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Brainstem Reticulospinal Neurons are Targets for Corticotropin-Releasing Factor-Induced Locomotion in Roughskin Newts

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Abstract

Stress-induced release or central administration of corticotropin-releasing factor (CRF) enhances locomotion in a wide range of vertebrates, including the roughskin newt, *Taricha granulosa*. Although CRF's stimulatory actions on locomotor behavior are well established, the target neurons through which CRF exerts this effect remain unknown. To identify these target neurons, we utilized a fluorescent conjugate of CRF (CRF-TAMRA **1**) to track this peptide's internalization into reticulospinal and other neurons in the medullary reticular formation (MRF), a region critically involved in regulating locomotion. Epifluorescent and confocal microscopy revealed that CRF-TAMRA **1** was internalized by diverse MRF neurons, including reticulospinal neurons retrogradely labeled with Cascade Blue dextran. In addition, we immunohistochemically identified a distinct subset of serotonin-containing neurons, located throughout the medullary raphé, that also internalized the fluorescent CRF-TAMRA **1** conjugate. Chronic single-unit recordings obtained from microwire electrodes in behaving newts revealed that intracerebroventricular (icv) administration of CRF-TAMRA **1** increased medullary neuronal firing and that appearance of this firing was associated with, and strongly predictive of, episodes of CRF-induced locomotion. Furthermore, icv administered CRF-TAMRA **1** produced behavioral and neurophysiological effects identical to equimolar doses of unlabeled CRF. Collectively, these findings provide the first evidence that CRF directly targets reticulospinal and serotonergic neurons in the MRF and indicate that CRF may enhance locomotion via direct effects on the hindbrain, including the reticulospinal system.

Keywords

CRF target neurons; stress-induced locomotion; serotonin neurons; chronic single-unit recording; amphibian

Introduction

Central administration of corticotropin-releasing factor (CRF) has potent locomotor-enhancing properties in vertebrates, including the roughskin newt, *Taricha granulosa* (Clements et al.,

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2002; Crespi and Denver, 2004; Lowry and Moore, 2006; Lowry et al., 1996). Previous investigations of rats, teleost fish and *Taricha*, have shown that intracerebroventricular (icv) CRF administration results in a dose and time-dependent increase in locomotion (Carpenter et al., 2007; Lowry et al., 1990; Sutton et al., 1982). Conversely, central administration of alpha-helical CRF (α hCRF₍₉₋₄₁₎), a competitive CRF receptor antagonist, greatly attenuates both exogenous CRF and stress-induced enhancement of locomotion (Britton et al., 1986; Lowry and Moore, 1991; Winslow et al., 1989). Furthermore, in rats and *Taricha* the locomotor-enhancing properties of CRF remain unaffected following hypophysectomy or systemic dexamethasone injection, lending support to the premise that CRF is targeting central sites to facilitate locomotor activation (Britton et al., 1985; Eaves et al., 1985; Moore et al., 1984).

Although CRF-enhanced locomotion is well documented, the neuronal phenotypes responsible for mediating CRF's facilitatory actions on locomotor behavior and the cellular mechanisms underlying this effect remain undetermined. To date, the majority of research has focused on CRF's upstream actions; on ascending serotonergic and noradrenergic neuromodulatory systems arising from midbrain and rostral pontine nuclei, such as the dorsal raphe nucleus (DRN), locus coeruleus (LC), and their respective targets in the ventral forebrain (Lowry et al., 1993,2000; Price et al., 1998; Tazi et al., 1987; Valentino et al., 1993). In contrast, possible CRF actions on the medullary reticular formation (MRF), a region densely populated by reticulospinal (RS) neurons that are critically involved in initiation and regulation of vertebrate rhythmic locomotion has received less investigation (Lowry and Moore, 2006; Lowry et al., 1991). Lowry, Rose, and Moore (1996) observed that icv CRF administration evoked a rapid (within minutes) and robust enhancement in rostromedullary neuronal firing, which was strongly associated with and predictive of, a simultaneous increase in locomotion. Furthermore, Rose et al. (1996) demonstrated that medullary CRF application rapidly enhanced membrane excitability and sensory responsiveness of antidromically identified RS neurons and unidentified non-RS neurons in the rostromedial MRF of awake, immobilized newts. These findings strongly suggest that CRF may target RS neurons within the MRF to facilitate locomotion. Due to the route of CRF administration in these studies, however, it remains unclear whether these effects were solely a result of the direct actions of CRF on rostromedial MRF neurons, including RS cells (Lowry et al., 1996; Rose et al., 1996).

Reticulospinal neurons play a critical role in the initiation and expression of rhythmic locomotion (Grillner and Whalen, 1999; Rossignol and Dubuc, 1994). They are well suited, in terms of their converging sensory inputs, as well as direct outputs to the spinal cord, to be primary targets for CRF's facilitatory actions on locomotor behavior (Brodin et al., 1988; Dubuc and Grillner, 1989; Grillner et al., 1995). Thus, the purposes of the present study were to: 1) determine whether, in fact, RS neurons are targets for CRF, 2) begin to identify the neurochemical phenotypes of hindbrain CRF target neurons and 3) identify the functional properties of neurons with activity related to CRF-activated locomotion. To this end, we used our recently developed fluorescent rhodamine conjugate of CRF, CRF-TAMRA **1** (Hubbard et al., 2009), to track the internalization of this peptide into MRF neurons. The CRF-TAMRA **1** probe is internalized into cells rapidly (within 5–10 min), in a time-dependent manner, thus permitting visualization of CRF target neurons. The question of whether RS neurons, in particular, were target cells for CRF was assessed by retrogradely labeling these neurons with Cascade Blue (CB) dextran prior to intraventricular (icv) administration of CRF-TAMRA **1** in awake, behaving newts. In addition, immunohistochemistry was employed to determine if CRF-TAMRA **1** was internalized by medullary serotonergic neurons. Lastly, we examined the functional efficacy of CRF-TAMRA **1** by comparing it to equimolar doses of unlabeled CRF using chronic single-unit recordings obtained from rostromedial MRF neurons while concurrently monitoring behavior-related neuronal activity. Our results allow us to propose a possible mechanism for CRF enhancement of locomotion via actions on RS and serotonin neuronal populations in the caudal brainstem.

