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Research paper

The involvement of Rhopr-CRF/DH in feeding and reproduction in the blood-gorging insect *Rhodnius prolixus*

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ABSTRACT

Rhodnius prolixus is a blood-gorging insect and a vector for human Chagas disease. The insect transmits the disease following feeding, when it excretes urine and feces contaminated with the *Trypanosoma cruzi* parasite. A corticotropin-releasing factor-like peptide acts as a diuretic hormone in *R. prolixus* (Rhopr-CRF/DH); however, its distribution throughout the insect's central nervous system (CNS) and the expression of its receptor in feeding-related tissue as well as the female reproductive system suggests a multifaceted role for the hormone beyond that of diuresis. Here we investigate the involvement of Rhopr-CRF/DH in feeding and reproduction in *R. prolixus*. Immunohistochemistry of the CNS showed diminished CRF-like staining in neurosecretory cells (NSCs) of the mesothoracic ganglionic mass (MTGM) immediately following feeding, and partial restocking of those same cells two hours later, indicating Rhopr-CRF/DH stores in this regions are involved in feeding. The results of the temporal qPCR analysis were consistent with the immunohistochemical findings, showing an increase in Rhopr-CRF/DH transcript expression in the MTGM immediately after feeding, presumably capturing the restocking of Rhopr-CRF/DH in the lateral NSCs following release of the peptide during feeding. Elevating haemolymph Rhopr-CRF/DH titres by injection of Rhopr-CRF/DH prior to feeding resulted in the intake of a significantly smaller blood meal in 5th instars and adults without an apparent effect on the rate of short-term diuresis. When adult females were injected with Rhopr-CRF/DH, they also produced and laid significantly fewer eggs. Finally, *in vitro* oviduct contraction assays illustrate that Rhopr-CRF/DH inhibits the amplitude of contractions of the lateral oviducts, highlighting a potential mechanism via which the hormone diminishes reproductive capacity. To conclude, the study of the Rhopr-CRF/DH pathway, its components and mechanisms of action, has implications for vector control by highlighting targets to alter feeding, diuresis, and reproduction of this disease vector.

1. Introduction

Rhodnius prolixus is a blood-gorging insect and a vector for human Chagas disease, caused by transmission of the parasite *Trypanosoma cruzi*. The disease affects the nervous, cardiovascular, and digestive systems and can be fatal in severe cases (World Health Organization, 2017). It is estimated that 6 to 7 million people are currently infected worldwide, with most cases occurring in Central and South America where *R. prolixus* is endemic (World Health Organization, 2017). Disease transmission occurs after feeding, when the insect takes a huge blood meal, up to ten times its initial body weight, triggering rapid diuresis to eliminate the excess water and salts (Martini et al., 2007). The *T. cruzi*-contaminated urine or feces enters the human host through the feeding wound or following scratching (Centers for Disease Control and Prevention, 2017). The haemolymph of a recently fed insect, as well as tissue homogenates from the mesothoracic ganglionic mass (MTGM) of the central nervous system (CNS), have been shown to potently stimulate Malpighian tubule secretion, leading to diuresis (Te Brugge et al., 2001; Coast et al., 2002; Orchard, 2006, 2009). Initially unidentified, a corticotropin-releasing factor-like hormone (Rhopr-CRF/DH) was later determined to be one of the diuretic hormones in *R. prolixus* contributing to the 1000-fold increase in fluid transport by the Malpighian tubules after feeding, working in concert with serotonin to act on the excretory system and orchestrate postprandial diuresis (Te Brugge et al., 2001; Coast et al., 2002; Orchard, 2006, 2009; Martini et al., 2007).

The mammalian corticotropin-releasing factor (CRF) is a neurohormone involved in the stress response, modulating a number of

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physiological processes in response to stressors, including feeding and reproduction (Majzoub, 2006). There is reason to believe that its ortholog in *R. prolixus* likewise has multifaceted roles, beyond diuresis (Lee et al., 2016).

Corticotropin releasing factor-like hormones have been identified and characterized as diuretics in a number of other insect species, including *Manduca sexta, Tenebrio molitor, Locusta migratoria and Schistocerca gregaria* (Coast et al., 2002; van Wielendaele et al., 2012). Additional roles for the insect CRF have been investigated in some of these insects. In *S. gregaria*, CRF/DH has been found to induce satiety, with insects injected with the peptide prior to feeding taking in a significantly smaller meal relative to water-injected controls (van Wielendaele et al., 2012). It is conjectured that because CRF/DH is released into the haemolymph as the insect feeds, that it may signal the end of feeding. In addition to its role in the regulation of feeding, CRF has been found to have a role in reproductive processes. In *L. migratoria*, Locmi-CRF/DH is co-localized with ovary maturating parsin (OMP) in neurosecretory cells (NSCs) in the pars intercerebralis, and sequence analysis shows these two neurohormones are encoded on the same gene in both *L. migratoria* and *S. gregaria* (Girardie et al., 1998; van Wielendaele et al., 2012). Further study showed that injection of mature *S. gregaria* females with CRF/DH resulted in the production of significantly smaller oocytes, and in reduced levels of ecdysteroids circulating in the haemolymph (van Wielendaele et al., 2012). Knockdown of Schgr-OMP reversed the adverse effects on feeding and reproduction.

