Journal of Insect Physiology 55 (2009) 435–440

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

The release of a pheromonotropic neuropeptide, PBAN, in the turnip moth *Agrotis segetum*, exhibits a circadian rhythm

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ARTICLE INFO

Article history:
Received 1 September 2008
Received in revised form 27 October 2008
Accepted 28 October 2008

Keywords:
Pheromone biosynthesis activating neuropeptide (PBAN)
Circadian rhythm
Agrotis segetum

ABSTRACT

In the female turnip moth, *Agrotis segetum*, a pheromone biosynthesis activating neuropeptide (PBAN) stimulates sex pheromone biosynthesis which exhibits a daily rhythm. Here we show data supporting a circadian rhythm in PBAN release from the corpora cardiaca, which we propose regulates the endogenous rhythm in sex pheromone biosynthesis. This conclusion is drawn as the observed daily rhythm in PBAN-like immunoreactivity in the hemolymph is persistent in constant darkness and is phase-shifted by an advanced light:dark cycle. PBAN-like immunoreactivity was found in the brain, the optic lobe, the suboesophageal ganglion and in the retrocerebral complex. In each hemisphere ca. 10 immunopositive neurons were observed in the *pars intercerebralis* and a pair of stained somata in the dorso-lateral protocerebrum. A cluster of cells containing PBAN-like immunoreactive material was found in the tritocerebrum and three clusters of such cells were found in the SOG. Their processes reach the corpora cardiaca via *nervi corporis cardiaci* and the dorsal surface of the corpora allata via the *nervi corporis allati*.

1. Introduction

Most species of nocturnal moths restrict mating activity and the preceding sex pheromone communication to discrete hours of the night. This enables them to synchronize reproductive behaviour and physiology both between the sexes and with predictable daily variation in the environment. Pheromone biosynthesis activating neuropeptide (PBAN), a 33 or 34 amino acid neuropeptide, stimulates sex pheromone production in female moths and supposedly plays a key role in temporal regulation of pheromone communication (Raina et al., 1989). Previous studies indicated that PBAN at least partly functions as a humoral factor, as the scotophase hemolymph shows both pheromonotropic activity and contains PBAN (Iglesias et al., 2002).

PBAN is produced in the suboesophageal ganglion (SOG) and is transported to the corpora cardiaca (CC) before its release into the hemolymph (Tillman et al., 1999; Rafaeli, 2002; Jurenka, 2004). Strong PBAN-like immunoreactivity (PBAN-ir) has been found in several clusters of neurosecretory cells in the mid-ventral SOG in several moth species, as well as in the CC (e.g. Kingan et al., 1992; Ma et al., 1998; Duportets et al., 1998; Choi et al., 2004). SOG extracts of several species, including *Agrotis segetum*, also show high pheromonotropic activity regardless of when the tissue was dissected (Raina and Klun, 1984; Marco et al., 1996; Zhu et al., 1995), while the noctuids *Spodoptera littoralis* and *Mamestra brassicae* show significantly higher pheromonotropic activity in the hemolymph during the scotophase than the light phase (Iglesias et al., 1999). Synthetic PBAN has been shown to induce pheromone biosynthesis in both isolated pheromone glands of *S. littoralis* (Raina et al., 1989; Fabriás et al., 1994) and in isolated abdomens of *A. segetum* (Rosén, 2002), while Iglesias et al. (2002) convincingly showed that PBAN is present in scotophase hemolymph from noctuid *S. littoralis*. These findings suggest that PBAN functions as a humoral factor and that pheromone production is regulated by a daily rhythm in PBAN release from the CC. Recently a G protein-coupled PBAN receptor was identified in *Helicoverpa zea* (Choi et al., 2003; Choi and Jurenka, 2004), further supporting a humoral route of action.

In the turnip moth, *A. segetum*, the daily rhythm in pheromone content of the pheromone gland is persistent in constant darkness and is phase-shifted by an advanced light:dark cycle, supporting that a circadian clock mechanism is involved in the pheromone biosynthesis rhythm (Rosén, 2002). This is further supported by

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doi:10.1016/j.jinsphys.2008.10.016

circadian firing patterns in PBAN-producing and releasing cells in *B. mori* (Tawata and Ichikawa, 2001), as well as immunoreactivity to several clock cell proteins in axons of brain neurons projecting to the CC (Sehadová et al., 2004; Sauman and Reppert, 1996; Wise et al., 2002; Závodská et al., 2003). This study aims to characterize the endogenous rhythm in PBAN-ir in female and male *A. segetum* hemolymph. We also present complementary immunocytochemistry data of cells expressing PBAN in the *A. segetum* brain-retrocerebral complex.

