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Insulin-like and testis ecdysiotropin neuropeptides are regulated by the circadian timing system in the brain during larval–adult development in the insect *Rhodnius prolixus* (Hemiptera)

Xanthe Vafopoulou *, Colin G.H. Steel

Department of Biology, York University, Toronto, Canada M3J 1P3

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ABSTRACT

Insulin-like peptides (ILPs) regulate numerous functions in insects including growth, development, carbohydrate metabolism and female reproduction. This paper reports the immunohistochemical localization of ILPs in brain neurons of *Rhodnius prolixus* and their intimate associations with the brain circadian clock system. In larvae, three groups of neurons in the protocerebrum are ILP-positive, and testis ecdysiotropin (TE) is co-localized in two of them. During adult development, the number of ILP groups increased to four. A blood meal initiates transport and release of ILPs, indicating that release is nutrient dependent. Both production and axonal transport of ILPs continue during adult development with clear cytological evidence of a daily rhythm that closely correlates with the daily rhythm of ILPs release from brains *in vitro*. The same phenomena were observed with TE previously. Double labeling for ILPs and pigment dispersing factor (PDF) (contained in the brain lateral clock cells, LNs) revealed intimate associations between axons of the ILP/TE cells and PDF-positive axons in both central brain and retrocerebral complex, revealing potential neuronal pathways for circadian regulation of ILPs and TE. Similar close associations were found previously between LN axons and axons of the brain neurons producing the neuropeptide prothoracicotropic hormone. Thus, the brain clock system controls rhythmicity in multiple brain neurohormones. It is suggested that rhythms in circulating ILPs and TE act in concert with known rhythms of circulating ecdysteroids in both larvae and adults to orchestrate the timing of cellular responses in diverse tissues of the animal, thereby generating internal temporal order within it.

1. Introduction

Internal temporal order in higher organisms involves regulation of the timing of diverse biochemical, physiological and behavioral events relative to each other and to the external world, such that they become orchestrated into adaptively significant sequences. Most of these sequences are repeated each solar day, but they are seldom direct responses to the environment. Rather, they are regulated by endogenous cellular mechanisms that generate a periodicity of about 24 h. This circadian periodicity is generated by specialized 'clock cells', usually located in the nervous and/or endocrine systems. These cells are synchronized (entrained) with the external solar world to precisely 24 h and generate rhythmic outputs in nervous pathways and/or release of hormones. These outputs drive rhythmic responses in target cells and tissues. Circadian timing systems have been most extensively studied in insects and mammals; many similarities exist in the two systems from molecular mechanisms, (e.g. [7,32]) to organizational principles, e.g. [4,53].

While the functional significance of rhythms in mammalian hormones has received extensive attention, relatively little information is available for insects (reviewed by [46]). This laboratory has focused on circadian control of hormone rhythms in insects, with emphasis on their regulation and functional significance. We employ the blood feeding bug, *Rhodnius prolixus*. This species catalyzed development of the field of insect physiology over 80 years ago (reviewed by [57]). Blood feeding is required to initiate each cycle of growth and development (in the larva) or reproduction (in the adult) and this results in very precise timing of these hormonally controlled processes, which greatly facilitates studies of their circadian control.

Abbreviations: CA, corpus allatum; CC, corpus cardiacum; DAB, 3,3-diaminebenzidine hydrochloride; FITC, fluorescein isothiocyanamide; ILPs, insulin-like peptides; LNs, lateral clock neurons; MNC, medial neurosecretory cells; NCC, nervus corpus cardiacum; PDF, pigment dispersing factor; PDH, pigment dispersing hormone; PGs, prothoracic glands; PNC, posterior neurosecretory cells; PPA, primary arborization area; PTTH, prothoracicotropic hormone; TE, testis ecdysiotropin; TRITC, tetramethylrhodamine isothiocyanate.

* Corresponding author. Address: Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J1P3. Fax: +1 416 736 5698.

E-mail address: xanthev@yorku.ca (X. Vafopoulou).

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The neuroanatomical organization of the circadian timing network in the brain of *Rhodnius* has been analyzed in both the larval and adult stages [48,51]. This network has been shown to regulate rhythmicity in the release of prothoracicotropic hormone (PTTH) from the brain in larvae [41,42] and testis ecdysiotropin (TE) from the brain in adults [45]. TE is a small neuropeptide first identified in the MNC of the moth *Lymantria dispar* [19] where it acts as a testis ecdysiotropin [16,55]. Synthetic TE stimulates production of the steroid hormones, ecdysteroids, by adult testes of *Rhodnius in vitro* [45]. Both PTTH and TE regulate production of ecdysteroids, which exhibit a pronounced circadian rhythm in the hemolymph of both larvae [2,40] and adults [49]. Ecdysteroids act on almost all developing cells of the insect, and circadian cycling of the ecdysteroid receptor (EcR) is also seen in numerous tissues ([47]). This wide range of targets of ecdysteroids makes them well suited to convey timing information to target cells that lack access to other temporal signals. We therefore regard ecdysteroids as 'messengers of time' to their targets (reviewed by [38].)

The present paper addresses the generality of the concept of hormones as 'messengers of time' by examining the neurons in the brain that produce insulin-like peptides (ILPs) and TE for evidence of rhythmicity and association with the clock cells in the circadian timing system. Bombyxin is an insulin related peptide [11] and bombyxin antibodies have been used widely to identify cells that produce ILPs in various insects e.g. [61,31,15,52]. Bombyxin has attracted considerable attention from physiologists due to its apparently diverse range of target tissues. It has been described as an ecdysiotropin [23], a regulator of growth [29,30], metamorphosis [14], hematopoiesis [26,35], sugar metabolism [36,37,18] and of ovarian development [5]. The diversity of reported targets of ILPs encourages the expectation that bombyxin and related ILPs may also be important hormonal messengers of time.