Materials and methods

Animals

Adult male roughskin newts ($N = 36$) were collected from Benton County, Oregon. Animals were housed in community tanks supplied with a continuous flow of aerated well water, fed a diet of chopped beef heart and maintained on a 12-h simulated photoperiod. All procedures were previously approved by The University of Wyoming Animal Care and Use Committee and conducted in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical Procedures for Hormone Delivery

All newts underwent surgical procedures involving implantation of a cannula guide for icv delivery of test substances to the right lateral ventricle. This set-up allowed for remote delivery of hormones or control substances during the experiment without handling of the newt (Hubbard et al., 2009). Prior to surgery, each newt was anesthetized by immersion in 0.1% tricaine methanesulfonate (MS-222). The newt's body was covered with cellulose tissue soaked in anesthetic to maintain anesthesia and facilitate transcutaneous respiration during surgery. The cannula guide, a 4.5-mm length of polyethylene tubing (PE 50), was implanted over a burr hole in the skull directly above the right lateral ventricle and anchored by dental cement to a stainless steel screw (0–90) inserted into the frontal bone. Following surgery, each newt was allowed to recover in cold ($13\text{--}14^{\circ}\text{C}$), aerated well water. Approximately 1–2 h into recovery and while still anesthetized, the newt was transferred to a 22 cm diameter circular glass arena containing enough cold, aerated well water to cover its entire body (Hubbard et al., 2009). A 26 ga stainless steel cannula, connected to a microsyringe by PE 50 tubing, was inserted into guide tube for remote icv delivery of test substances to the awake, freely behaving newt during the experiment.

CRF-TAMRA 1 Labeling and Retrograde Tracing Experiments

For experiments involving quantification of CRF-TAMRA 1 internalization in MRF neurons, each newt ($n = 8$) received icv infusion of 14.24 ng CRF-TAMRA 1 in 4 μl of amphibian Ringer's (9.6 g/L Tyrode's Salts, Sigma-Aldrich, T2145; supplemented with 1 g/L NaHCO_3) with an infusion rate of about 2 $\mu\text{l}/\text{min}$. This dose was chosen based on our previous findings demonstrating significant locomotion enhancing effects in the newt (Hubbard et al., 2009). After a 30 min post-treatment exposure period, the newt was immediately sacrificed by rapid decapitation and the brain was processed using the histological procedures described below.

To determine whether RS neurons were targets for CRF, 6 additional newts were used for retrograde labeling of RS neurons 12–14 days prior to CRF-TAMRA 1 administration. Each newt was anesthetized as described above. The spinal cord was exposed at the level of the first cervical vertebra and either the right or left side of the cord was hemisected. Cascade Blue dextran (10,000 MW; Invitrogen, D1976) crystals were inserted into hemisected cord, and Gelfoam (Upjohn) moistened with an amphibian Ringer's solution was placed over the exposure and skin was sutured closed. The newt was maintained on a normal feeding schedule for a two-week period prior to conducting the recording experiment. On the day of the experiment, the newt was anesthetized in 0.1% MS-222 and a cannula guide implanted for remote delivery of CRF-TAMRA 1. Following recovery from anesthesia, the newt was administered 14.24 ng of CRF-TAMRA 1 icv in 4 μl amphibian Ringer's and behavior was monitored for an additional 30 min. At the end of this 30 min post-treatment period, the newt was immediately sacrificed by rapid decapitation and the brain was processed as described below.

Serotonin(5-HT) Immunohistochemistry

To determine if serotonin-containing medullary raphe neurons were cellular targets for CRF internalization, immunohistochemistry was conducted on serial sections obtained from the hindbrains of 4 newts, each of which had received icv administration of 14.24 ng CRF-TAMRA **1** in 4 μ l of Ringer's. Following CRF-TAMRA **1** administration, each newt's behavior was monitored for an additional 30 min post-treatment, after which the animal was sacrificed by rapid decapitation. The brain was processed using standard histological procedures and cut serially into 20 μ m thick sections (see histological procedures section for details). Immunohistochemical labeling for serotonin was performed by rinsing slides with 0.6% H₂O₂ in PBS (0.01 M) for 30 min at room temperature (RT). Sections were rinsed twice in PBS (0.01 M) and then washed in 50% ETOH for 10 min (RT). Slides were then pre-incubated with normal goat serum diluted in PBS (0.01 M) containing 10% Triton X-100, 2% BSA and 50 mM Tris-buffered saline (PBST-BSA) for 2 h at RT followed by incubation with a primary antibody against serotonin (generated in rabbit; 1:5000 diluted in PBST; Immunostar, 20080) for 18 h at 4°C. Sections were then rinsed in PBS (0.01 M) and incubated in a secondary antibody conjugated to Alexa Fluor 488 (goat anti-rabbit IgG; 1:1000 diluted in PBS; Invitrogen, A11034) for 45 min at RT. Sections were given a final rinse in PBS and air dried for 8 h prior to coverslipping using Vectashield Mounting Medium with DAPI (DAPI; 4',6-diamidino-2-phenylindole) (VECTOR, H-1200) to fluorescently stain cell nuclei. Medullary sections from three additional newts were used for positive, negative and preabsorption (5-HT BSA conjugate, 10 μ g/ml; Immunostar, 20081) control experiments. Omission of the primary antibody showed an absence of staining verifying the specificity of the 5-HT antiserum.

Chronic Single-Unit Recording and Behavioral Experiments

Experiments involving chronic single-unit recording of MRF neurons in awake, behaving newts (n = 15) employed the following surgical procedures. The newt was anesthetized and a cannula guide was implanted over the right lateral ventricle as described previously. The dorsal hindbrain and fourth ventricle (4v) of the medulla were exposed by removal of overlying tissue. The recording electrode assembly consisted of two twisted pairs of 50- μ m Diamel-insulated nichrome microwires (impedances of 100–500 k Ω at 1 kHz). Electrodes were positioned on the medulla under visual control such that the electrode pairs straddled the midline and made contact with the dorsal surface of the rostromedial MRF, which lies just beneath the ependymal layer in *Taricha*. Sites for electrode placement varied across newts, but always encompassed the paramedial regions of the right or left MRF. To provide structural support for the recording electrode assembly, dental cement was used to anchor the implant to the stainless steel skull screw (0–90) and a stainless-steel wire suture through the neck skin, just rostral to the shoulder blades. The electrode assembly was fabricated so the microwires, twisted together and reinforced with cyanoacrylate glue, extended above the skull implant for several cm, to terminate in an Amphenol microminiature connector strip (Lowry et al., 1996; Rose et al., 1998). In this way, the entire implant was low in mass, flexible and provided very stable recordings.