2. Materials and methods

2.1. Insects

A laboratory culture of *A. segetum*, originating from Denmark and southern Sweden, was maintained in a reversed 17:7 light:dark (L:D) cycle with lights-on at 19:00 (Zeitgeber time 0, ZT 0), lights-off at 12:00 (50% R.H., 23 °C). Larvae were reared on an artificial bean-based diet (Zhu et al., 1996). Pupae were separated by sex, maintained in separate climate chambers and transferred to a new box 1–2 h before each scotophase. Newly eclosed animals were maintained in a fresh box with access to ca 15% honey water.

2.2. Hemolymph sample collection and preparation

Hemolymph samples were collected from 3-day-old animals from four different treatment groups: females reared under ordinary L:D cycle, males in an ordinary L:D cycle, females subjected to constant darkness since lights-off (12:00) the previous day and females exposed to a shifted light:dark cycle. In the last group, females were transferred to a scotophase advanced by 4 h 1 day after eclosion (defined as 1-day old). The third shifted scotophase was then prolonged into constant darkness until the insects were sacrificed the following day (when 4-day-old).

Animals were sacrificed during the day at nine different time points: ZT 0, ZT 2, ZT 6, ZT 10, ZT 14, and ZT 16 (the light phase) and ZT 18, ZT 20, ZT 22 (the dark phase). Hemolymph samples were collected according to Marco et al. (1995). Decapitated individuals were placed individually, thorax downwards, in 500 μl Eppendorf tubes with the bottom tips removed, which were inserted in larger 1.5 ml Eppendorf tubes. Animals were subsequently centrifuged at 2 000 rpm for 6 min at 4 °C. Hemolymph samples of 10 μl from each individual were collected and diluted in 180 μl of 80% ethanol and vortexed for at least 5 s. Samples were centrifuged at 12 000 rpm for 5 min at 4 °C, supernatants were collected and briefly frozen (−80 °C) before freeze-drying. Hemolymph samples were stored at −80 °C until analyzed by ELISA (see Section 2.4 below). Scotophase animals, and animals in constant darkness, were handled under red light until the centrifugation step.

2.3. Quantitative enzyme-linked immunosorbent assay (ELISA) of PBAN

2.3.1. Chemicals and buffers

The PBAN contents of all samples were determined by a competitive ELISA procedure, developed and described in further detail by Marco et al. (1995). The rabbit PBAN antibodies used were produced targeting *H. zea* PBAN (Hez-PBAN) linked to keyhole limpet hemocyanin by a cysteine residue and N-succinimidyl-4-(maleidimidomethyl)-cyclohexane carboxylate (Marco et al., 1995). The following buffers were used: coating buffer: 0.5 M carbonate–bicarbonate buffer, pH 9.6; PBST: 0.2 M phosphate buffer, 0.8% NaCl, 0.05% Tween-20, pH 7.5; substrate buffer: 0.1 M sodium citrate buffer, pH 5.5.

2.3.2. ELISA of hemolymph samples

A total of 13 microtiter plates with hemolymph samples were run and 10 of these were complete, i.e. contained exactly one sample from each time point and treatment group. Care was taken, as far as possible, to include an equal number of samples from each time point in every treatment series represented on a plate. To avoid systematic errors, equivalent samples were not applied to equivalent wells on different plates. The total data set includes 14 samples from each time point and treatment group except males ZT10 (10 samples), males ZT20 (13 samples) and males ZT18 (13 samples).

Each ELISA microtiter plate was coated overnight with Cys-Hez-PBAN (20–33) linked to bovine serum albumine (BSA) by a dimethylpimelimidate unit (0.3 μg/ml in coating buffer, 50 μl/well, 4 °C). The same day, each freeze-dried hemolymph sample, and a series of standard Hez-PBAN solutions with concentrations ranging from 0.1 pM to 100 nM, were diluted in 25 μl of PBST and added to 25 μl of PBAN antibody (1:25,000 in PBST) before incubation overnight at 4 °C. The following day, microtiter plates were washed thoroughly with PBST and unspecific binding sites blocked by 100 μl of 3% non-fat milk in PBST for 1 h at room temperature (RT). After an additional wash step with PBST, samples and standards were added to the wells for at least 90 min at room temperature. After washing with PBST, horseradish peroxidase-conjugated anti-rabbit IgG (50 μl, 1:6000 in PBST; Jackson Immunoresearch) was added to each well and incubated for 1 h at room temperature. After another wash step (in PBST), 50 μl of 0.01% tetramethylbenzidine with 0.004% H_2O_2 in substrate buffer was added to each well for approximately 20–30 min. The colour reaction was stopped by addition of 25 μl 4 M sulphuric acid and absorbance values obtained at 465 nm using a Labsystems Multiscan Multisoft ELISA plate reader.

