Bernier and Peter, 2001; mammals: Krahn et al., 1986). The current results with *S. hammondii* are consistent with our previous finding that intracerebroventricular αhelCRF$_{(9\text{-}41)}$ injection increases meal size relative to unhandled controls in *X. laevis* juveniles (Crespi and Denver, 2004).

Our studies of *S. hammondii* and *X. laevis* suggest two roles for CRF in the regulation of food intake in amphibians that develop at different times. First, CRF injections acutely inhibited foraging in all developmental stages. Similar acute increases in endogenous CRF production leading to activation of the hypothalamic-pituitary-interrenal (HPI) axis are expected in response to physical or psychological stressors. This is supported by findings from our laboratory of increased CRF immunoreactivity (CRF-ir) in the preoptic nucleus (homolog to the mammalian paraventricular and supraoptic nuclei) in juvenile *X. laevis* in response to physical stress (Yao et al., 2002). We also showed that water volume reduction, an environmental stressor, activates the HPI axis in prometamorphic tadpoles, and this activation likely drives stress-induced acceleration of metamorphosis (Denver, 1993, 1997; Denver et al., 1998). Premetamorphic tadpoles also appear to have an active HPI axis as they can mount a CORT response to stressful environmental conditions, such as increased population density, food restriction, and water volume reduction (Denver, 1998; Glennemeier and Denver, 2002b; Hayes, 1997). From the time tadpoles hatch, they must deal with potentially harmful environmental conditions, such as reduced resource availability and predation. Therefore, it is not surprising that the ability to alter foraging behavior in response to stress develops early in amphibians.

Secondly, the stimulatory effect of αhelCRF$_{(9\text{-}41)}$ injection suggests that endogenous CRF secretion modulates foraging rate in relatively nonstressful conditions. This result supports a growing body of evidence in rodents suggesting that hypothalamic CRF secretion is involved in the regulation of appetite independent of its ability to suppress food intake in times of stress (Cone, 2000). However, the stimulatory effect of αhelCRF$_{(9\text{-}41)}$ was not observed until prometamorphic tadpole stages. The blockade of CRF receptors in premetamorphic tadpoles did not affect foraging possibly because CRF secretion is low at this developmental stage (Carr and Norris, 1990) or because the neural circuitry controlling food intake is not complete until prometamorphic stages. Further investigations of the ontogeny of CRF neurons in the tadpole brain and of the interaction between CRF neurons and other hypothalamic neurons involved in food intake (e.g., neuropeptide-Y neurons) are needed to fully understand the mechanisms behind the ontogeny of CRF regulation of foraging.

Interestingly, neuroendocrine controls of food intake do not fully develop until after the suckling phase in rats: CRF and NPY expression and secretion are low in neonatal rats, and NPY neuronal connections among hypothalamic feeding centers are not in place until postnatal days 15–16 when pups initiate independent ingestion of solid food (Grove and Smith, 2003). Leptin, a potent inhibitor of food intake in adult mammals, does not affect food intake in the neonatal rat (Proulx et al., 2002) or suckling behavior in the late-gestation sheep (Ross et al., 2003). Similarly, we show that the tonic appetite suppressing effect of CRF also does not develop until later stages in *S. hammondii* tadpoles. In each case, it appears that the development of more complex neuroendocrine controls that stop or reduce feeding takes

place after a phase of rapid growth and maximal food intake. We propose that the development time of such controls is determined in accordance with specific life history transitions: at the time of independent feeding in the rat, and at the time when metamorphosis no longer depends on growth rate in the tadpole (Wilbur and Collins, 1973).

Sites of CRF action

We were able to compare the behavioral effects of CRF and αhelCRF$_{(9\text{-}41)}$ administered to two different brain regions in prometamorphic tadpoles: third ventricle injections spread only through fore- and midbrain regions, while ventricle injections were restricted to the hindbrain region. From this experiment, we showed that injection of CRF in the fore-midbrain region, but not the hindbrain region, inhibited foraging. This result suggests that CRF either directly or indirectly inhibits food intake behaviors via hypothalamic neurons, as shown in mammals (Heinrichs and Richard, 1999). One possible mechanism linking CRF secretion with food intake in the hypothalamus involves stimulation of monoamine neurons in the dorsomedial hypothalamus (DMH). In a study of CRF regulation of locomotion in amphibians, Lowry et al. (2001) showed that third ventricle CRF injection increased both dopamine and serotonin concentrations specifically in the DMH of rough-skinned newts. Although Lowry et al. did not measure foraging behavior, others have shown that dopamine and serotonin signaling in the DMH inhibits feeding (Schwartz et al., 2000). Furthermore, the DMH is thought to be an intermediary among the primary feeding centers in the rat (Bernardis and Bellinger, 1998). Given these data, we are interested in testing the hypothesis that CRF inhibition of foraging is mediated by monoaminergic neurons in the DMH.