Following surgery, the newt was allowed to recover (1–2 h) in a container of cold, aerated well water. Prior to recovery, the newt was transferred to a circular glass dish containing cold, aerated well water and a cannula connected to a microsyringe, via a length of polyethylene (PE 50) tubing, was inserted in the guide tube for icv delivery of hormone/s or control substances during the experiment. Leads from the microwire electrode implant assembly were connected through Microdot Mininoise coaxial cables to a Grass Model P55 preamplifier where the signal was amplified and filtered with a bandpass of 100–1 kHz. Unit activity and behavior were continuously monitored before the start of the experiment to determine when the animal had fully recovered from anesthetic.

Activity of single rostromedial MRF neurons was recorded for 5 min prior to (pre-treatment) and 30 min after (post-treatment) icv administration of CRF-TAMRA **1** (14.24 ng/4 μ l Ringer's; $n = 4$), an equimolar dose of CRF (12.82 ng/4 μ l Ringer's; $n = 4$), a vehicle control (VEH; 4 μ l Ringer's; $n = 4$), or the TAMRA dye (1.72 ng/4 μ l Ringer's; $n = 3$) alone. In addition, locomotor behavior was recorded continuously throughout each experiment. For behavior quantification purposes, the onset and offset of locomotion were identified by a combination of voice narration and video recordings.

For synchronization of behavioral and neurophysiological data signals, voice narratives as well as a 1 Hz tone were recorded on video and neurophysiological data tapes. Analog signals from neuronal activity, voice narratives and 1 Hz tones were digitized with a Neurodata Neurocorder (Model DR-484; Cygnus Technology, Inc.) and stored on pulse-coded VHS (Neurodata Neurocorder) tapes for offline analyses. Captured raw data from each experiment was re-sampled at 25,000 Hz using a Cambridge Electronic Design data acquisition system (Micro 1401 mk II, Cambridge Electronic Design; CED). Spike identification, counting and sorting were conducted using Spike 2, version 5.13 software program (CED). All single neuron spikes were sorted from templates by a combination of visual inspection of individual spike waveforms and in some cases, Principal Components Analysis. Once templates were sorted and all units identified, video recordings of behavior, voice narratives, and 1 Hz tones were played back in synchrony with sampled unit activity and the onset and offset of locomotion were manually coded with keyboard event markers in Spike 2.

To determine if central administration of CRF-TAMRA **1** altered neuronal activity of MRF neurons in a fashion similar to unconjugated CRF, each unit's mean frequency of firing (10 s bins) occurring 5 min prior to- (pre-treatment) and extending 30 min after treatment (post-treatment) was analyzed using the Change-Point Test for Continuous Variables (210 time points; two-tailed; $\alpha = 0.05$) across the four different treatment conditions (CRF-TAMRA **1**, CRF, VEH, and TAMRA). For units showing significant changes in activity following treatment, the direction of change was established by visual inspection of the data records. Units were then classified according to their direction of change (increase or decrease) and the proportions of units that changed (increase or decrease) activity were compared statistically to those that did not change across treatment conditions using 2×2 Chi-Square or Fisher's Exact Tests.

Behaviors categorized as either walking or swimming (locomotion) were analyzed for each 10 sec interval beginning 5 min prior to and extending to 30 min following treatment with CRF-TAMRA **1**, CRF, VEH or TAMRA alone. Episodes of walking or swimming consisting of at least 2 s duration were defined as locomotor bouts. To determine the magnitude of change for the number of locomotor bouts pre- to post-treatment for a given newt, difference scores were derived by subtracting the mean number of locomotor bouts occurring prior to treatment from the mean number of locomotor bouts occurring post-treatment. Significance tests were conducted on the number of locomotor bouts across treatment conditions with one-way ANOVAs and post-hoc tests using Tukey's HSD ($\alpha = .05$; two-tailed).

Histological Procedures and Fluorescence Microscopy

After each test procedure, the newt was immediately decapitated, the exposed brain rinsed twice in ice-cold PBS (0.01 M), and fixed in situ for 24 h in a solution of 30% sucrose and 10% formalin. The brain was then removed, rinsed and fixed for an additional 24–48 h. Brains were embedded in O.C.T. (Tissue Tek) and transversely sectioned at 20 μ m with a cryostat. Serial sections were thaw-mounted onto glass slides and air-dried for at least 8 h prior to coverslipping. For experiments involving retrograde labeling with Cascade Blue dextran, slides were coverslipped using Vectashield (VECTOR). For all other experiments, Vectashield

Mounting Medium with DAPI was used as a coverslipping medium to fluorescently stain cell nuclei.

Digital images of brain sections were acquired with an epifluorescence microscope (Olympus Bx51) and a SPOT RT-KE® digital video imaging system with accompanying software. Appropriate filter cube sets were used for TAMRA (Chroma 31004; DM 595 nm, BP 520–600 nm), Cascade Blue dextran and DAPI (Olympus 31009; DM 400 nm, BP 320–400 nm) visualization. To quantify the number of neurons internalizing CRF-TAMRA **1**, monochromatic grayscale images were taken of every tenth section (200 μ m intervals) extending from the most rostral portion of the medulla to the obex at 20 $\times$ magnification. Parameters for signal gain, gamma, and exposure time for image acquisition were identical across all animals. Image J (NIH) was used to count the number of neurons that showed CRF-TAMRA **1** internalization. A neuron was counted as CRF-TAMRA **1** positive if at least 10 fluorescent endosomal units were visible within a DAPI-stained nucleated neuron or a clearly defined cell body in the case of RS neurons retrogradely labeled with Cascade Blue dextran. An endosome was defined as a spot of fluorescence greater than 0.1 μ m but less than 0.5 μ m in diameter. These values are consistent with other studies in which endosomes have been counted for quantification purposes (Lewis et al., 2004; Lutz et al., 1991; Hubbard et al., 2009). Cell counting was accomplished by overlaying each image with a 300 $\times$ 300 μ m grid directly over the midline of the tissue section using Adobe Photoshop (v. 6.0). Only cells meeting the above criteria that fell within this grid were counted. Statistical analysis on the numbers of neurons showing CRF-TAMRA **1** internalization was conducted using one-way analysis of variance (ANOVAs) and Tukey's HSD post-hoc tests ($\alpha = .05$; two-tailed).