In *Rhodnius* larvae, synthetic bombyxin has mild ecdysteroidogenic activity on prothoracic glands (PGs) [43], indicating that receptors for ILPs exist in *Rhodnius*. Moreover, *Rhodnius* brains *in vitro* release ILPs with a daily rhythm [44]. The present paper reports that there are several groups of cells in the brain of *Rhodnius* that produce ILPs, all of which have associations with the brain circadian timing system. All the cells exhibit structural evidence of rhythmicity. The cells of two groups express both ILPs and TE. The findings are discussed in relation to the growing repertoire of neurohormones that potentially convey temporal information to target tissues.

2. Materials and methods

2.1. Animals

Male fifth (last) instar larvae of *Rhodnius* were reared at 28 ± 0.5 °C in a cycle of 12 h light:12 h dark. Unfed larvae are developmentally arrested. A blood meal given to larvae initiates adult development and a blood meal given to adults initiates a cycle of reproduction. The day of feeding was designated day 0. Ecdysis to the adult is under circadian control [1] with a gate median on day 21 after feeding. For localization and developmental studies, brain complexes (the brain, corpus cardiacum-corpus allatum (CC–CA) and suboesophageal ganglion (SEG), were dissected in scotophase at 7 h after lights-off at different days of development as detailed in Results. For studies of daily rhythms, brain complexes were dissected twice a day, in the middle of scotophase (7 h after lights-off) and in the middle of photophase (7 h after lights-on). At least five animals were examined at each time point within a day or during development.

2.2. Antibodies and chemicals

A guinea pig polyclonal antibody against a synthetic decapeptide corresponding to the N-terminus (GIVDECCLRP) of the A-chain of bombyxin II [20] was a generous gift of Dr. A. Mizoguchi (Nagoya University, Japan). This decapeptide represents a highly conserved region of all bombyxin variants (references in [20]). Synthetic bombyxin II [24] has a mild steroidogenic activity on *Rhodnius* prothoracic glands *in vitro* showing that *Rhodnius* possesses receptors for bombyxin [43]. The specificity of the bombyxin antibody to recognize native ILP(s) in *Rhodnius* has been validated by Vafopoulou and Steel [44]; for example, this antibody removed all bombyxin-like peptides released into incubation media by brains *in vitro*. For the present work, we also found that double immunoprecipitation of brain incubation medium with excess anti-bombyxin and anti-guinea pig IgG removed all immunoreactivity from Western blots.

A rabbit polyclonal antibody against synthetic pigment dispersing hormone (β-PDH) of *Uca pugilator* (NSELINSILGLPKVMNDA) has been used extensively in our laboratory to trace the axonal projection of *Rhodnius* clock cells in both larvae [51] and adults [48]. The antibody was a generous gift of Dr. K. Rango Rao (University of West Florida, Florida). This antibody recognizes the insect homologues of PDH (known as pigment dispersing factors, PDFs) and has also been used in tracing axonal projections of putative clock cells in various other insects e.g. [9,13]. The PDH antiserum has been shown to recognize the native PDFs of *Rhodnius* [51]; for example, pre-adsorption of the antiserum with excess PDH peptide completely abolished staining in immunohistochemistry.

A rabbit polyclonal antibody against amino acids 1-11 (ISDFDEYEPLN) at the N-terminal of *Lymantria* testis ecdysiotropin (TE) was a kind gift from Dr. M. Loeb (USDA, Beltsville, MD). This antibody recognizes the native TE in its host *Lymantria dispar* [19]. In *Rhodnius*, the antibody recognizes TE-related peptides in the brain-retrocerebral complex and the synthetic peptide acts as a testis ecdysiotropin *in vitro* [45]; see also Section 4.

BLAST searches of the trace archive of the *Rhodnius* genome revealed that the genome contains specific nucleotide sequences with 96% identity to the coding sequences for the amino acid sequences used for production of the bombyxin, PDF and TE antibodies, respectively.

Goat anti-guinea pig IgG conjugated to horseradish peroxidase, goat anti-guinea pig IgG, goat anti-rabbit IgG conjugated to the green fluorophore fluorescein isothiocyanate (FITC), goat anti-guinea pig IgG conjugated to the red fluorophore tetramethylrhodamine isothiocyanate (TRITC) and 3,3-diamine benzidine hydrochloride (DAB) were all purchased from Sigma–Aldrich (St. Louis, MO). Vectashield mounting medium was purchased from Vector Laboratories, Burlington ON, Canada. Kaleidoscope standards and Tris–Tricine–SDS gels were purchased from BioRad (Hercules CA).

2.3. Immunohistochemistry and imaging

Brain complexes were fixed in 4% buffered paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) and processed using standard procedures (see [51,48]). All primary antibodies were used at 1:1000 dilution. For single label with anti-bombyxin, a goat anti-guinea pig IgG conjugated to FITC was used as a secondary antibody at a 1:100 dilution. For double label experiments with anti-bombyxin and anti-PDF, or anti-bombyxin with anti-TE a goat anti-guinea pig IgG conjugated to TRITC and a goat anti-rabbit IgG conjugated to TRITC were used, both at 1:100 dilution. When the primary antibody was replaced with non-immune serum no autofluorescence was detected, and when the secondary antibody was replaced with PBS fluorescence levels were indistinguishable from