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An intrinsic CRF signaling system within the optic tectum

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ABSTRACT

Previous work indicates that CRF administration inhibits visually guided feeding in amphibians. We used the African clawed frog *Xenopus laevis* to examine the hypothesis that CRF acts as a neurotransmitter in the optic tectum, the major brain area integrating the visual and premotor pathways regulating visually guided feeding in anurans. Reverse transcriptase PCR revealed that cells in the optic tectum express mRNA for CRF and the CRF R1 receptor but not the CRF R2 receptor. Radioligand binding studies indicated that specific binding of [^{125}I]-Tyr-oCRF to tectal cell membranes can be displaced by the CRF R1 antagonists antalarmin or NBI-27914. CRF increased the expression of mRNA encoding regulator of G-protein signaling 2 (*rgs*2) in tectal explants and this effect was blocked by antalarmin. CRF had no effect on basal glutamate or gamma-aminobutyric acid (GABA) secretion but inhibited secretion of norepinephrine from tectal explants, an effect that completely blocked by antalarmin. Using a homologous radioimmunoassay we determined that CRF release from tectal explants in vitro was potassium- and calcium-dependent. Basal and depolarization-induced CRF secretion was greater from optic tectum than hypothalamus/thalamus, telencephalon, or brainstem. We concluded that the optic tectum possesses a CRF signaling system that may be involved in modulating communication between sensory and motor pathways involved in food intake.

1. Introduction

Corticotropin-releasing factor (CRF) was first isolated as the major hypothalamic peptide regulating ACTH secretion from the pituitary gland during stress (Spiess et al., 1981; Vale et al., 1981). In the 30 + years since CRF was first isolated there have been many advances in our understanding of CRF receptor subtypes and the interaction between CRF and its receptors in the control of adaptive behavior (Ronan and Summers, 2011). A major role for CRF receptor agonists during stress is the inhibition of food intake and appetite, which appears to involve CRF and related peptides interacting with both CRF R1 and R2 receptors at different sites within the CNS (Bakshi et al., 2007; Chen et al., 2010) and periphery (Fekete et al., 2011).

We previously suggested (Carr, 2006) an alternative theory to explain CRF effects on food intake that involves direct modulation of sensory cues for recognizing food. This theory is based on data that CRF inhibits visually guided feeding and prey capture in at least three anuran species: the Texas toad (*Bufo speciosus*) (Carr et al., 2002), the Western spadefoot toad (*Spea hammondii*) (Crespi and Denver, 2004) and the bullfrog (*Rana catesbeiana*) (Morimoto et al., 2011). The fact that these anuran species rely on visual cues to locate and capture prey suggests that CRF may directly or indirectly modulate subcortical visual processing. In fact, work from our laboratory (Carr et al., 2010) and from others (Boorse and Denver, 2004; Calle et al., 2005; Yao et al., 2004) has provided anatomical and biochemical evidence for the existence of a population of CRF producing cells within the anuran optic tectum, the major brain area involved in integrating visual cues regarding predators and prey. Measurable quantities of CRF have been reported in the optic tectum of *Xenopus laevis* (Boorse and Denver, 2004) and *Bufo marinus* (Carr et al., 2010). We have demonstrated that the CRF content of the tectum most likely comes from local sources, as retinal deafferentation has no effect on tectal CRF content in *B. marinus* (Carr et al., 2010). Furthermore, immunohistochemical studies have shown the existence of CRF-immunoreactive (ir) neurons within the tecti of *X. laevis* (Calle et al., 2005; Yao et al., 2004) and *B. marinus* (Carr et al., 2010). Within the tectum CRF-ir neurons appear to be strategically located to interact with retinal afferents that form the bulk of tectal layer 9 (Carr et al., 2010).

At present it is unknown whether CRF is released as a neurotransmitter by tectal neurons. Furthermore, it is not known if CRF acts on receptors within the tectum to modulate the release of other neurotransmitters. CRF R1 mRNA expression has been observed in the optic tectum using in situ hybridization (Calle et al.,

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2006), but it is unknown whether the neurons expressing CRF R1 mRNA are interneurons or projection neurons. In this study we extend our previous work to determine if tectal neurons express mRNA for CRF and its receptors and if measurable quantities of CRF are released from tectal neurons after depolarization in a calcium dependent fashion. Furthermore, we investigated whether CRF acts on local receptors to modulate tectal neurotransmitter release in the optic tectum.

2. Methods

2.1. Reagents

Antalarmin was purchased from Tocris Bioscience (Minneapolis, MN). [^{125}I]-Tyr-oCRF (2200 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA). Bacitracin, dihydroxybenzylamine, dopamine, epinephrine, gamma-aminobutyric acid (GABA), L-glutamate, NBI-27914, norepinephrine, orthophthaldialdehyde (OPA), polyethylenamine, and sodium sulfite were purchased from Sigma–Aldrich Co. (St. Louis, MO).

2.2. Animals and care

Six-month old South African Clawed Frog (*X. laevis)* juvenile frogs (20–30 g) were used for all studies except the radioligand binding studies, where young adult female frogs (60–80 g) were used to maximize tissue recovery. Juvenile frogs were obtained from our in-house colony and were reared in deionized water containing 0.33 g/L Instant Ocean in a large tank (178 cm L × 46 cm W × 51 cm D, 300 L) at a maximum stocking density of 60 per 300 L. Adult frogs were maintained in dechlorinated tap water in a flow-through system (Aquatic Habitats, 160-L tanks, 20 animals per tank) as described previously (Goleman and Carr, 2006). Frogs were maintained at a temperature of 22–23 °C on a 12L:12D light regimen. Frogs were fed NASCO floating (juveniles) or sinking (adults) *Xenopus* chow three times per week. The water was changed and the tank were cleaned three times per week. All procedures were approved by the Texas Tech Animal Care and Use Committee.