Additionally, a Bio-Rad laser-scanning confocal microscope with accompanying Bio-Rad Laser 2100 software, provided by The University of Wyoming Microscopy Core Facility, was used to capture high-resolution images of RS and non-RS neurons displaying internalization of the CRF-TAMRA **1** conjugate, and to visualize CRF-TAMRA **1** internalization in serotonin immunoreactive (-ir) neurons. Sections were scanned using appropriate laser settings corresponding to the excitation wavelengths for TAMRA (green He/Ne laser, ex: 658 nm), Alexa 488 (red argon laser, ex: 488 nm), Cascade Blue dextran and DAPI (blue diode laser, ex: 405 nm) visualization with an optical thickness of 2 μ m. All sections were scanned a total of 6 times using Kalman filtering and in some cases, z-stacks were generated from a single section through the MRF using 2 μ m steps resulting in a total of 10 images per stack.

Results

CRF Targets Reticulospinal and Non-Reticulospinal Neurons in the MRF

Consistent with our previous findings (Hubbard et al., 2009) a 30 min exposure to CRF-TAMRA **1** resulted in a high degree of fluorescence internalization into DAPI-labeled MRF neurons following icv conjugate administration. CRF-TAMRA **1** was internalized into somata as well as primary and secondary dendrites of morphologically diverse neurons distributed throughout the rostrocaudal MRF. The majority of DAPI-labeled neurons internalizing CRF-TAMRA **1** were located in the perikaryal zone of the MRF, just ventral to the ependymal layer of the fourth ventricle. Most notable, however, was the internalization of the CRF-TAMRA **1** conjugate into large DAPI-labeled medullary neurons (≥ 30 μ m in diameter) lying deep within the neuropil of the medial and paramedial MRF, a region known to contain a high density of axon terminals, en passant fiber tracts, and extensive dendritic arborizations. Statistical analysis conducted on cell counts for the number of DAPI-labeled neurons internalizing CRF-TAMRA **1** (One-way ANOVA, $P > 0.05$) across rostral and caudal sections of the medulla revealed no significant differences. Thus, cell counts across all sections for each animal were combined and the total number of DAPI-labeled neurons with and without CRF-TAMRA **1** internalization was quantified across animals. Results obtained from cell counts revealed that

33.9% (1246/3679) of DAPI-labeled neurons in the MRF showed internalization of CRF-TAMRA **1** conjugate, whereas the other 66.1% (2433/3679) showed no evidence of CRF-TAMRA **1** internalization (data not shown).

A major impetus for this study was to determine if RS neurons, known to play a key role in regulating locomotion, were targets for CRF actions. To directly test this hypothesis, RS neurons were identified with a retrograde tracer and subsequently labeled with CRF-TAMRA **1** following icv conjugate administration in behaving newts. As expected, a heterogeneous MRF neuronal population internalized CRF-TAMRA **1** suggesting actions of this peptide on diverse neurons in the brainstem MRF. Neuronal targets of CRF-TAMRA **1** included a significant proportion of retrogradely labeled RS neurons (19%; 47/253) as well as large numbers of non-RS medullary neurons (47%; 119/253). The remaining neurons (34%; 87/253) were retrogradely-labeled RS neurons that showed no CRF-TAMRA **1** internalization. Retrogradely labeled RS neurons internalizing CRF-TAMRA **1** were located predominantly ipsilateral to the spinal cord hemisection where the fluorescent tracer was applied, although some contralateral labeling was seen in more rostrally placed sections. Identified RS neurons internalizing CRF-TAMRA **1** were principally found within the perikaryal zone, but in some cases, these neurons were also seen within the MRF neuropil. In general, punctate CRF-TAMRA **1** internalized fluorescence was seen dispersed throughout the somata and proximal dendrites of RS and non-RS neurons in the medial and paramedial regions of the MRF (Fig. 1 and Fig. 2). RS neurons showing CRF-TAMRA **1** internalization were typically clustered together with non-RS neurons also showing internalization of the conjugate as well as RS neurons without CRF-TAMRA **1** internalization (Fig. 2). Many of the paramedially placed RS neurons had long dendritic projections extending ventrolaterally deep with the MRF neuropil (Fig. 1f and Fig. 2g). In general, cell nuclei were devoid of CRF-TAMRA **1** internalization, although on some occasions, diffuse nuclear labeling was evident after 30 min of exposure to the conjugate in a small portion of CRF-TAMRA **1** labeled neurons (Hubbard et al., 2009). In addition, we saw no clear evidence of CRF-TAMRA **1** internalization into retrogradely labeled RS axons located within the medial longitudinal fasciculus (MLF) or in any other axons located in the neuropil.

Serotonin-Containing Neurons in the Medullary Raphé Internalized CRF-TAMRA **1**

Intraventricular CRF-TAMRA **1** administration resulted in punctate fluorescent labeling within a distinct subset of serotonin-ir neurons located throughout the caudal medullary raphé. At 30 min after icv CRF-TAMRA **1** administration, conjugate internalization was principally located in the soma and primary dendrites of serotonin-ir neurons. These neurons had cell bodies located in the perikaryal regions of the medullary raphé with long dendrites reaching ventrally (Fig. 3). CRF-TAMRA **1** fluorescence was also internalized into non-serotonin containing neurons located in the paramedial zone of the MRF. It is noteworthy that many of these latter cell types were found in close proximity to each other, often surrounding serotonin-ir raphé neurons.

