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Brainstem Neuronal and Behavioral Activation by Corticotropin-Releasing Hormone Depend on the Behavioral State of the Animal

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Abstract

Central administration of corticotropin-releasing hormone (CRH) is known to enhance locomotion across a wide range of vertebrates, including the roughskin newt, *Taricha granulosa*. The present study aimed to identify the CRH effects on locomotor-controlling medullary neurons that underlie the peptide's behavioral stimulating actions. Single neurons were recorded from the rostral medullary reticular formation before and after intraventricular infusion of CRH in freely behaving newts and newts paralyzed with a myoneural blocking agent. In behaving newts, most medullary neurons showed increased firing 3–23 min after CRH infusion. Decreases in firing were less common. Of particular importance was the finding that in behaving newts, medullary neurons showed a cyclic firing pattern that was strongly associated with an increase in the incidence of walking bouts, an effect blocked by pretreatment with the CRH antagonist, alpha-helical CRH and not seen following vehicle administration. In contrast, the majority of medullary neurons sampled in immobilized newts lacked temporal cyclicity in their firing patterns following intraventricular infusion of CRH. That is, there was no evidence for a fictive locomotor activity pattern. Our results indicate that the actual expression of locomotion is a critical factor in regulating the behavior-activating effects of CRH and underscore the importance of using an awake, unrestrained animal for analysis of a hormone's neurobehavioral actions.

Keywords

in vivo recordings; hormone actions; locomotion; rostral hindbrain; medullary neurons

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Introduction

A broad range of research has shown that corticotropin-releasing hormone (CRH) is involved in regulating behavioral responses to stressors in diverse vertebrate taxa, including mammals, birds, amphibians and fishes (reviewed by Lowry and Moore, 2006). CRH has been implicated in fear and anxiety-like behaviors in rats (Abrams et al., 2005) and psychopathology of anxiety and depression in humans (Binder and Nemeroff, 2010). Suppression of feeding by CRH has been observed in fish (Bernier and Peter, 2001; Volkoff et al., 2005), amphibians (Crespi and Denver, 2004; Crespi et al., 2004), birds (Denbow et al., 1999; Furuse et al., 1997; Ohgushi et al., 2001; Richardson et al., 2000; Zhang et al., 2001a, b) and mammals (reviewed in Dunn and Berridge, 1990). CRH also decreases reproductive behaviors in birds (Maney and Wingfield, 1998) and rats (Sirinathsinghji, 1985; Sirinathsinghji, 1987).

One of the most pronounced and reliable behavioral effects of CRH is stimulation of locomotion. Stress-induced release or central administration of CRH has potent locomotor-enhancing properties across a wide range of vertebrates including the roughskin newt, *Taricha granulosa* (Crespi and Denver, 2004; Dunn and Berridge, 1990; Lowry and Moore, 2006; Moore et al., 1984). Previous behavioral investigations of rodents, teleost fish, and *Taricha*, showed that intracerebroventricular (icv) CRH administration results in a rapid and dose-related increase in locomotion, an effect blocked by central administration of the CRH receptor antagonist, alpha-helical CRH (ahCRH) (Britton et al., 1986; Carpenter et al., 2007; Clements et al., 2002; Lowry et al., 1990; Lowry and Moore, 1991; Lowry et al., 1996). In addition, CRH-enhanced locomotion depends on numerous ethologically relevant and species-specific contextual factors. These include the prevailing physiological and behavioral state of the animal, as well as its interactions with external cues and stressors imposed by its environment including conspecifics, social hierarchies, and predator-prey relationships (Carpenter et al., 2009; Carr et al., 2002; Dunn and Berridge, 1990; Lowry and Moore, 2006; Strome et al., 2002). Environmental factors, such as an animal's housing conditions and the novelty of the environment in which the experimental stressor occurs or CRH is administered, also have been shown to differentially effect locomotor and arousal-related behaviors in a variety of vertebrates including fish, amphibians, rodents, and non-human primates (Britton et al., 1982; Clements and Schreck 2004; Lowry and Moore, 2006; Strome et al., 2002; Sutton et al., 1982). For example, icv administered CRH stimulates locomotor activity in rats tested in a familiar environment (e.g., animal's home cage), whereas in a novel, unfamiliar environment, such as represented by an open field arena, CRH administration results in decreased locomotion (Britton et al., 1982; Sutton et al., 1982). Similarly, in juvenile chinook salmon (*Oncorhynchus tshawytscha*), centrally administered CRH (icv) has been shown to increase downstream movement in a simulated saltwater stream while decreasing movement into a novel environment (a trap entered by a narrow opening) (Clements and Schreck, 2004). Another example of context-dependent CRH effects is the fact that this peptide's actions depend upon an animal's current behavioral state. A few studies conducted in non-human primates have demonstrated differential behavioral and neuroendocrine effects of CRH administration in rhesus monkeys allowed to freely roam in their home cage compared to chair-restrained monkeys (Kalin et

al., 1983a, b) or monkeys housed socially with conspecifics versus those housed in isolation (Strome et al., 2002). Thus, the neural actions of CRH that mediate the peptide's behavioral effects are likely to be observable and understandable only when viewed in a realistic and appropriate functional context.

While the locomotion-enhancing neural actions of CRH are poorly understood, there is a great deal known about the neural control of locomotion, per se, making this pronounced behavioral effect of CRH especially amenable to a mechanistic neurophysiological analysis. The rhythmic pattern of vertebrate locomotion is generated within the spinal cord, by a network of interconnected motor neurons and interneurons, known as the locomotor central pattern generator (Grillner, 1981; Grillner and Wallén, 1985; Rossignol and Dubuc, 1994). Although the rhythmic pattern of locomotion is generated spinally, the initiation and speed of locomotion are under descending control by a heterogeneous group of reticulospinal (RS) neurons and interneurons within the medullary reticular formation (Grillner, 1981; Drew and Rossignol, 1984; Drew et al., 1986).

Much of what is currently known about the neural control of locomotion comes from *in vitro* electrophysiological experiments conducted in semi-intact fish or amphibian preparations where fictive locomotion can be evoked, or *in vivo* studies of partially restrained, anesthetized or pharmacologically immobilized mammals. Although techniques for recording in awake, unrestrained animals have been available for many years (Deliagina et al., 2000; Drew et al., 1986; Rose, 1986; Rose and Weishaar, 1979; Trulson and Jacobs, 1979), reduced, partially-restrained, or immobilized preparations have been more commonly used due to the greater technical control and ease with which recordings can be obtained. However, this convenient simplicity comes at the cost of losing completely naturalistic information in regard to the behavior under investigation, which can lead to contradictory findings and in some instances, even spurious results (McGinty, 1973; Steriade, 2001; Trulson, 1984). Locomotion, like any behavior, is dynamic and relies on integration of incoming sensory input and motor feedback from both central and peripheral afferents. Moreover, the functional properties of neurons have immense plasticity, which is only evident when an animal is displaying the behavior of interest (Rose, 1986; Steriade, 2001).