While the inhibitory effect of CRF on foraging only occurred with injections into the fore-midbrain region, injection of αhelCRF$_{(9\text{-}41)}$ into either brain region stimulated foraging compared with uninjected controls. Taken alone, our finding with αhelCRF$_{(9\text{-}41)}$ suggests that in nonstressful conditions, CRF acts on both hypothalamic and hindbrain neurons to suppress food intake. Considering the lack of effect of CRF injection into the hindbrain, activity may be maximal in rostrally projecting inhibitory neurons originating from the hindbrain (neurons that express CRF receptors) under the conditions of our assay. Consequently, only by inhibiting this pathway can we observe a change in behavior. We have identified strongly staining CRF-ir neurons in the hindbrain of *X. laevis* (e.g., locus coeruleus, Yao et al., 2002), but we have not yet mapped projections from these neurons or the locations of CRF receptor expression. However, the potent stimulatory effect of αhelCRF$_{(9\text{-}41)}$ injected into this region suggests that hindbrain neurons that express CRF receptors play some role in the regulation of foraging. Hindbrain nuclei have been implicated in the regulation of food intake in rodents, but the mechanisms and neural circuitry of this control are not understood (Schwartz et al., 2000). In rodents, brainstem noradrenergic neurons extend fibers rostrally (Koob, 1999; Swanson and Sawchenko, 1983), and norepinephrine release from presynaptic terminals in the PVN stimulates food intake (Capuano et al., 1992). Although these neurons have not been studied in amphibians, we hypothesize that inhibition of hindbrain noradrenergic neurons might be one mechanism by which CRF regulates food intake in the tadpole.

Effect of CORT on food intake

Short-term treatment with CORT stimulated foraging in *S. hammondii* tadpoles, as has been shown in rodents and fish (Bernier et al., 2004; Dallman et al., 1993; Tataranni et al., 1996). Conversely, treatment with metyrapone, a CORT synthesis inhibitor, decreased foraging and increased resting time. Metyrapone treatment did not result in a statistically significant decrease in whole-body CORT content; however, we did not expect that such short-term treatment with the corticosteroid synthesis inhibitor would affect the total pool of CORT in the body. This treatment could have reduced plasma CORT concentration, as suggested by the significant decrease in foraging that we observed with metyrapone. Further evidence for the stimulatory action of endogenous CORT on appetite is the increase in foraging that we observed 6 h after CRF intracerebroventricular injection. This increase in foraging was associated with elevated whole-body CORT content and was completely blocked by treatment with the GR antagonist RU486.

CRF and CORT functions in energy balance

When the effects of CRF and CORT on foraging and locomotion in prometamorphic tadpoles are considered together, they support the hypothesis that CRF and CORT regulate behavioral responses to acute stressors in tadpoles to promote survival and the maintenance of energy balance. The immediate behavioral effects of CRF within the CNS are to stimulate locomotion and to inhibit foraging. These behavioral responses are highly adaptive and serve to promote survival. At the same time, CRF activates the HPI axis leading to an increase in plasma adrenocorticotropic hormone and CORT. The delayed stimulatory effect of CORT on foraging observed after CRF injection in this study is consistent with the suppressive and preparative roles of glucocorticoids in the stress response as proposed by Sapolsky et al. (2000). First, CORT suppresses (or overrides) the immediate anorexigenic actions of CRF, and thus limits the duration of the anorectic response. This effect of CORT would prevent the animal from exceeding its energetic reserves. Second, CORT stimulation of feeding aids in the restoration of energy reserves that were depleted during the anorexic period and prepares the tadpole for future responses to stress.

Although we did not specifically investigate the effects of chronic stress on food intake in this study, we can make some predictions based on our findings. Our results suggest that in the unstressed state, CRF modulates food intake such that greater secretion would exert stronger inhibition on foraging. If CRF secretion increases due to chronic stress, as has been shown in mammals (Herman et al., 1995; Ma and Aguilera, 1999), we predict that basal foraging rates will decrease and result in a reduction in tadpole growth and development. Chronic elevation of circulating CORT could also have negative effects on foraging and energy balance as tadpoles treated with CORT (via addition to the aquarium water) for ≥14 days exhibited reduced growth and slowed development (Glennemeier and Denver, 2002b; Hayes, 1997).

In prometamorphic *S. hammondii* tadpoles, water volume reduction causes a cessation of foraging as the animals accelerate metamorphosis (Denver et al., 1998; M. Phillips and R. Denver, unpublished data), and this acceleration is associated with increased hypothalamic CRF content (Denver, 1997). This result led to the hypothesis (Denver, 1997) that CRF functions at multiple levels in the developmental and behavioral response of spadefoot toad tadpoles to habitat desiccation. CRF may act centrally to inhibit appetite and foraging while activating the thyroid and HPI axes to accelerate metamorphosis. Results presented herein strongly support this hypothesis as we have shown that exogenous CRF has potent inhibitory effects on foraging, and endogenous CRF plays an important role in modulating foraging behavior in prometamorphic *S. hammondii* tadpoles.

Acknowledgments

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