Neurophysiological and Behavioral Effects of CRF-TAMRA **1**

A total of 54 MRF neurons was recorded from 15 newts in the CRF-TAMRA **1** (n = 14 neurons), unconjugated CRF (n = 14 neurons), VEH (n = 12 neurons), and TAMRA (n = 14 neurons) treatment conditions (Fig. 4a). Change-Point Tests conducted on pre- and post-treatment mean firing rates across these four conditions showed that central CRF-TAMRA **1** and unconjugated CRF administration significantly altered firing of the majority of neurons sampled. Specifically, identical numbers of neurons showed significant changes (increase or decrease) in firing following icv CRF-TAMRA **1** (71.3%, 10/14) or an equimolar dose of CRF (71.3%, 10/14) compared to VEH (33.3%, 4/12) or TAMRA (21.4%, 3/14) control conditions. For neurons showing significant changes in activity as determined by the Change-Point Test,

further examination of the direction (increase or decrease) of change was conducted for each unit. Analysis revealed that 64.3% (9/14) of these neurons increased firing following icv CRF-TAMRA **1** compared to 57.1% (8/14) after administration of an equimolar dose of CRF, demonstrating that the conjugate acted in a functionally similar manner as unconjugated CRF (Fig. 4b). Moreover, a significantly larger proportion of neurons showed increases in their firing rate following CRF-TAMRA **1** or unlabeled CRF administration compared to VEH (25%; 3/12) and TAMRA (21.4%; 3/14) conditions (Fig. 4b). The remaining units either decreased [CRF-TAMRA **1**: 7.1% (1/14) vs. CRF: 14.3% (2/14) vs. VEH: 8.3% (1/12) vs. TAMRA: 0% (0/14)] their firing rate or did not change [CRF-TAMRA **1**: 28.6% (4/14) vs. CRF: 28.6% (4/14) vs. VEH: 66.7% (8/12) vs. TAMRA: 78.6% (11/14)] their activity following treatment (Fig. 4b).

The excitatory effect of both CRF-TAMRA **1** and CRF on medullary neuronal firing was extremely rapid, with mean latencies for change occurring 8–9 min after icv administration (Fig. 5), a time course typical of G protein-coupled receptor signaling and receptor-mediated endocytosis (Berry et al., 1996; Mantyh et al., 1995; Perry et al., 2005; Rasmussen et al., 2004). Fisher's Exact Test on the proportion of units exhibiting significant changes (increase or decrease) in mean frequency of firing compared to those showing no change revealed no significant difference between CRF-TAMRA **1** and unconjugated CRF treatments. Likewise, Fisher's Exact Test showed no significant differences between proportions of units that changed activity compared to those without change in the VEH (28.6%) vs. TAMRA (21.4%) treatment conditions. Consequently, data from CRF-TAMRA **1** and unlabeled CRF conditions were combined as were data from VEH and TAMRA conditions. The resulting two groups were compared using a 2×2 Chi Square test, which showed that CRF-treated newts had a significantly greater proportion of neurons that displayed changes in firing rate compared to the combined VEH and TAMRA control animals (20/28, 71.4% vs. 7/26, 26.9%; $\chi^2 = 10.68$, $df = 1$, $P = 0.001$).

A one-way ANOVA on difference scores for the mean number of locomotor bouts revealed a significant effect across the four treatment conditions [$F(df = 3, 11) = 11.04$, $P = 0.001$]. Tukey post-hoc tests showed that the mean number of locomotor bouts significantly increased in groups of animals receiving icv CRF-TAMRA **1** (mean = 47.75 ± 4.53) or an equimolar dose of CRF (mean = 38.75 ± 11.09) compared to groups of animals treated with VEH (mean = 3.25 ± 3.25) or the TAMRA dye (mean = 6.33 ± 3.28) alone, suggesting that CRF-TAMRA **1** is acting like the native CRF peptide, in its capacity to stimulate locomotion (Fig. 6).

Discussion

Prior research on CRF-enhanced locomotion has focused primarily on ascending neuromodulatory systems, originating from rostral brainstem structures. In contrast, little research has investigated CRF effects on descending locomotor control systems, such as the caudal brainstem reticulospinal system. Accordingly, a primary goal of this study was to determine if MRF neurons, including RS neurons were targets for CRF actions. In addition, we sought to determine if serotonin neurons, previously implicated in ascending neural effects of CRF (Clements et al., 2003; Kirby et al., 2000; Lowry et al., 2000; Price et al., 1998; Summers et al., 2003), were also targets for CRF in the hindbrain. To accomplish these objectives, we utilized our recently developed, fluorescent CRF-TAMRA **1** conjugate (Hubbard et al., 2009) as a tool for tracking CRF internalization into RS and other raphe neurons in the hindbrain MRF of the roughskin newt, *T. granulosa*. We also used chronic single unit recordings to examine the functional changes induced by icv CRF-TAMRA **1** or unconjugated CRF administration on MRF neuronal populations while concurrently monitoring locomotor behavior. Our results showed CRF-TAMRA **1** internalization by a widespread MRF neuronal population, including a substantial number of retrogradely labeled RS neurons. Furthermore,

we identified serotonin-ir neurons in the medullary raphé of the medial MRF, that also internalized CRF. Finally, icv CRF-TAMRA **1** significantly increased locomotion and changed medullary neuronal firing, primarily increasing it, at a similar time course and dosage as unconjugated CRF. These new findings suggest that a direct CRF effect on hindbrain MRF neurons may underlie the peptide's behavioral activating effects in addition to likely CRF effects on more rostral brain systems.

The hindbrain MRF targets for CRF were diverse neuronal phenotypes, including a significant number of retrogradely labeled RS neurons. RS neurons displaying CRF-TAMRA **1** internalization were typically clustered in the paramedial region of the rostral MRF, but were also found medially, deep within the neuropil of the caudal medullary raphé. In addition to CRF-internalizing RS neurons, a large number of unidentified non-RS neurons also displayed CRF-TAMRA **1** internalization. These results are in line with previous studies demonstrating CRF receptor expression and CRF-like immunoreactivity in somata and dendrites of diverse neuronal populations in the mammalian MRF (Chalmers et al., 1995; Cummings et al., 1983; De Souza et al., 1985; Milner et al., 1993; Potter et al., 1994; Sakanaka et al., 1986). However, contrary to previous reports by Milner et al. (1993) in the rostral ventrolateral medulla of the rat hindbrain we saw no clear evidence of CRF-TAMRA **1** labeling in axonal processes or terminal varicosities in the rostromedial medulla of the newt. Although the latter could not be determined due to an inability to resolve axon terminal ultrastructure with confocal microscopy, the former may have been attributable to the different animal models and brain regions investigated.