Two logarithmic standard curves with synthetic PBAN, with concentrations ranging from 0.1 pM to 100 nM PBAN (25 μl/sample) and a buffer blank, were calculated for each microtiter plate (Fig. 1). First of the standard curve was obtained using synthetic Hez-PBAN diluted in PBST (Fig. 1; black curve). Second standard curve was prepared with each reference sample containing both synthetic Hez-PBAN and 10 μl of freeze-dried hemolymph, collected and treated to an ethanol:water solution as described above (Fig. 1; grey line). This hemolymph was collected 5 h before the onset of the scotophase, when very low amounts of PBAN were expected, and averaged by physical pooling of the hemolymph of 18 individuals. Addition of hemolymph to the samples used for the reference curve decreased the obtained absorbance values at many of the plates but the relative decrease in absorbance values (*R*-value in Fig. 1) varied substantially between plates. A theoretical standard curve was therefore calculated for each plate by visually fitting it to the two standard curves not spiked with hemolymph (equation is found in Marco et al., 1995). This approach may have resulted in calculated PBAN contents higher than the actual values and skewed the relative differences in PBAN content between samples. It does not, however, alter the ranking order of PBAN content between samples on the same plate. On the two plates run without a reference curve spiked with hemolymph (in total three samples from almost all time points and treatment groups), the highest absorbance values of the reference graphs were much lower than absorbance values obtained in the sample wells. The reference graphs for these two plates with samples were therefore adjusted (increased) to an estimated value. This may also have affected the amounts of PBAN obtained but not the relative ranking order of samples on the same plate.

The upper and lower reliable detection limits were defined from each theoretical reference graph. The end points of this reliable

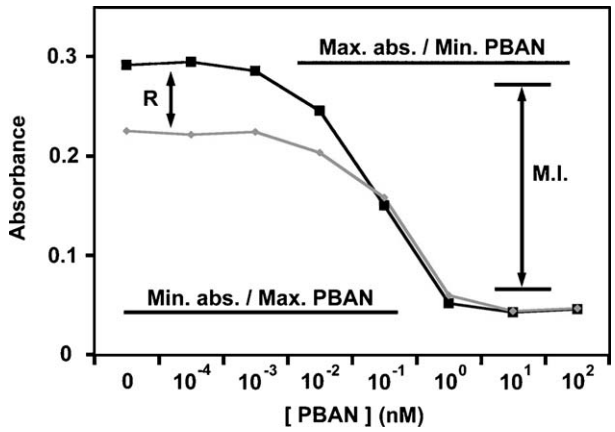

Fig. 1. Example of reference graph for ELISA measurements (black curve). A low PBAN amount in a sample results in a high absorbance value, and vice versa, within a limited absorbance interval. For very high and low PBAN concentrations, the slope of the reference graph is too flat and only samples with absorbance values within the measurement interval (M.I.) were considered reliable. The highest and lowest reliable absorbance values, i.e. the end points of the M.I. interval, were calculated as the absorbance value 10% below/above the maximal/minimum absorbance limit of the theoretical standard curve. In some of the reference graphs, the PBAN reference samples were spiked with cell culture medium or daytime hemolymph. This usually resulted in a reduction (R) of the high absorbance limit (grey curve), though the relative decrease often varied between different runs. The PBAN concentrations at the *x*-axis refer to the reference solutions used to obtain the reference graphs.

measurement interval (M.I.) were calculated as the absorbance value 10% below/above the maximal/minimum limit of the theoretical standard curves (as shown in Fig. 1). The microtiter plates with hemolymph samples showed higher variation than plates with dissected samples with a lower detection limit of 9 ± 4 pM (S.D.) and high limit of 635 ± 128 pM (S.D.). This corresponds to 0.2–16.3 fmol PBAN-like affinity per 10 μl of hemolymph. No samples exceeded the high detection limit on any plate. Three samples with absorbance values just outside the reference curve were recorded by zero PBAN (DD CT10, CT2 and CT6).

2.4. Immunocytochemistry on paraplast sections

Immunocytochemistry of *A. segetum* brains, including the SOG, and ovipositors was performed with the same primary PBAN antibody (Hez-PBAN)) as for the ELISA measurements.