To fully understand how a hormone such as CRH acts on neurons to affect behavior, it is imperative that investigations be conducted in the fully intact and behaving animal. Electrophysiological recording in freely-behaving animals has been used effectively in this laboratory to identify behavior-controlling actions of gonadal and corticosteroid hormones (Rose, 1986, 1992; Rose et al., 1998). In an earlier study, we determined that central CRH evokes diverse changes in activity, notably cyclic increases in firing, of neurons in the rostromedial medullary reticular formation (rMRF) that are temporally linked with locomotor activation in roughskin newts (Lowry et al., 1996). Subsequently, we determined that many medullary neurons are targets for CRH (Hubbard et al., 2009, 2010). In the present study, in order to identify CRH effects that were likely to be causal in locomotor activation, we compared the brainstem neural effects of central CRH in behaving newts with the effects in pharmacologically-immobilized newts. In this way, we were able to determine whether the cyclic increases in rMRF neuronal activity, which appeared to be causally related to episodes of locomotion in our previous study, were an obligatory neural

consequence of CRH or whether the behavioral state of the animal (mobile or immobilized) was a determining factor in the peptide's action. In addition, we sought to identify functional changes in rMRF neurons, such as increased bursting or correlated firing, which might contribute to the cyclic increases in firing that appear to be driving cyclic episodes of locomotion. With this approach, we determined that CRH effects on rostromedial medullary neurons in behaving newts differed greatly from effects in pharmacologically immobilized animals, showing that this neuropeptide's actions depend on the prevailing functional state of the animal's brain and behavior. These results are another example of context-dependent neurobehavioral effects of hormones like those described previously for vasotocin and corticosterone (Coddington and Moore, 2003; Rose, 2000; Rose et al., 1995).

Materials and Methods

Animals and surgical procedures

Adult male roughskin newts (*Taricha granulosa*, N=37) were collected from Benton County, Oregon. Newts were housed in a community tank supplied with a continuous flow of cold, aerated well water (13-14 °C), fed chopped beef heart and maintained on a simulated natural light/dark cycle. 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 Guidelines for the Care and Use of Laboratory Animals.

Surgical procedures were similar to those routinely used in this laboratory (Lowry et al., 1996; Hubbard et al., 2010). Prior to undergoing surgery, each newt was anesthetized by immersion in 0.1% MS-222 (tricaine methanesulfonate). During surgery the newt was covered with cellulose tissue soaked in anesthetic to maintain anesthesia and facilitate transcutaneous respiration. The dorsal surface of the skull and upper cervical vertebrae were exposed and a cannula guide, consisting of a 4.5 mm length of polyethylene tubing (PE 50), was implanted through a burr hole directly over the right lateral ventricle. Dental cement was used to anchor the cannula guide to a stainless steel screw (0-90) inserted into the left frontal skull bone and a stylette was placed into guide to occlude it. The recording electrode assembly consisted of two pairs of twisted 50- μ m Diamel-insulated nichrome microwires (impedances of 100-500 k Ω). Surgical implantation of recording electrodes was accomplished by removal of connective and meningeal tissues overlying the fourth ventricle of the medulla. Electrodes were positioned so that the electrode pair straddled the midline to make contact with the surface of the rostromedial medulla in proximity to the dorsally-positioned neurons of the paramedian reticular formation. Sites for electrode placement varied slightly across animals, but always encompassed the paramedian regions of the right or left rMRF (Fig. 1). Dental cement anchored the recording electrodes to the skull, the cannula guide, and a stainless-steel wire suture through the skin at the back of the skull. Following surgery, the newt was allowed to recover in cold, aerated well water for 2-3 h.

Hormone administration

In order to examine the neurophysiological and associated behavioral effects of CRH and to establish the receptor specificity of CRH actions, each newt received icv infusion (described further below) of one of three solutions: (1) CRH (CRH_{rat/human}, Sigma: C-3042; 1 μ l of 50

ng/ μ l in Ringer's), (2) pretreatment with the CRH receptor antagonist, alpha-helical CRH (ahCRH, Sigma: C2917; 500 ng/0.5 μ l Ringer's), followed 5 minutes later by CRH (100 ng in 0.5 μ l Ringer's) (ahCRH + CRH) or (3) a vehicle control (VEH; 1 μ l 90% amphibian Ringer's). Infusions were delivered at approximately 1 min/ μ l of solution.

Electrophysiological recording and behavioral testing procedures

Prior to full recovery from anesthesia, the newt was transferred to the recording apparatus. Recording was conducted with the newt immersed in cold, aerated well water in a 22 cm diameter circular glass arena. The stylette was removed from the guide tube and a cannula, connected to a microsyringe via polyethylene tubing, was inserted for icv delivery of hormone/s or VEH to the right lateral ventricle. This arrangement allowed for test compound delivery during the experiment without handling the newt. Leads from the microwire electrode assembly were connected to Microdot Mininoise coaxial cables which led to a Grass Model P55 preamplifier where the signal was amplified with a bandpass of 100-1 kHz in accordance with the relatively low frequency composition of newt single neuron action potentials.

Neuronal activity was continuously monitored prior to the start of the experiment to determine when the animal had fully recovered from anesthesia. For all experimental conditions, medullary single-neuron activity was sampled for 5 min prior to- (pre-treatment period), and 30 min after (post-treatment period) icv administration of CRH, ahCRH + CRH or VEH, to the right lateral ventricle.

In order to distinguish behavior-dependent neural effects of CRH from neural effects not specifically related to behavior, newts were randomly assigned to one of two groups: the behaving group (n=19) or the immobilized group (n=18). Procedures for hormone/s or vehicle treatment were identical for both groups of newts, with the only difference being that newts in the immobilized group received intraperitoneal (ip) injections of a myoneural blocking agent, gallamine triethiodide (2% gallamine triethiodide in amphibian Ringer's, 0.2 ml/newt, ip, supplemented as necessary), prior to recovery from anesthesia and the start of the experiment. Previous research has shown that this immobilization procedure is not stressful for the newt (Rose et al., 1993).

For experiments in which the animal was freely behaving and not paralyzed by gallamine triethiodide, behavior was recorded with a video camera onto 8 mm video tapes. Experiments conducted with immobilized newts were also monitored and recorded onto video tape, but for the purposes of verifying that no movements occurred during an experiment. Analog signals from neuronal activity and voice narratives were digitized with a Neurodata Neuro-corder (Model DR-484; Cygnus Technology, Inc.) and stored onto VHS tape for later offline analyses.

Analysis of behavioral and neurophysiological data

Synchronization between video records of behavior and electrophysiological signals was achieved by means of a 1 Hz tone generated by a metronome that was concurrently recorded on the audio channels of the video camera as well as the Neurocorder. Onset and offset of locomotion prior to and following hormone/s or VEH administration was manually signaled

with an electronic marking signal in addition to the voice narrative and video recordings. Electrophysiological signals were analyzed offline with a Cambridge Electronic Design system (Micro 1401 mk II, Cambridge Electronic Design; CED). Single neuron spike identification, counting and sorting was conducted with CED Spike 2, version 5.13 software program, in some cases assisted with principle components analysis. Once single neurons were identified, videotape recordings, voice narratives, and 1 Hz tones were played back in synchrony with sampled neuron activity to identify relationships between behavioral and neural events.

For the behaving group of newts, the onset and offset of locomotion was manually coded using keyboard event markers in Spike 2. Samples of behavior categorized as either walking (locomotion) or immobility (absence of locomotor movement) were statistically analyzed for each 10 sec interval occurring 5 min prior to- and extending 30 min following treatment with CRH, VEH or ahCRH + CRH. Episodes of locomotor-related movements were defined as discrete events or 'locomotor bouts' consisting of at least 2 s of walking. The duration (s) of each bout as well as the time intervals between bouts of locomotion for each animal was also quantified pre- and post-treatment. To determine the magnitude or degree of change due to the effects of CRH treatment compared to pre-treatment conditions, difference scores were calculated for the mean number of locomotor bouts, bout duration (s), and latencies(s) between bouts, by subtracting each animal's mean pre-treatment score from its mean post-treatment score (post – pre). Significance was evaluated with Kruskal-Wallis Test and post-hoc tests were conducted using Mann-Whitney unless otherwise stated. In addition, the time of occurrence of a CRH-induced change in locomotor activity was assessed by the Change-Point Test for Continuous Variables (Siegel and Castellan, 1988). The Change-Point Test was conducted on the number of locomotor bouts occurring 5 min before (pre-treatment) and 30 min after CRH treatment (post-treatment). A total of 35 time points were analyzed (1 min time bins) with the exception of one newt, where only 22 time points were available for analysis.