Immunohistochemistry revealed that serotonin-containing neurons in the medullary raphé of the MRF were also targets for CRF's action. Visualization of CRF-TAMRA **1** internalization using confocal microscopy demonstrated that fluorescent CRF-TAMRA **1** puncta were largely distributed throughout the soma and primary dendrites of these medially placed serotonin-containing neurons. These data indicate that CRF is directly targeting serotonergic neurons in the medullary raphé, raising the possibility that CRF effects on hindbrain serotonergic neurons are involved in the peptide's locomotor activation. However, our findings cannot address the question of whether these CRF-internalizing, serotonergic neurons project to the spinal cord, although many serotonergic hindbrain neurons are known to project spinally (Davis et al., 1989; Faslo et al., 1986; Kwait and Basbaum, 1992; Sims, 1977). Future studies could investigate this possibility by utilizing the CRF-TAMRA **1** probe to track CRF internalization into retrogradely labeled reticulospinal neurons within the medullary raphé in combination with serotonin-immunohistochemistry.

Consistent with previous findings with CRF from this laboratory (Lowry et al., 1996; Rose et al., 1996), rostromedullary neurons displayed diverse activity changes, principally increases, but also decreases, following icv CRF-TAMRA **1**. Increased MRF neuronal firing was cyclic, as were episodes of increased locomotion. The neuronal effects of CRF-TAMRA **1** were comparable in pattern, magnitude and time course to rostromedial MRF neurons recorded in newts administered higher doses of unconjugated CRF (Lowry et al., 1996), indicating that CRF-TAMRA **1** acted similarly in its capacity to potentiate locomotor-related neuronal firing in the newt MRF as the unlabeled peptide. An important point regarding fluctuations of increased MRF neuronal firing is that the onset of activity slightly anticipated and temporally overlapped bouts of locomotion. A second significant point is that while increases in MRF neuronal activity were closely associated with bouts of locomotion, MRF neuronal firing was not rhythmically time-locked with specific stepping movements. These two observations argue that this increase in firing was not secondary to locomotion-related feedback from sensory neurons or from the spinal stepping CPG. Moreover, the fact that CRF-TAMRA **1** induced increases in MRF neuronal firing often preceded the onset of locomotion and was not synchronized with specific stepping movements strongly suggests that CRF is critical to the

initiation of locomotor behavior, but not necessary for the actual expression of rhythmic locomotion. Lastly, centrally administered CRF-TAMRA **1** or an equivalent dose of CRF induced a rapid and robust enhancement in locomotor activity in *Taricha*, an effect not observed in VEH- or TAMRA-treated newts. The rapid onset of the neurophysiological effect of CRF-TAMRA **1** is significant because it provides a better temporal marker for conjugate action than is attainable with histological information alone. In addition, the neurophysiological data suggest that the early stages of internalization are sufficient for initiation of the neuronal processes leading to CRF's behavioral effect. These data match our previous demonstration of the specificity of CRF-TAMRA **1** actions on locomotor behavior in the newt, providing further evidence that CRF-TAMRA **1** conjugate has functionally similar neurobehavioral effects at equivalent doses to the unlabeled CRF peptide (Hubbard et al., 2009).

Conclusion

The present study provides the first evidence that CRF is directly targeting RS and medullary raphe serotonergic neurons in the caudal brainstem MRF. Although prior research on neurobehavioral actions of CRF in vertebrates has focused primarily on rostral brainstem neurons, particularly serotonergic and noradrenergic circuitry and their ascending connections, a direct CRF action on the hindbrain RS system has received less attention. Given the well-established role of RS neurons in the initiation and expression of rhythmic locomotion in vertebrates and the fact that RS neurons and serotonin-containing neurons in the medullary raphe are targets for CRF in the newt MRF, it stands to reason that CRF action on the hindbrain RS-raphé motor system is a likely mechanism by which this peptide simulates locomotion. In summary, use of our CRF-TAMRA **1** probe, coupled with recordings of MRF neuronal activity during CRF-induced locomotor activation, have yielded novel information implicating a direct action of CRF on hindbrain neurons, including reticulospinal and serotonergic raphe cells as causal factors in the behavioral activating effects of CRF in the roughskin newt.

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References

- Berry SA, Shah MC, Khan N, Roth BL. Rapid agonist-induced internalization of the 5-Hydroxytryptamine_{2A} receptor occurs via the endosome pathway *in vitro*. *Mol. Pharmacol* 1996;50:306–313. [PubMed: 8700138]
- Britton KT, Lee G, Dana R, Risch SC, Koob GF. Corticotropin-releasing factor (CRF) receptor antagonist blocks activating and 'anxiogenic' actions of CRF in the rat brain. *Brain Res* 1986;369:303–306. [PubMed: 3008937]
- Britton DR, Varela M, Garcia A, Rosenthal M. Dexamethasone suppresses pituitary-adrenal but not behavioral effects of centrally administered CRF. *Life Sci* 1985;38:211–216. [PubMed: 3001465]
- Brodin L, Grillner S, Dubuc R, Ohta Y, Kasicki S, Hokfelt T. Reticulospinal neurons in lamprey: Transmitters, synaptic interactions and their role during locomotion. *Archiv. Ital. Biol* 1988;126:317–345.
- Carpenter RE, Watt MJ, Forster GL, Øverli Ø, Bockholt C, Renner KJ, Summers CH. Corticotropin-releasing factor induces anxiogenic locomotion in trout and alters serotonergic and dopaminergic activity. *Horm. Behav* 2007;52:600–611. [PubMed: 17826776]