During feeding, Rhopr-CRF/DH is released from lateral NSCs of the MTGM to initiate diuresis in *R. prolixus* (Maddrell, 1964; Te Brugge et al., 2001; Orchard, 2009; Lee et al., 2016); however, this peptide is also found in medial NSCs in the brain, with axons projecting to the neurohaemal organ, the corpus cardiacum (CC) (Te Brugge et al., 1999). The NSCs of the MTGM release Rhopr-CRF/DH from neurohaemal sites on abdominal nerves into the haemolymph and can therefore have widespread targets, in addition to those related to diuresis. A Rhopr-CRF/DH receptor is expressed in many tissues in *R. prolixus*, with pronounced expression observed in the testes, the foregut, and the ovaries of 5th instars (Lee et al., 2016). In adults, a significant increase in expression is observed in the mature ovaries, relative to the immature ovaries of 5th instars, suggesting Rhopr-CRF/DH's activity increases in the sexually mature female. The existing data on CRF drives speculation of its potentially complex, and yet to be explored, role in the physiology of *R. prolixus*.

Thus, this study aimed to describe (1) the time course of expression of Rhopr-CRF/DH from pre-to post-feeding through immunohistochemistry and qPCR analysis; (2) the effect of Rhopr-CRF/DH on feeding behaviour through feeding assays; and (3) the effect of Rhopr-CRF/DH on reproductive processes through egg-laying assays and in vitro oviduct contraction assays. Given the current literature we hypothesized that Rhopr-CRF/DH would exhibit inhibitory effects on both feeding and reproduction in *R. prolixus*. Additionally, the effect of serotonin, another diuretic hormone in *R. prolixus* released at feeding (Orchard, 2006), was tested in the feeding assays.

2. Materials and methods

2.1. Animals

Newly-emerged juvenile (5th instar) and adult *R. prolixus*, approximately 1.5 months post-feed on defibrinated rabbit blood (Cedarlane, Burlington, CA) as 4th and 5th instars, respectively, were taken from a colony maintained at 25 °C and high humidity (50%).

2.2. Immunohistochemistry

Fixation and staining were performed as described in Te Brugge et al. (1999). Insects (5th instars and adults) were dissected in physiological saline (150 mM NaCl, 8.6 mM KCl, 2 mM $CaCl_2$, 4 mM $NaHCO_3$, 34 mM glucose, 8.5 mM $MgCl_2$, 5 mM HEPES, H_2O, pH 7), their CNSs removed and fixed in 2% paraformaldehyde for 1 h at room temperature. After fixation, the tissues were washed in phosphate-buffered saline (PBS) before being incubated in 4% Triton for 1 h at room temperature. The tissues were again washed in PBS and then transferred to primary antiserum, containing a polyclonal antisera raised in rabbit against the *L. migratoria* CRF-like hormone (Locmi-DH) at a concentration of 1:1000 in 0.4% Triton and normal goat serum (NGS). The tissues were kept in the primary antiserum solution on a rotator for 48 h at 4 °C. After another round of washing, the tissues were placed in secondary antibody, a 1:600 Cy3-labelled sheep anti-rabbit immunoglobulin in NGS and PBS. Control tissues were prepared following the same protocol with the primary antiserum solution pre-absorbed with 10^{-3} M Rhopr-CRF/DH for 48 h.

Confocal images were analyzed using ImageJ Software (Ferreira and Rasband, 2012) to quantify changes in Rhopr-CRF/DH-like expression (i.e., staining) from pre- to post-feed (0 h and 2 h). Staining intensity was determined by tracing the stained region of the medial NSCs in the brain and the lateral NSCs in the MTGM and assigning grayscale values ranging from 0 (minimum intensity) to 255 (maximum intensity). To ensure accuracy, all images were treated consistently with respect to scale and number of slices in the Z-stacks.

2.3. Feeding assay

Fifth instars were injected with 1 μL 10^{-4} M Rhopr-CRF/DH, 1 μL 10^{-4} M serotonin or 1 μL physiological saline and weighed to record pre-feed weight. Adults were injected with 2 μL 10^{-4} M Rhopr-CRF/DH or physiological saline and also weighed prior to feeding. Twenty-four or 2–3 h after injection, insects were fed on defibrinated rabbit blood for 20 min. This is the typical time for blood-gorging and whilst diuresis is underway during this time, the insect does not eliminate any urine until feeding has ceased. Immediately after feeding, insects were weighed to measure the size of the meal. They were weighed again 30 min later, and then at hourly intervals for four hours to record rate of diuresis.

2.4. Egg-laying assay

Fifth instars were sexed, keeping males and females in separate jars. They were then fed as 5th instars and left in an incubator at 25 °C following a 12-h light/dark cycle. One to 2 weeks after emergence into adults, they were again fed and returned to the incubator. Seventy-two hours later, males and females were placed into vials for mating (1 female: 2 males). Another 48 h later, males were removed from the vials. Females were left alone in the vials and the following day were injected with 2 μL 10^{-3} M Rhopr-CRF/DH or physiological saline. The number of eggs laid by control and exper-

Table 1
Primers for qPCR.

Target	Sequence (5′-3′)
Rhopr-CRF/DH	Fw: CAACCACCATTACAGGAATC Rv: GTCAGCCTGTTTGTATGTCG
Rhopr-beta-actin	Fw: TCATCTCACCAATCTAACCCAC Rv: CATTCCTACCATTACACCCTGAT
Rhopr-rp49	Fw: AGGAGAAATTGGCGCAAG Rv: GAAACCAGTAGGAAGCATGTGT

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