For the behaving group of newts, neuronal activity (10 s bins) was quantified during periods of locomotion (walking) and periods of immobility (no locomotion). The criterion for the former type of activity was defined as neuronal activity associated with at least 2 s of locomotion whereas the latter type was defined as any activity occurring in the absence of locomotion, which included but was not limited to, non-locomotor related movements such as head turns, tail flicks, or eye closures. To afford a proper comparison between behaving and pharmacologically immobilized groups of newts, since the latter group lacked any behavior from which to categorize neural activity, we quantified "overall neuronal activity" (10 s bins) by taking each neuron's mean frequency firing rate occurring 5 min (pre-treatment) prior to and extending 30 min (post-treatment) following hormone/s or VEH treatment. These data were then used for all subsequent quantitative analyses and statistical comparisons made between the behaving and immobilized groups of newts including those performed for autocorrelation, cross-correlation and burst detection analyses.

To determine if CRH altered rMRF neuronal activity relative to control conditions (i.e., VEH or ahCRH + CRH) in the behaving group of newts, pre- and post-treatment activity during periods of locomotion and periods of immobility were analyzed using the Change-

Point Test (210 time points). In addition, we used the Change-Point Test to also compare the effects of CRH treatment on overall neuronal activity in behaving and immobilized groups of animals. Statistical analyses conducted on the proportion of neurons that changed activity compared to those that did not show any changes following treatment were performed using Chi-square Tests (two-tailed; $\alpha=0.05$). In cases where multiple Chi-square Tests were performed, adjustments to the alpha level were made using a Bonferroni correction.

Hormone effects on periodicity and synchronicity of firing of single medullary neurons and pairs of simultaneously recorded neurons were analyzed by autocorrelation and cross-correlation for behaving and immobilized group of newts, respectively. Autocorrelations were conducted in Statistical Package for the Social Sciences software (v. 12) on 10 sec bins of activity pre- and post-hormone/s or VEH treatment for each neuron. Of the 138 neurons sampled, six neurons were excluded from the autocorrelation analyses due to a lack of activity prior to treatment, leaving a total of 132 neurons in which autocorrelational analysis was conducted. Cross-correlation analysis was also performed on the pre- and post-treatment discharge patterns for the behaving and immobilized groups of newts. Six neurons were excluded from this analysis due to an absence of activity prior to treatment and an additional 18 neurons were excluded either because of extremely low frequency firing rates or were recorded in isolation with no pairing possible. Both autocorrelograms and cross-correlograms were generated in Spike 2 (CED) using 1 s bins, 1000 and 500 s bin widths and time ranges of ± 500 s and ± 250 s, respectively.

Burst analysis was conducted to determine whether CRH altered the bursting patterning of rMRF neurons in behaving compared to immobilized groups of animals using a CED script in Spike 2 (bursts.s2s). A burst was defined as an event consisting of two or more spikes with intervals between two spikes equal to or less than 0.05 s. The number of bursts, inter-burst intervals (IBIs; in ms) and burst durations (ms) were calculated and difference scores were derived by subtracting each neuron's mean post-treatment score from its mean pre-treatment score. Since multiple neurons were sampled from the same animal within groups, significance tests were performed on difference scores using a linear mixed model analysis approach (reported type 3 tests of fixed effects). Between group contrasts were conducted on parameter estimates using Bonferroni adjusted pairwise comparisons.

Results

Effects of CRH administration on locomotor behavior

In the behaving group of animals, icv CRH infusion produced a rapid and pronounced increase in locomotor behavior, an effect which was significantly attenuated by pre-treatment with the CRH receptor antagonist, ahCRH, to levels seen following administration of a VEH control solution (amphibian Ringer's). Change-Point Tests revealed that CRH infusion significantly enhanced the incidence of locomotion in 7 out of 9 newts (CRH1, $p<0.01$; CRH3, $p=0.008$; CRH4, $p=0.054$; CRH5, $p=0.05$; CRH6, $p<0.001$; CRH8, $p=0.01$; CRH9, $p=0.001$) with mean latency for change occurring approximately 13 min and 15 sec following CRH infusion. A qualitative change in locomotion also occurred in three animals, which exhibited bouts of swimming, a form of locomotion not observed in any animals prior to CRH administration.

To determine the magnitude of change in locomotor activity due to CRH treatment compared to VEH and ahCRH + CRH control conditions, difference scores were calculated by subtracting the mean number of locomotor bouts occurring pre-treatment from the mean number of locomotor bouts that occurred post-treatment. One animal was dropped from the analysis due to insufficient data points post-treatment as a result of video camera malfunction, yielding a sample of 18 newts (CRH=8 newts; VEH=5 newts; ahCRH + CRH=5 newts). Analysis of difference scores for the number of locomotor bouts showed a significant treatment effect across the CRH, VEH, and ahCRH + CRH conditions (Kruskal-Wallis, $\chi^2=7.51$, $df=2$, $p=0.023$). Post-hoc analyses (Mann-Whitney) demonstrated that icv CRH (mean=70.13, ± 13.41) significantly enhanced the number of locomotor bouts compared to treatment with VEH alone (mean=20.4, ± 10.98 ; $p=0.023$) and ahCRH pre-treatment (ahCRH + CRH; mean=24.8, ± 9.8 ; $p=0.028$). Subsequent analyses conducted on difference scores for locomotor bout duration and time between bouts revealed no significant treatment effects.

Neuron sample and location of recorded neurons

The results presented below are based on recordings from 138 neurons captured at 37 different recording sites located throughout the paramedial rMRF ($n=37$ newts; Fig. 1). Recordings from an individual newt typically yielded 2-4 well-identified single neurons. Of the 138 neurons sampled, 72 neurons were recorded in the 19 behaving newts and 66 neurons were recorded in the 18 immobilized newts. For the behaving newts, 35 neurons were sampled from 9 newts for the CRH condition, 15 neurons from 5 newts in the VEH condition, and 22 neurons from 5 newts in the ahCRH + CRH condition. For the immobilized animals, 25 neurons were sampled from 7 newts in the CRH condition, 21 from 5 newts in the VEH condition, and 20 from 6 newts in the ahCRH + CRH condition.

Relationship of rMRF neuronal activity to locomotor behavior

One of the most striking features of the rostral medullary neurons in this study was the extent to which their activity was related to locomotion (Fig. 2). Prior to any treatment, the majority of neurons sampled in the behaving animals (81.7%; 58/71) showed increases in activity associated with transitions between immobility and walking (1 neuron was excluded from analysis due to insufficient data points). The remaining neurons either fired infrequently or were quiescent during locomotion (18.3%; 13/71). Of the neurons displaying walking-related firing, 39.7% (23/58) fired prior to the onset of a bout of walking (mean latency=0.40 s, ± 0.084), whereas the other 60.3% (35/58) fired after walking onset (mean latency=-0.57 s, ± 0.085). A large proportion of neurons (69%, 49/71) were also tonically active to some degree during immobility. Other cells either fired occasionally (14.1%, 10/71) or not at all (16.9%, 12/71) during periods of immobility.