- Chalmers DT, Lovenberg TW, De Souza EB. Localization of novel corticotrophin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J Neurosci* 1995;15:6340–6350. [PubMed: 7472399]
- Clements S, Larsen DA, Dickhoff WW, Schreck CB. Central administration of corticotropin-releasing hormone stimulates locomotor activity in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). *Gen. Comp. Endocrinol* 2002;125:319–327. [PubMed: 11884077]
- Clement S, Moore FL, Schreck CB. Evidence that acute serotonergic activation potentiates the locomotor-stimulating effects of corticotropin-releasing hormone in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) (small star, filled). *Horm. Behav* 2003;43:214–221. [PubMed: 12614652]
- Crespi EJ, Denver RJ. Ontogeny of corticotropin-releasing factor effects on locomotion and foraging in the Western spadefoot toad (*Spea hammondi*). *Horm. Behav* 2004;46:399–410. [PubMed: 15465525]
- Cummings S, Elde R, Ellis J, Lindall A. Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat: an immunohistochemical study. *J. Neurosci* 1983;3:1355–1368. [PubMed: 6345725]
- Davis BM, Duffy MT, Simpson SB. Bulbospinal and intraspinal connections in normal and regenerated salamander spinal cord. *Exp. Neurol* 1989;103:41–51. [PubMed: 2912749]
- De Souza EB, Insel TR, Perrin MH, Rivier J, Vale WW, Kuhar MJ. Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: An autoradiographic study. *J. Neurosci* 1985;5:3189–3201. [PubMed: 3001239]
- Dubuc R, Grillner S. The role of spinal cord inputs in modulating the activity of reticulospinal neurons during fictive locomotion in the lamprey. *Brain Res* 1989;483:196–200. [PubMed: 2650805]
- Eaves M, Thatcher-Britton K, Rivier J, Vale W, Koob GF. Effects of corticotropin-releasing factor on locomotor activity of hypophysectomized rats. *Peptides* 1985;6:923–926. [PubMed: 3878503]
- Faslo A, Franzoni MF, Gaudino G, Steinbusch HWM. The organization of serotonin-immunoreactive neuronal systems in the brain of the crested newt, *Triturus cristatus carnifex* Laur. *Cell Tiss. Res* 1986;243:239–247.
- Grillner S, Deliagina T, Ekeberg O, El Manira A, Hill RH, Lansner A, Orlovsky GN, Wallen P. Neural networks that co-ordinate locomotion and body orientation in lamprey. *Trends Neurosci* 1995;18:270–279. [PubMed: 7571002]
- Grillner S, Wallen P. On the cellular bases of vertebrate locomotion. *Prog. Brain Res* 1999;123:297–309. [PubMed: 10635725]
- Hubbard CS, Dolence EK, Shires JA, Rose JD. Identification of brain target neurons using a fluorescent conjugate of corticotropin-releasing factor. *J. Chem. Neuroanat* 2009;37:245–253. [PubMed: 19481009]
- Kirby LG, Rice KC, Valentino RJ. Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. *Neuropsychopharmacology* 2000;22(2):148–162. [PubMed: 10649828]
- Kwait GC, Basbaum AI. Origin of brainstem noradrenergic and serotonergic projections to the spinal cord dorsal horn of the rat. *Somatosens. Mot. Res* 1992;66:235–251.
- Lewis CM, Dolence EK, Zhang Z, Rose JD. Fluorescent vasotocin conjugate for the identification of the target cells for brain actions of vasotocin. *Bioconjug. Chem* 2004;15:909–914. [PubMed: 15264881]
- Lowry CA, Deviche P, Moore FL. Effects of corticotropin-releasing factor (CRF) and opiates on amphibian locomotion. *Brain Res* 1990;513:94–100. [PubMed: 2161698]
- Lowry CA, Kedzie KA, Renner KJ, Moore FL. Flouxetine potentiates the effects of corticotropin-releasing factor on locomotor activity and the serotonergic system. *Soc. Neurosci. Abstr* 1993;19:175.
- Lowry CA, Moore FL. Corticotropin-releasing factor (CRF) antagonist suppresses stress-induced locomotor activity in an amphibian. *Horm. Behav* 1991;25:84–96. [PubMed: 1646155]
- Lowry CA, Moore FL. Regulation of behavioral responses by corticotropin-releasing factor. *Gen. Comp. Endocrinol* 2006;146:19–27. [PubMed: 16426606]
- Lowry CA, Renner KJ, Laughlin LM, Moore FL. CRF alters monoamine and monoamine metabolite levels in discrete motor areas in the amphibian brain. *Soc. Neurosci. Abstr* 1991;17:1418.
- Lowry CA, Rose JD, Moore FL. Corticotropin-releasing factor enhances locomotion and medullary neuronal firing in an amphibian. *Horm. Behav* 1996;30:50–59. [PubMed: 8724179]