Tissues were dissected under red light during the last 5 h of the scotophase and were instantly fixed at 4 °C overnight in modified Bouin–Hollande solution (0.7% mercuric chloride, no acetic acid). All tissue was then washed in 70% ethanol and treated by a series of dehydrating ethanol solutions, ending with chloroform before transfer to melted paraplast. After incubation at 58 °C overnight in a vacuum oven, the paraplast samples were cooled to room temperature (RT), 7 μm sections were attached to microscope slides and dried on a hot plate (42 °C) for at least 48 h. After deparafinisation in xylene, the sections were rehydrated through a series of ethanol:water solutions, incubated in Lugol's iodine (3 min) followed by 7.5% sodium thiosulfate (5 min) to remove residual heavy metal ions, and subsequently washed in distilled water and phosphate-buffered saline (0.3% Tween-20 [PBST]). Blocking with normal goat serum (10% in PBST, 30 min at RT) was followed by incubation with the primary antibody diluted in PBST overnight at 4 °C in sealed humidified boxes. After rinsing with PBST (3× 10 min at RT), samples were incubated for 1 h at RT with goat anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, 1:1000 in PBST). The slides were then washed in PBST (3× 10 min at RT), followed by a final wash in 0.05 M Tris–HCl buffer, pH 7.4, for 10 min at RT. Staining was obtained with hydrogen peroxidase (0.005%) and 3,3′-diaminobenzidine tetrahydrochloride (0.25 mM) as substrates in 0.05 M Tris–HCl, pH 7.4 (25–30 min;). After staining, the sections were washed in distilled water, dehydrated in an ethanol series and mounted in

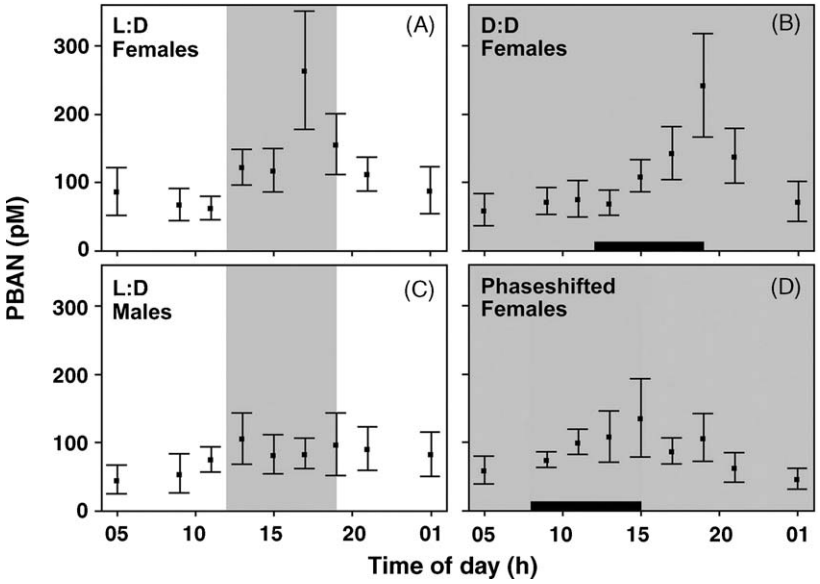

Fig. 2. Daily variation in PBAN amount in *Agrotis segetum* hemolymph estimated by ELISA. Concentration values refer to the reference solution used in the ELISAs and 100 pM correspond to approximately 2.5 fmol PBAN-like immunoreactivity per 10 μl of hemolymph. Error bars show the 95% confidence interval for samples from the same time point. $N = 14$ samples for almost all time points and treatment groups. (A) Females maintained in an L:D cycle. (B) Females subjected to constant darkness since lights-off (12:00) the previous day. The black bar at the bottom of the graph shows the scotophase the animals were reared in. (C) Males maintained in a L:D cycle. (D) Phase-shifted females exposed to an L:D cycle advanced by 4 h for 3 days. Samples were collected after an additional day in constant darkness. The black bar at the bottom of the graph shows the shifted scotophase, to be compared with the unshifted scotophase shown in the graph above (B).

DPX mounting medium. All sections were examined by light microscopy, either by a Zeiss Axioplan (Zeiss) or Olympus BX41 microscope. Photographs were obtained by an Olympus DP200 digital camera.