Relationship of CRH effects on medullary neuronal activity to behavioral state

For neurons recorded in all newts, Change-Point Test analysis (two-tailed, $\alpha=0.05$), was conducted on each neuron's pre- and post-treatment mean frequency firing rates (210 time points; 10 sec binwidths) for the CRH, VEH, and ahCRH + CRH conditions. For neurons showing a significant change in activity, the time of change (mean latency in minutes) and

the direction of change (increased or decreased) was also determined. Change Point Tests revealed that most neurons showed significant changes in their mean frequency of firing following treatment with CRH (28/35; 80%) compared to VEH (8/15; 53.3%) or ahCRH + CRH (9/22; 40.9%) administration (Fig. 3). Furthermore, a significantly greater proportion of neurons showed changes in their overall activity following CRH (28/35, 80%) administration compared to treatment with ahCRH + CRH (9/22, 40.9%; $\chi^2=9.06$, $df=1$, $p=0.003$) (Fig. 3; Bonferroni corrected α level=0.025). An increase in the proportion of neurons showing changes in their overall neuronal activity in the CRH compared to the VEH treatment condition approached significance (8/15, 53.3%; $\chi^2=3.70$, $df=1$, $p=0.054$) (Fig. 3; Bonferroni corrected α level=0.025). In addition, changes in firing following CRH was, on average, extremely rapid and occurred within 8 minutes and 36 s after CRH administration and ranged from 3 min to 23 min after peptide infusion.

To distinguish CRH effects on neuronal activity related specifically to locomotion from activity unrelated to locomotion (e.g., spontaneous discharge) in behaving newts, we compared each neuron's firing during locomotion (Fig. 3) and periods of immobility (Fig. 3). The majority of neurons displaying locomotor-related activity showed significant changes in their firing rates following CRH (27/35, 77.1%) administration compared to VEH (2/15, 13.3%; $\chi^2=17.55$, $df=1$, $p<0.001$) or ahCRH + CRH (3/22, 13.6%; $\chi^2=21.85$, $df=1$, $p<0.001$) treatment (Fig. 3; Bonferroni corrected α level=0.025). In contrast, CRH (14/35, 40%) had no effect on neural activity during immobility compared to VEH (8/15, 53.3%; $\chi^2=0.75$, $df=1$, $p=0.38$) or ahCRH + CRH (10/22, 45.5%; $\chi^2=0.16$, $df=1$, $p=0.68$) control conditions (Fig. 3). The fact that icv-administered CRH increased rostromedullary neuronal firing during locomotion but not during periods of immobility compared to VEH or ahCRH treatment indicates that this effect is somehow specific to locomotion and not due to a general increase in spontaneous activity.

We also compared mean latencies for onset of locomotor-related firing to determine if CRH administration altered firing onsets compared to VEH and pre-treatment with ahCRH (ahCRH + CRH). Difference scores were derived by subtracting each neuron's mean pre-treatment locomotion-related firing onset latency from its mean post-treatment onset latency. Linear mixed models analysis conducted on difference scores revealed no significant differences in mean latency onset for locomotor-related firing across the three different treatment conditions.

Effects of CRH administration on medullary neuronal activity in behaving compared to immobilized animals

To determine if CRH administration altered the activity of neurons in the behaving compared to immobilized group of animals, the Change-Point Test was conducted on each neuron's overall activity prior to and following treatment with CRH, VEH or ahCRH + CRH. As stated previously, in behaving animals, CRH resulted in a significantly greater proportion of neurons displaying activity changes (increases or decreases) compared to VEH or ahCRH + CRH (Fig. 3 and 4) conditions. Similarly, in the immobilized group of animals, a greater proportion of neurons showed significant changes in activity in the CRH condition (20/25, 80%) compared to VEH control (8/20, 40%; $\chi^2 = 7.56$, $df = 1$, $p=0.006$) or ahCRH +

CRH treatment (7/21, 33.3%; $\chi^2=10.25$, $df=1$, $p=0.001$) (Fig. 4). The mean latency of change in activity for these neurons as determined by the Change-Point Test was 10 min and 42 s after CRH infusion whereas in the behaving group of animals, the average time of change was approximately 8 min and 36 s post-CRH infusion.

To determine if CRH administration differentially altered overall neuronal activity in medullary neurons sampled in behaving compared to immobilized newts, we compared proportions of neurons showing changes versus those that showed no change in activity following CRH administration using 2×2 Chi-square Tests. No significant differences were found ($\chi^2=0$, $df=1$, $p=1$) (Fig. 4). The direction of change in activity for each neuron following icv CRH was also determined by Change-Point Test for the behaving and immobilized groups of newts. Of the neurons recorded in the behaving newts that showed significant changes following CRH, 46% (16/35) increased firing, 34% (12/35) decreased firing and 20% (7/35) showed no change. Likewise, 40% (10/25) of neurons recorded in the immobilized newts showed increased firing, 40% (10/25) decreased firing, and 20% (5/25) did not change. Taken together, these findings show that icv CRH results in rapid and diverse changes in the activity of medullary neurons in both behaving and immobilized newts.

Effects of CRH administration on periodicity of neuronal firing

Previous studies have shown that CRH-induced locomotion and locomotor-related medullary neuronal activity in newts tends to be cyclic or rhythmic (Lowry et al., 1996; Hubbard et al., 2010). This finding suggests that a change in the rhythmicity of rMRF neuronal activity might play a role in the CRH effect on locomotion. This possibility was examined by autocorrelation analysis of discharges from 132 neurons recorded across each of the treatment conditions for both behaving and immobilized groups of newts.

Autocorrelational analysis was conducted across four time lags, and neurons displaying significant autocorrelations for 3 of the 4 respective lags were categorized as displaying periodicity (Box and Jenkins, 1970), whereas those that did not meet this criterion were categorized as displaying no periodicity. Under pre-infusion conditions, the majority of neurons in both the behaving and immobilized groups of animals lacked firing periodicity and the incidence of periodicity was not significantly different from the control treatment conditions ($p>0.05$; Table 1). Chi-square Tests conducted on post-treatment frequency scores for neurons showing firing periodicity compared to those that showed no periodicity revealed that in behaving newts, CRH produced a greater proportion of neurons with firing periodicity (28/32, 87.5%) than VEH (7/13, 53.8%) treatment ($\chi^2=6.06$, $df=1$, $p=0.01$). However, the proportion of neurons displaying firing periodicity following CRH for behaving newts was not significantly different from the proportion displaying periodicity following ahCRH + CRH (18/22, 81.8%) treatment (ahCRH + CRH; $\chi^2=0.33$, $df=1$, $p=0.56$). Additionally, the proportion of neurons in the behaving group of newts that showed periodicity following treatment with CRH (28/32) was significantly different from the immobilized group for CRH (14/24, 58.3%; $\chi^2=6.22$, $df=1$, $p=0.012$), VEH (3/20, 15%; $\chi^2=26.87$, $df=1$, $p<0.001$), and ahCRH + CRH (9/21, 42.9%; $\chi^2=11.99$, $df=1$, $p<0.001$) treatment conditions (Bonferroni corrected $\alpha=0.0125$). In contrast to the findings seen in the

behaving group of newts, there were no significant differences in firing periodicity following ahCRH + CRH treatment in the immobilized group of newts (Table 1).