- Lowry CA, Rodda JA, Lightman ST, Ingram CD. Corticotropin-releasing factor increases in vitro firing rates of serotonergic neurons in the rat dorsal raphe nucleus: evidence for activation of a topographically organized mesolimbocortical serotonergic system. *J. Neurosci* 2000;20:7728–7736. [PubMed: 11027235]
- Lutz W, Salisbury JL, Kumar R. Vasopressin receptor-mediated endocytosis: current view. *Am. J. Physiol* 1991;261:F1–F13. [PubMed: 1650143]
- Mantyh PW, Allen CJ, Ghilardi JR, Rogers SD, Mantyh CR, Lius H, Basbaums AI, Vigna SR, Maggio JE. Rapid endocytosis of a G protein-coupled receptor: Substance P-evoked internalization of its receptor in the rat striatum *in vivo*. *Proc. Natl. Acad. Sci. U S A* 1995;92:2622–2626. [PubMed: 7535928]
- Milner TA, Reis DJ, Pickel VM, Aicher SA, Giuliano R. Ultrastructural localization of afferent sources of corticotropin-releasing factor in the rat rostral ventrolateral medulla: Implications for central cardiovascular regulation. *J. Comp. Neurol* 1993;333:151–167. [PubMed: 7688383]
- Moore FL, Roberts J, Bevers J. Corticotropin-releasing factor (CRF) stimulates locomotor activity in intact and hypophysectomized newts (amphibia). *J. Exp. Zool* 1984;31:331–333. [PubMed: 6334126]
- Perry SJ, Junger S, Kohout TA, Hoare SRJ, Struthers RS, Grigoriadis DE, Maki RA. Distinct conformations of the corticotropin releasing factor type 1 receptor adopted following agonist and antagonist binding are differentially regulated. *J. Biol. Chem* 2005;280:11560–11568. [PubMed: 15653688]
- Potter E, Sutton S, Donaldson C, Chen R, Perrin M, Lewis K, Sawchenko PE, Vale W. Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. *Proc. Natl. Acad. Sci. USA* 1994;91:8777–8781. [PubMed: 8090722]
- Price ML, Curtis AL, Kirby LG, Valentino RJ, Lucki I. Effects of corticotrophin-releasing factor on brain serotonergic activity. *Neuropsychopharmacology* 1998;18(6):492–502. [PubMed: 9571657]
- Rasmussen TN, Novak N, Nielsen SM. Internalization of the human CRF receptor 1 is independent of classical phosphorylation sites and of β -arrestin 1 recruitment. *Eur. J. Biochem* 2004;271:4366–4374. [PubMed: 15560778]
- Rose JD, Marrs G, Moore FL. CRF alters corticosterone effects on medullary sensorimotor integration and neural excitability. *Soc. Neurosci. Abstr* 1996;22:1149.
- Rose JD, Marrs GS, Moore FL. Rapid, corticosterone-induced disruption of medullary sensorimotor integration related to suppression of amplexic clasping in behaving roughskin newts (*Taricha granulosa*). *Horm. Behav* 1998;34:268–282. [PubMed: 9878276]
- Rossignol S, Dubuc R. Spinal pattern generation. *Curr. Opin. Neurol* 1994;4:894–902.
- Sakanaka M, Shibasaki T, Lederis K. Corticotropin releasing factor-like immunoreactivity in the rat brain as revealed by modified cobalt-glucose oxidase-diaminobenzidine method. *J. Comp. Neurol* 1986;260:256–298. [PubMed: 3497182]
- Sims TJ. The development of monoamine-containing neurons in the brain and spinal cord of the salamander, *Ambystoma mexicanum*. *J. Comp. Neurol* 1977;173:319–335. [PubMed: 856887]
- Summers CH, Kampshoff JL, Ronan PJ, Lowry CA, Prestbo AA, Korzan WJ. Monoaminergic activity in subregions of raphe nuclei elicited by prior stress and the neuropeptide corticotropin-releasing factor. *J. Neuroendocrinol* 2003;15:1122–1133. [PubMed: 14636174]
- Sutton RE, Koob GF, Le Moal M, Rivier J, Vale W. Corticotropin-releasing factor produces behavioural activation in rats. *Nature* 1982;294:331–333. [PubMed: 6978997]
- Tazi A, Swerdlow NR, LeMoal M, Rivier J, Vale W, Koob GF. Behavioral activation by CRF: Evidence for the involvement of the ventral forebrain. *Life Sci* 1987;41:41–49. [PubMed: 3496511]
- Valentino RJ, Foote SL, Page ME. The locus coeruleus as a site for integrating corticotropin-releasing factor and noradrenergic mediation of stress responses. *Ann. NY Acad. Sci* 1993;697:173–188. [PubMed: 7903030]
- Winslow JT, Newman JD, Insel TR. CRH and α -Helical-CRH modulate behavioral measures of arousal in monkeys. *Pharm. Behav* 1989;32:919–926.

Figure 1.

A confocal z-stack image (A–C) of a retrogradely labeled reticulospinal neuron (RS) neuron in the paramedial medullary reticular formation (MRF) displaying CRF-TAMRA **1** internalization. A: Retrograde labeling with Cascade Blue (CB) dextran. B: CRF-TAMRA **1** internalization. C: Overlay of images A and B with pseudocolors blue designating Cascade Blue dextran and red designating CRF-TAMRA **1**. D: High resolution image of a single optical scan illustrating the typical punctate appearance of CRF-TAMRA **1** internalization in the soma and proximal dendrites. This punctate internalization is consistent with receptor-mediated endocytosis. E: Diagram of a transverse section through the caudal medulla corresponding to the level from which images A–C were taken. Insert is a reverse-contrast image showing location of the neuron. F: Reverse-contrast confocal image showing a large retrogradely labeled RS neurons in the paramedial MRF displaying CRF-TAMRA **1** internalization and sending a long dendritic projection terminating in the medullary raphe. G: Diagram of the newt brainstem

depicting a transverse section through the caudal medulla and an insert containing the reverse-contrast image of the neurons shown in F. Scale bars: (A–C) 50 μ m.

Figure 2.

Confocal images of retrogradely-labeled RS and non-RS neurons in the medial and paramedial MRF that did and did not internalize CRF-TAMRA 1. A: RS neurons in the medullary raphe retrogradely labeled with Cascade Blue dextran (blue). B: CRF-TAMRA 1 internalization (red) in medullary raphe neurons. C: Overlay of images A and B illustrating RS neurons that did (solid white arrows) and did not internalize (broken white arrow) CRF-TAMRA 1. D: Diagram depicting the level at which images A and B were taken with insert of a reverse-contrast epifluorescent image of same section taken at a lower resolution. E: Retrogradely labeled RS neurons (blue). F: Medullary neurons internalizing CRF-TAMRA 1 (red). G: Overlay of images E and F illustrating RS and non-RS neurons that internalized CRF-TAMRA 1

conjugate. H: Diagram showing the level at which images E–F were taken. Insert illustrating a reverse-contrast confocal image of same section shown in image E. Scale bars: (A–C) 30 μm , (E–G) 50 μm .

Figure 3.

Confocal microscopic images illustrating CRF-TAMRA **1** internalization by serotonin-ir medullary raphe neurons. Left (A–D) and right (E–H) panels depict DAPI-labeled (A, E) and serotonin-ir neurons (B, F) clustered along the midline, some of which showed CRF-TAMRA **1** internalization in somata and proximal dendrites (C and G). D and H represent the overlays of images A–C and E–G, respectively. Scale bars: (A–H) 50 μ m.

Figure 4.

A: Diagram of the newt brainstem showing recording sites in the MRF (54 neurons; 15 sites) and an example of a typical neuron's spike waveform. B: The percentage of neurons that showed increases, decreases, or no change in mean frequency firing across the CRF-TAMRA 1, CRF, VEH or TAMRA treatment conditions. Vertical scale bar: (A) 50 μ V.

Figure 5.

Firing rate records from representative MRF neurons, one (A) recorded 5 min prior to- (pre-treatment) and 30 min after (post-treatment) icv CRF-TAMRA **1** and a second (B) recorded at similar times before and after icv unconjugated CRF. CRF-TAMRA **1** significantly enhanced mean frequency (Hz) firing and concurrently increased locomotion activity (black bars), a pattern similar to that seen following the equimolar icv dose of CRF.

Figure 6.

Mean ($\pm$ SEM) difference scores illustrating the magnitude of change for the number of locomotor bouts across the CRF-TAMRA 1, CRF, VEH and TAMRA treatment conditions. Intraventricular administration of CRF-TAMRA 1 or equimolar dose of unconjugated CRF significantly increased locomotion compared to VEH- or TAMRA-treated newts.