2.3. Reverse transcriptase PCR (RT-PCR)

Juvenile frogs were euthanized with tricane methanesulfonate (MS-222) (1 g/L dH2O) buffered with $NaHCO_3$. Brain areas (telencephalon, hypothalamus/thalamus, optic tectum, brainstem) were transferred to TRIZOL® reagent (Invitrogen, CA), sonicated, and RNA extracted according to the manufacturer's instructions. The resulting pellet was re-suspended in RNAase free water and immediately stored in −80 °C for subsequent use. The purity and concentration of extracted RNA was checked using a NanoDrop 2000 micro-volume spectrophotometer (Thermo Scientific, Wilmington, DE). The sample purity was obtained from the A260/280 ratio and all samples used for cDNA synthesis had a A260/280 ratio between 1.80 and 2. RNA samples were DNase (Sigma–Aldrich) treated prior to reverse transcription carried out from 1 μg of total RNA using the ProtoScript® M-MuLV First Strand cDNA Synthesis kit (New England BioLabs MA USA) according to manufacturer's protocol. cDNA was stored at −20 °C. Primers (Table 1) were used based upon sequences reported by Boorse and Denver (2004) or Carr et al. (2008). *Rpl*8 was used as a reference gene since it has been shown by Shi and Liang (1994) that *rpl*8 has a constant and ubiquitous expression in *X. laevis*.

PCR was carried out using HotStart-IT™ Taq DNA Polymerase (Affymetrix, California, USA) in a PTC-200 (Peltier Thermal Cycler MJ Research., Nevada, USA) using 30 cycles of annealing and 72 °C elongation. PCR products were separated on 2% agarose gels and viewed in a Kodak High Performance Fluorescent Transilluminator. Two–three samples were analyzed for each brain region from each animal. Amplicon identity was confirmed by sequencing.

Table 1
Oligonucleotide primers used to amplify *X. laevis* genes.

Gene	Oligonucleotide Sequence from 5′ to 3′	Reference
crf	F: TCTCCTGCCTGCTCTGTCCAA R: CTTGCCATTTCTAAGACTTCACGG	Boorse and Denver, 2004
crfr1	F: GATCTAAATAGCAGGATGCTGTTGGC R: GCCGTTCAGGTGACATTCTCTGTAC	Boorse and Denver, 2004
crfr2	F: GTACCTCCCACATCCCACAGCTTCAC R: GACGCCCAGGTTCCATTCTCAAAGC	Boorse and Denver, 2004
crfbp	F: TGACTCCTGCTTCCAGACCT R: TGACCTTGTAATGCTCCCCAC	Boorse and Denver, 2004
rgs2	F: CCCGTCTGAGCTACTTCCTG R: CTGAATGCTGCTACGCCATA	NM_001095045
rpl8	F: CACAGAAAGGGTGCTGCTAAG R: CAGGATGGGTTTGTCAATACG	Carr et al., 2008

2.4. Radioligand binding studies

Adult female frogs ($n = 23$) were anesthetized with MS-222, brain tissues were removed and homogenized in 10 vol homogenization buffer (50 mM Tris–HCl/250 mM sucrose, pH 7.4). Homogenates were centrifuged at 1,000*g* for 10 min at 4 °C. The supernatant was decanted and centrifuged at 35,000*g* for 25 min at 4 °C. The pellet containing isolated cell membranes were reconstituted in binding buffer (50 mM Tris–HCl, 10 mm $MgCl_2$, 2 mM EGTA, 0.1% BSA, 100 units/mL aprotinin with 1% bacitracin, pH 7.4 at 19 °C). Magnesium concentrations were based on (Holmes et al., 1984) for optimal binding. Membranes were kept on ice while preparing tubes for binding assays. Assays were performed in a 25 °C shaking water bath for 2 h. All assays were carried out in a final volume of 0.5 mL in 12 × 75 mm polypropylene tubes. Final assay volumes contained 150–350 μg protein in 200 μL buffer, 50 μL radioligand (0.1–0.3 nM) and 250 μL binding buffer. Nonspecific binding were determined using 10 μM concentrations of the CRF R1 selective antagonists NBI-27914 (Baram et al., 1997) or antalarmin (Webster et al., 1996). Incubations were terminated by placing tubes in an ice bath for 10 min. Membranes were harvested and rapidly filtered onto Whatman GF/C filters using a membrane harvestor (Brandel, Gaithersburg, MD). Filters were pretreated with 0.5% polyethylenamine (PEI) to minimize nonspecific binding of the radioligand to the filter. Washes were performed with ice cold 50 mM Tris–HCl buffer pH 7.4 for an experimentally determined length of time. Following washes, filters were air dried approximately 15 min. Filter punches were placed in clean polypropylene tubes and counted on a Packard gamma counter.

Final protein content was determined in duplicate using a modification (Markwell et al., 1981) of the Lowry method (Lowry et al., 1951). Specific binding was defined as the total radioligand binding minus the nonspecific radioligand binding. Wash experiments were performed to empirically determine the wash time needed to remove excess nonspecific radioligand binding to membranes and filters. Single concentrations of [^{125}I]-Tyr^0 oCRF (1 nM) were used in each assay. Filters were washed with ice cold 50 mM Tris buffer using one of six different wash times, ranging from single 1 to 6 s washes to multiple 4 s washes. Wash times yielding at least 70% specific binding of the total radioligand binding were used in binding assays.