To summarize, the above results indicate that CRH increased the number of medullary neurons displaying rhythmic firing in behaving newts, whereas icv administered CRH in immobilized animals had no such effect. Autocorrelograms for neurons recorded in behaving and immobilized newts following CRH treatment are depicted in Figs. 5 and 6 and illustrate this relationship (1 s bins; 1000 bin widths; 500 and 250 ms offset, respectively). Qualitatively speaking, autocorrelograms in the behaving group of animals following CRH administration generally appeared cyclic, consisting of obvious peaks and valleys, whereas there was an absence of cyclicity in autocorrelograms for most of the neurons sampled in the immobilized newts. Rather, the majority of neurons recorded in the immobilized group had autocorrelograms that appeared flat or lacked an obvious central peak (Fig. 5), a finding consistent with results from our autocorrelational analyses.

Effects of CRH on correlated firing patterns between neurons

In addition to the question of possible CRH effects on firing periodicity of individual neurons, there is also the question of how CRH acts upon neuronal populations within the rMRF and whether an increase in CRH-induced correlated firing patterns underlies this peptide's locomotor enhancing effects. We evaluated this possibility by examining the cross-correlational discharge patterns of simultaneously recorded neurons prior to and following treatment with CRH, VEH, or ahCRH + CRH. Out of a total of 214 possible neuronal combinations, 127 pairs were from the behaving newts and 87 pairs were from the immobilized newts (Table 2). Prior to any treatment, correlated discharge patterns (both negative and positive) were found in 51/127 (40%) pairs of neurons in the behaving group of animals and 8/87 pairs (9%) in the immobilized groups of newts. The remaining pairs showed no correlated firing patterns. In the behaving newts, the proportions of neuronal pairs showing correlated (negatively and positively correlated) and uncorrelated firing patterns were compared. Prior to CRH, VEH, or ahCRH + CRH treatment in the behaving group of animals, there were no significant differences in the proportions of neuronal pairs with correlated firing patterns ($\chi^2=0.824$, $df=2$, $p=0.39$). Similarly, in immobilized newts proportions of neuronal pairs showing significant positive, negative or uncorrelated discharges were also not statistically different across treatment conditions ($\chi^2=2.31$, $df=2$, $p=0.315$). However, group comparisons revealed that the proportions of neuronal pairs showing correlated firing patterns differed significantly between the behaving and immobilized groups of newts prior to any hormone treatment ($\chi^2=26.22$; $df=5$, $p<0.001$), with the former group showing a greater proportion of positively-correlated pairs, indicative of synchronous firing (Table 2). In contrast, the immobilized newts showed the opposite pattern; a greater proportion of neuronal pairs in this group showed no correlated firing patterns prior to hormone/s or VEH treatment (Table 2). This finding indicates that prior to treatment the immobilized groups of newts differed greatly in the degree of synchronicity present in their neuronal discharge patterns compared to behaving newts.

Analysis of the effect of CRH treatment on the correlated firing patterns of neuronal pairs recorded in behaving compared to immobilized groups of newts yielded no significant

difference between the proportion of correlated and uncorrelated pairs for behaving newts following CRH (36/64) compared to VEH (7/12; $\chi^2=0.018$, $df=1$, $p=0.894$) or ahCRH + CRH (34/51; $\chi^2=1.293$, $df=1$, $p=0.256$) administration (Table 2). For immobilized newts, there was also no significant difference between the proportion of pairs showing correlated firing following CRH (14/36) compared to ahCRH + CRH (8/25) ($\chi^2=0.3$, $df=1$, $p=0.58$), but there was for the VEH (3/26) ($\chi^2=5.68$, $df=1$, $p=0.017$) condition. For the latter result, CRH (14/36) treatment in the immobilized group produced a significantly greater proportion of uncorrelated neuronal pairs than VEH treatment (3/26).

Since the above analyses did not take into account the direction of correlative firing patterns, we also examined numbers of positively and negatively correlated pairs and pairs that showed no correlated firing (Table 2). For cases in which there were no negatively correlated neuronal pairs to compare between treatment conditions, when the direction of correlation was taken into account, results showed that the proportion of positively correlated pairs was significantly greater in the behaving groups of newts treated with CRH, compared to immobilized newts administered CRH ($\chi^2=15.28$, $df=2$, $p<0.001$), VEH ($\chi^2=19.31$, $df=2$, $p<0.001$), or ahCRH + CRH ($\chi^2=4.23$, $df=1$, $p=0.04$) (Table 2). Cross-correlograms of representative pairs of simultaneously recorded medullary neurons in behaving and immobilized groups of newts are shown in Fig. 6 and illustrate the differences in correlated discharge patterns prior to and following CRH treatment. The majority of neuronal pairs (36/64, 56%) in the behaving animals showed positively correlated firing patterns following CRH, whereas the other 44% (28/64) were uncorrelated (Table 2). Conversely, after CRH in the immobilized group of newts, the majority of neuronal pairs (22/36, 61%) showed uncorrelated firing patterns while the remaining pairs showed either positive (9/36, 25%) or negatively correlated firing patterns (5/36, 14%) (Table 2). This finding indicates that CRH had differential effects in the behaving compared to immobilized newts, which may have led to a desynchronicity in neuronal firing patterns in the latter group, something not seen in behaving animals prior to or following treatment with CRH.

Effects of CRH on neuronal bursting patterns

Burst analysis was conducted on the firing of 137/138 neurons to determine if CRH altered the bursting patterns in behaving compared to immobilized newts. One neuron was not used due to insufficient post-treatment activity. Linear mixed models analyses of difference scores for the mean number of bursts revealed significant treatment effects ($F(5, 131)=5.28$, $p<0.001$; Fig. 7A). For the behaving newts, post-hoc tests showed significantly more bursts following CRH (mean=142±16) compared to VEH (mean=54 ±24, $p=0.039$) or ahCRH + CRH (mean=47±20, $p=0.004$) treatment conditions. In contrast, the number of bursts in the immobilized newts yielded no significant differences in bursting across the CRH (mean=63±18), VEH (mean=67±21) or ahCRH + CRH (mean=30±20) conditions. Comparisons of difference scores for the mean number of bursts following CRH demonstrated a significant increase in bursting between behaving (mean=142±16) compared to immobilized newts (mean=63±20) ($p=0.022$; Fig 7A).

Linear mixed models analysis of difference scores for IBIs revealed significant treatment effects across groups ($F(5, 131)=3.20$, $p=0.009$). Further analyses of bursting patterns using

pairwise comparisons showed that CRH significantly decreased IBIs for medullary neurons recorded in behaving newts compared to immobilized animals (Fig 7B). In general, the mean latency between bursts for behaving animals following CRH was much shorter (7 ± 18 s), than the immobilized group (mean latency = 111 ± 21 s; $p=0.004$). No significant group differences were found for VEH or ahCRH + CRH treatment conditions. Analysis of burst duration, also showed no significant differences across treatment conditions, either within or between groups.

Discussion

As explained in the Introduction, there is now substantial evidence that CRH is a critical mediator of stress effects on diverse behaviors in vertebrates. Stimulation of locomotion by CRH has been particularly studied as a robust model for this peptide's behavioral effect. Prior research has focused on CRH's upstream actions; on ascending serotonergic and noradrenergic neuromodulatory systems arising from midbrain and rostral pontine nuclei, such as the dorsal raphe nucleus, locus coeruleus, and their respective targets in the ventral forebrain (Lowry et al., 2009, 2000; Price et al., 1998; Tazi et al., 1987; Valentino et al., 1993). On the other hand, potential CRH actions on the medullary reticular formation (MRF), a region containing reticulospinal (RS) neurons critically involved in initiation and regulation of vertebrate rhythmic locomotion has been less investigated (Lowry and Moore, 2006; Lowry et al., 1996).

An obvious critical strategy in an electrophysiological investigation of the neurobehavioral action of hormones is to record from neurons likely to control the behavior of interest, while these hormonal effects are transpiring. Most electrophysiological studies on the neural effects of hormones and other neurally-active substances that affect behavior have been conducted in anesthetized or immobilized whole animals or, increasingly, brain slices, preparations where behavior could not be concurrently expressed. These approaches may be more convenient or in some cases provide more technical options, like whole cell recording, but ultimately, behaving animals must be studied in real time to reveal the specific neural effects of hormones that underlie their behavioral effects. For example, previous studies of the effects of ovarian hormones on lordosis responses in freely-moving golden hamsters revealed striking changes in sensorimotor properties of brainstem, hypothalamic and limbic system neuronal function that were closely tied to emergence of hormone-dependent behavior (Rose, 1986, 1992). Similar types of behavior-related hormone actions were obtained in a study of hindbrain neurons and corticosterone-induced suppression of courtship clasping in behaving roughskin newts (Rose et al., 1998). Studies of this type have invariably revealed neuronal functional changes that appeared causal in the behavioral effects of hormones. These changes could not have been accurately predicted without recording from neurons in a behaving animal.

Medullary neurons and locomotor control

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. Rostromedial medullary neurons

were the focus of this study due to the extensive evidence in lamprey, including our own previous findings in *Taricha*, that neurons in this brain region are responsible for controlling onset, offset and speed of locomotion (Dubuc et al., 2008; Grillner et al., 1995; Lowry et al., 1996; Rose et al., 1998). Confirming our earlier results in freely-behaving newts (Lowry et al., 1996; Rose et al., 1998), the great majority of rMRF neurons showed locomotion-related firing before any hormone administration. These neurons tended to have some degree of activity during immobility and then exhibited firing increases just prior to or in synchrony with locomotion. Because episodes of increased rMRF neuronal activity tended to precede and overlap the overt occurrence of an episode of increased locomotion and because neurons in this brain region, including RS neurons that we have previously investigated (Hubbard et al., 2010; Lowry et al., 1996; Rose et al., 1998) have been implicated in locomotor control, it seems likely that these rMRF neurons could be critically involved in controlling these bouts of locomotion. Since we saw little evidence in our recordings of any firing that was temporally coincident with specific stepping movements (e.g. footfalls) and because the neuronal firing was often phase-advanced in relation to episodes of walking (Fig. 2), it is unlikely that the increased locomotor-related neuronal firing was secondary to sensory feedback from locomotion or from activity in a central locomotor pattern generator.

CRH neuronal effects related to enhanced locomotion

The present study's primary aim was to identify CRH effects on rMRF neurons that might be causal in producing locomotor stimulation. In addition, we sought to determine how the locomotor-activating effect of CRH, specifically CRH actions on locomotion-controlling hindbrain neurons, might depend on and interact with the behavioral state of the animal. As in our earlier study (Lowry et al., 1996), intraventricular CRH rapidly enhanced locomotion in most newts, including the more extreme effect of producing swimming in some animals. In the present study, this effect, like nearly all the effects of CRH, was blocked by ahCRH, showing the receptor specificity of CRH actions.

In association with the stimulation of locomotion, CRH caused a significant change in neuronal activity in immobilized as well as freely-moving newts, but the proportions of neurons showing activity changes in these two groups of newts did not differ. However, for neurons with locomotion-related firing, those likely to have some role in locomotor control, the CRH-induced change in firing was specific to periods of locomotion (Fig. 3). There was no difference in activity before and after CRH when neuronal activity during immobility was examined (Fig. 3). This result underscores the functional specificity of the behavioral CRH effect, that it was not a consequence of increased spontaneous or tonic neuronal activity independent of behavioral state.

In behaving newts the change in firing after CRH was predominantly an increase, with a mean latency of 8.5 minutes, well in advance of the 13 min 15 sec mean latency for increased locomotion. This is a critical point since it suggests a causal effect for the increase in locomotion after CRH. Previously (Hubbard et al., 2010; Lowry et al., 1996), we observed that neuronal activity and concurrent bouts of locomotion were cyclic and the cyclicity of neuronal activity and locomotion were closely associated. A similar pattern in these variables was evident in the present study (Fig. 2). For example, a significant

proportion of the rMRF neurons (39.7%) that displayed a CRH-induced enhancement in locomotor-related activity had firing onsets that preceded and overlapped with the onset of walking (Fig. 2).

As assessed by autocorrelograms, rhythmic firing (not to be confused with cyclic episodes of locomotion-related firing described above) was initially absent in most neurons for both behaving and immobilized newts. However, after CRH, rhythmicity was present in the great majority of neurons in moving animals and significantly more than in behaving, vehicle treated or the CRH treated immobilized newts. An unexpected finding was that ahCRH pretreatment enhanced rhythmic firing in a significant proportion of rMRF neurons in the behaving group of newts, similar to levels observed following CRH administration (Table 1). One possible explanation is that ahCRH can act as a partial agonist at higher doses (Baldwin et al., 1991; Menzaghi et al., 1994; Smart et al., 1999). However, the dose for ahCRH (500 ng/0.5 μ l ahCRH in Ringer's) used in the present study was identical to that administered in previous investigations conducted by Lowry and others (Lowry and Moore, 1991) in *Taricha*, and has been shown to reliably block the CRH-induced enhancement of locomotion. Furthermore, ahCRH pretreatment here successfully abolished CRH-enhanced locomotor-related firing and subsequent increases in locomotor behavior. Thus, ahCRH may be acting as a partial agonist but at a subthreshold level, by increasing firing rhythmicity of individual neurons an effect insufficient to alter other neuronal firing properties, including bursting characteristics or the necessary increase in firing synchrony at the network level, required to give rise to locomotor behavior.

Correlated firing between neuronal pairs was present in approximately 38% of such pairs before CRH treatment in behaving newts, but in immobilized newts was present in only 8%, significantly fewer (Table 2). The most salient finding of the correlational firing analysis seems to be in the higher degree of correlated firing in the behaving newts, both before and after CRH or control treatments (Table 2). Neurons in behaving newts also showed more post-CRH bursting than controls, an effect not seen in immobilized newts and likely to be of functional importance (Fig. 7A). Inter-burst intervals were shortened by CRH in behaving versus immobilized newts, such that IBIs were about 7 sec in behaving and 111 sec in immobilized newts (Fig. 7B).

Insights from the comparisons between neurons recorded in moving and immobilized newts

In both behaving and immobilized newts, the neuronal actions of CRH typically regarded to be vital to the peptide's neural functional effects, binding with receptors, internalization and initiation of signaling in second-messenger pathways occurred. Yet, there were many salient differences in the neuronal effects produced by CRH that apparently stemmed from the functional differences between these groups of animals, such as the presence of muscle tone, posture and locomotor function in moving newts.

As stated above, CRH in the behaving newts caused a pronounced increase in locomotion that was not continuous, but exhibited in frequent bouts separated by periods of immobility (Fig. 2). These bouts of locomotion were preceded by increased neuronal firing, which also waxed and waned with locomotion. No such cyclic surges in neuronal activity were seen in

immobilized newts, that is, there was no suggestion of fictive locomotor activity. This finding differs from results with the isolated brainstem-spinal preparation with lampreys, an extensively used *in vitro* model for analysis of locomotor control (Grillner et al., 1987; Dubuc et al., 2008), where the neural activity associated with swimming is expressed in the absence of a capacity of overt movement. In addition, CRH differentially increased the neuronal firing specifically to episodes of locomotion, increased rhythmic firing and bursting in moving newts. Lastly, the incidence of correlated firing between neurons was higher in behaving newts. Consequently, the receptor binding, internalization and signaling cascade initiated in CRH target neurons is just the beginning of a suite of neuronal functional consequences that emerge subsequent to the peptide's direct effect on signaling mechanisms and depends critically on the neurobehavioral state of the animal.

Neuronal targets for CRH

Ideally, in an electrophysiological investigation of hormone-dependent behavior, it would be desirable to sample activity of neurons that are verified target cells for the hormone in order to distinguish direct from indirect hormone effects. This goal is not achievable with the techniques we employed for single neuron recording in behaving newts, but with our development of a fluorescent CRH conjugate we have been able to separately identify CRH target neurons in the brainstem (Hubbard et al., 2009; 2010). Due to route in which CRH was administered in this study (icv), we cannot ascertain whether these CRH effects were the result of the direct actions of the peptide on rMRF neurons, including RS neurons, or instead due to indirect CRH actions at effector sites located upstream, such as in the mesencephalic locomotor region and the pontomedullary locomotor strip, or downstream from the rMRF, at the level of the spinal cord locomotor central pattern generator. The potential for a CRH effect on locomotion-controlling serotonin neurons is also likely, as shown by work in mammals, birds, salmonids, as well as *Taricha* (Cazalets et al., 1992; Clements et al., 2003; Grillner et al., 1987; Harris-Warrick and Cohen, 1985; Hubbard et al., 2010; Lowry et al., 2009). In rainbow trout (*Oncorhynchus mykiss*), icv administered CRH at high doses increases both locomotor activity and serotonin concentrations in subpallial and brainstem regions (raphé nuclei) known to play a role in stress-induced behavioral arousal and locomotor activation (Carpenter et al., 2007). In chinook salmon and *Taricha*, concurrent icv administration of CRH with fluoxetine, a selective serotonin re-uptake inhibitor, potentiated CRH-induced locomotor stimulation whereas in the former species, co-administration of serotonin 1A receptor antagonist (NAN-190) decreased CRF-induced locomotion (Lowry et al., 2009; Clements et al., 2003). In our previous investigation of CRH effects on medullary neurons and locomotion in *Taricha* (Lowry, et al., 1996), we recorded from midline and paramedian medullary locations in an effort to sample activity from raphé serotonin neurons, but none of those neurons displayed the highly regular firing patterns characteristic of serotonin neurons that have been identified in mammals (see Lowry et al., 1996 for further discussion). As stated previously, the neurons recorded in that earlier study showed activity properties and behavior-related CRH effects very similar to those of neurons recorded from somewhat more lateral recording sites in the present work.

Using our recently-developed fluorescent CRH conjugate we have demonstrated that a large and diverse population of hindbrain neurons in *Taricha*, showed receptor-specific conjugate

internalization (Hubbard et al., 2009). Included in these putative CRH target neurons were substantial numbers of identified RS and raphe serotonin neurons (Hubbard et al., 2010). These two neuronal phenotypes are especially important because of extensive evidence linking RS and serotonin neurons to locomotor control (Brodin et al., 1988; Dubuc et al., 2008; Lowry and Moore, 2006; Viana Di Prisco et al., 1992; Viana Di Prisco et al., 1994). For example, studies conducted in lamprey have demonstrated that RS neuronal cell bodies within the caudal brainstem lie in close proximity to serotonin immunoreactive fibers (Viana DiPrisco et al., 1994) and serotonin immunofluorescence in retrogradely labeled RS neurons have been verified (Brodin et al., 1986, 1988). Further evidence implicating serotonin and the descending RS system in locomotor facilitation derives from studies conducted in rat and lamprey isolated brainstem-spinal cord preparations demonstrating that serotonin application induces fictive locomotion (Cazalets et al., 1992; Harris-Warrick and Cohen, 1985). Moreover, rhythmic locomotor activity, induced by treadmill walking in adult behaving rats, is associated with increased levels of serotonin and the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in the ventral funiculus of the spinal cord, a region which receives dense projections from RS neurons arising from the caudal brainstem (Gerin et al., 1995). Although we did not electrophysiologically identify RS neurons in the present study, it is highly likely, based on the locations and movement-related firing properties seen in our previous recordings from RS neurons (Rose et al., 1998) that some were in our sample. We have also previously shown that CRH application to the medulla in immobilized newts results in rapidly enhanced RS neurons membrane excitability (Rose et al., 1996). Collectively, the results of the present and previous studies show that CRH has rapid and powerful effects on a large population of medullary neurons. In addition the target neuron population for CRH is diverse, including raphe serotonin neurons, non-serotonergic RS neurons and many other, presently unidentified phenotypes of medullary reticular neurons. Due to the presence of CRH receptors in such a large and diverse population of medullary neurons (Hubbard et al., 2009), CRH effects are most likely initiated directly at that level, including direct actions on RS neurons, with the most powerful and direct influence on locomotion, as well as more indirect effects mediated through serotonin neurons or neurons at higher levels of the brain.

Neuronal actions of CRH that stimulate locomotion in *Taricha*

On the basis of the present results together with other recent findings, we propose the following explanation for the locomotion-enhancing action of CRH in roughskin newts. Bouts of locomotion appear to occur when firing of rMRF neurons (including RS neurons) increase to a critical level. Intraventricular CRH infusion results in rapid internalization and binding with CRH receptors in widespread brain regions, but critical for locomotion is internalization of receptor binding in a large population of rMRF neurons (including RS neurons) as well as hindbrain raphe serotonin neurons. Second messenger signaling, through unknown mechanisms, initiates multiple changes in the firing of rMRF/RS neurons, including substantially increased bursting, increased correlated firing and increased firing during locomotion. The first two of these changes in firing, when summated across a population of rMRF neurons, would likely produce shifts in firing that could reach threshold for locomotion, particularly when accompanied by locomotion-specific increased firing. Although the locomotion-specific firing was statistically elevated above firing during

immobility, our numerous observations that increased firing during episodes of locomotion were frequently associated with firing that began immediately prior to movement, that is, it is really not meaningfully inseparable from locomotion-correlated firing. Although the underlying cellular mechanisms for these specific types of increases in hindbrain neuronal firing are not known, we have previously shown that medullary CRH application increases the membrane excitability of RS neurons (Rose et al., 1996). In addition, in our studies of retrogradely labeled RS neurons (Lewis et al., 2005; Hubbard et al., 2010) we observed that the neurons have long dendritic processes that frequently run in close proximity in parallel with each other, a configuration that would be conducive to ephaptic coupling and correlated firing.

Insights regarding the neuronal actions of CRH

As stated above, the initial steps in the cellular action of CRH, internalization, receptor binding and initiation of second messenger signaling, would have transpired in moving as well as immobilized newts, but clearly, the consequences for neuronal function of these processes are state-dependent and not simply a lock and key process that initiates fixed changes in neuronal function. CRH targets a very diverse neuronal population (Hubbard et al., 2009, 2010), including non-RS and RS neurons, with properties we have characterized in *Taricha* (Rose et al., 1996, 1998). These neurons have very robust, dynamic functional properties that are, nonetheless, modifiable by corticosteroids, arginine vasotocin and CRH. We have shown, for example, that the potent functional effects of vasotocin on rMRF neurons can be reversed by prior exposure of the brain to corticosterone (Rose et al., 1996). A comparable reversal of function, in this case of CRH, is seen with prior exposure to corticosterone (Rose et al. 1996). Thus, the functional neuronal effects of CRH are not invariant, but highly dependent on the chemical environment of the brain as well as the behavioral state of the animal. This type of functional plasticity of neuropeptide action provides for a diverse and adaptive range of behavioral control by these hormones (Coddington and Moore, 2003; Rose et al., 1995, 1996; Rose, 2000). This state-dependent functional plasticity of CRH action, once again, points to the importance of studying the neuronal effects of hormones in intact, fully functional animals.

The present results also augment previous findings indicating a rostral brainstem involvement in CRH-stimulated locomotion (Lowry et al., 2009, 2000; Price et al., 1998; Tazi et al., 1987; Valentino et al., 1993; Waselus et al., 2011), where ascending projections from the rostral brainstem would modulate forebrain processes such as cognitive and emotional effects of CRH, whereas the peptide's action on hindbrain neurons, many of which internalize CRH (Lowry and Moore, 2006; Hubbard, et al., 2010), would more directly regulate locomotion.

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Highlights

- > We examine whether CRH neuronal effects depend upon the behavioral state of an animal
- > Single neurons were recorded from the rostral medulla in behaving and paralyzed newts
- > Neural activity was recorded before and after intracerebroventricular CRH infusion
- > We found that the actual expression of locomotion is critical for CRH neural effects

Figure 1.

Diagram of roughskin newt brainstem and recording sites (black dots) in the rMRF. A total of 138 neurons were isolated in 19 behaving and 18 immobilized newts. Abbreviations: MB, midbrain; and rMRF, rostral medullary reticular formation.

Figure 2.

Traces of recordings from three representative rMRF neurons recorded simultaneously from a single newt 5 min prior to and 30 min after icv CRH. CRH significantly changed activity in all 3 neurons, enhancing mean firing frequency in 2 of 3 neurons, decreasing it in one (middle trace) and concurrently increasing locomotion (bars at bottom of figure and each inset). Neuronal activity during locomotion was phasic and consisted of frequent bursting with increased firing often preceding and overlapping with bouts of locomotion (inserts). The black horizontal bars represent bouts of locomotor activity, i.e., walking. Although locomotion may include both walking and swimming in a urodele amphibian such as *Taricha*, this animal only displayed walking behavior (no swimming was observed).

Figure 3.

Percentage of total neurons from behaving newts showing changes in overall neuronal activity (i.e. sampled during all behavioral states), during locomotion, and during immobility. Chi-square Tests (two-tailed; $\alpha=0.05$) as a function of treatment condition, revealed that the proportion of neurons showing changed activity was significantly greater for the CRH condition than the VEH ($p<0.05$) or ahCRH + CRH ($p<0.001$) treatment conditions for both overall neuronal activity and activity during locomotion. In contrast, the proportion of neurons that showed changed activity during immobility did not differ across CRH (white bars), VEH (light gray bars) or ahCRH + CRH (dark gray bars) treatment conditions. * $p<0.01$. ** $p<0.001$.

Figure 4.

Percentages of total neurons, across all states combined, that displayed changes in overall neuronal firing following CRH (white bars), VEH (light gray bars), or ahCRH + CRH (dark gray bars) in the behaving (left) and immobilized (right) newts. The proportion of neurons with changed activity was significantly greater following CRH than treatment with VEH or ahCRH + CRH for both behaving and immobilized newts. * $p < 0.05$ ** $p < 0.01$

Figure 5.

Representative autocorrelograms of discharges from 18 neurons recorded in behaving (9 neurons, 3 newts, Neurons A-I) and immobilized (9 neurons, 3 newts, Neurons J-R) newts. Each autocorrelogram was generated from 5 min of pre-CRH and 30 min post-CRH recording. Note the lack of cyclicity of in the autocorrelogram outputs for most neurons from the immobilized group.

Figure 6.

Representative autocorrelograms and cross-correlograms of discharges from two pairs of simultaneously recorded rMRF neurons (A) neuron pairs recorded in behaving and (B) in immobilized newts prior to (pre-treatment) and after (post-treatment) CRH administration.

Figure 7.

Mean (± 1 standard mean error) difference scores for the number of bursts (A) and inter-burst intervals (B) across the behaving (white bars) and immobilized (black bars) groups of newts following CRH, VEH and ahCRH + CRH. (A) Central CRH administration of in behaving newts significantly enhanced neuronal bursting compared to that in immobilized newts. CRH infusion increased the mean number of bursts compared to VEH but this effect was blocked by pre-treatment with ahCRH (see results). CRH administration enhanced the mean number of bursts displayed by neurons sampled in the behaving group compared to immobilized animals treated with CRH (see results). (B) Significant treatment effects for mean IBI latencies were found. CRH administration in behaving newts resulted in a

significant reduction in the mean latencies compared to immobilized animals treated with CRH. * $p < 0.05$. ** $p < 0.01$.

^aSignificant difference between CRH and VEH treatment conditions within behaving group of newts ($p = 0.039$).

^bSignificant difference between CRH and ahCRH + CRH treatment within behaving group of newts ($p = 0.004$).

ns = no significant difference.

Table 1

Effects of CRH treatment on firing periodicity of neurons (n=132) recorded in behaving and immobilized newts.

	Behaving Newts			Immobilized Newts		
	CRH	VEH	ahCRH + CRH	CRH	VEH	ahCRH + CRH
Pre-Treatment						
% periodicity	9 (28.1%)	1 (7.7%)	8 (36.4%)	5 (20.8%)	0 (0%)	5 (23.8%)
% no periodicity	23 (71.9%)	12 (92.3%)	14 (17.3%)	19 (79.2%)	20 (100%)	16 (76.2%)
Post-Treatment						
% periodicity	28 (87.5%)	7 (53.8%)	18 (81.8%)	14 (58.3%)	3 (15%)	9 (42.9%)
% no periodicity	4 (12.5%)	6 (46.2%)	4 (18.2%)	10 (41.7%)	17 (85%)	12 (57.1%)

CRH effects on cross-correlated discharges of neuron pairs (n=214) recorded in behaving and immobilized newts.

Table 2

	Behaving Newts			Immobilized Newts		
	CRH	VEH	ahCRH + CRH	CRH	VEH	ahCRH + CRH
Pre-Treatment						
% positively correlated	24 (37.5%)	5 (41.7%)	21 (41.2%)	3 (8.3%)	1 (3.9%)	4 (16%)
% negatively correlated	0 (0%)	0 (0%)	1 (1.96%)	0 (0%)	0 (0%)	0 (0%)
% uncorrelated	40 (62.5%)	7 (58.3%)	29 (56.8%)	33 (91.7%)	25 (96.2%)	21 (84%)
Post-Treatment						
% positively correlated	36 (56.3%)	7 (58.3%)	33 (64.7%)	9 (25%)	2 (7.7%)	8 (32%)
% negatively correlated	0 (0%)	0 (0%)	1 (1.96%)	5 (13.9%)	1 (3.9%)	0 (0%)
% uncorrelated	28 (43.7%)	5 (41.7%)	17 (33.3%)	22 (61.1%)	23 (88.5%)	17 (68%)