PBAN antiserum (dilution 1:1000) was used for staining the brain-SOG complexes of seven females and four males; three female brain-SOGs and two ovipositors were dissected in light at ZT12 (5 h before the scotophase). The PBAN results are primarily

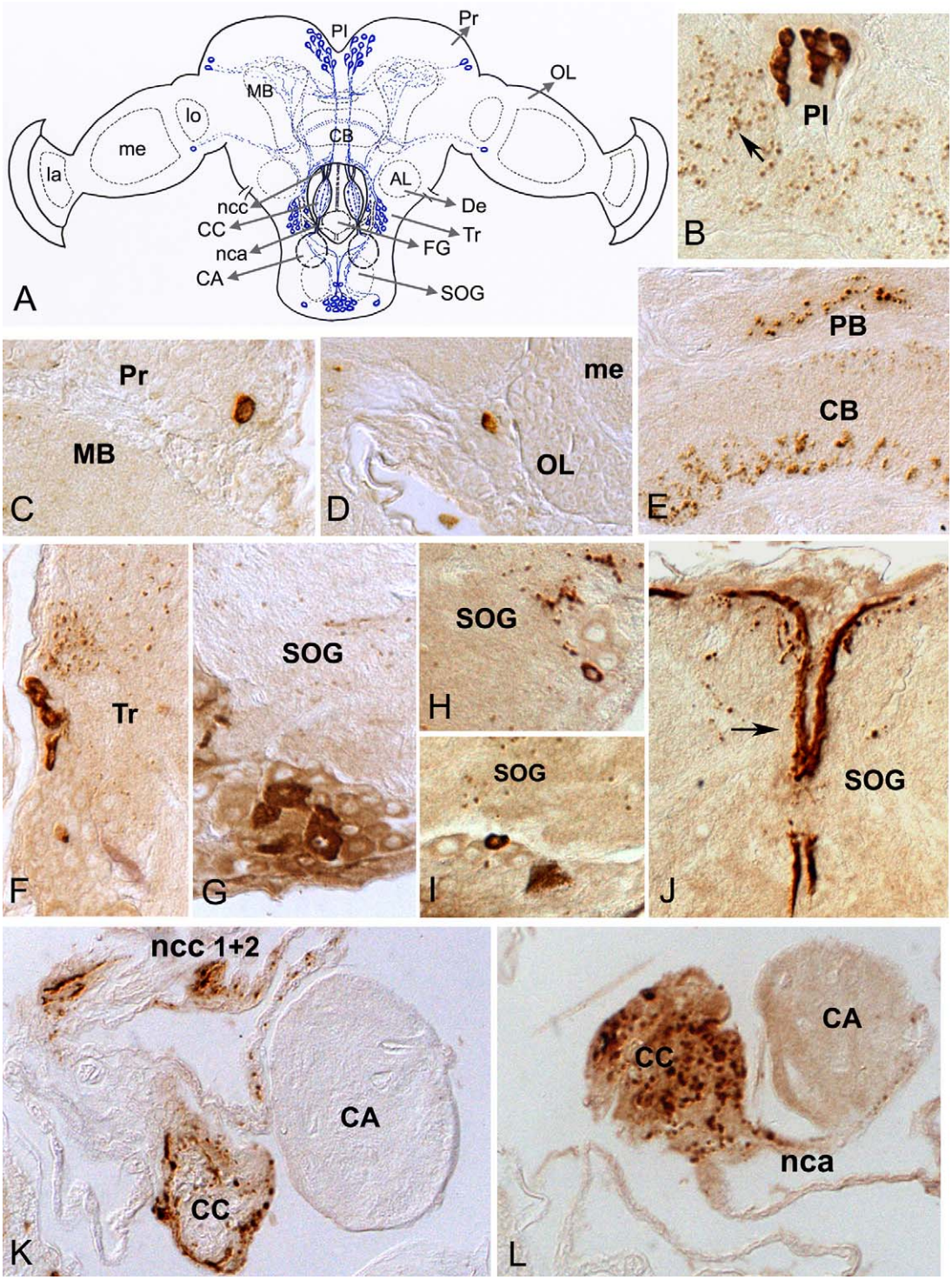

Fig. 3. PBAN-immunoreactive cells in the brain, the suboesophageal ganglion and neurohemal organs of the female *A. segetum*. (A) A schematic diagram illustrating the topography of PBAN-ir cells and axons. (B) Strongly stained cells in the *pars intercerebralis*. (C) An immunopositive cell located in the dorso-lateral region of the protocerebrum. (D) PBAN-ir in a single cell located fronto-ventrally at the proximal edge of the medulla in the optic lobe. (E) Axonal projections in the region of the protocerebral bridge and the mushroom body in the frontal protocerebrum. (F) Stained cells in the tritocerebrum. (G) Large, strongly stained cells in the labial neuromere of the SOG. (H) A small cell in the ventro-lateral region of the SOG. (I) A pair of perikarya located in the maxillary neuromere of the SOG. (J) Heavily stained fibres in the middle part of the SOG. (K) PBAN-ir in the corpora cardiaca. (L) Strong staining in the corpora cardiaca and no PBAN-ir in the corpora allata. *Abbreviations*: corpora cardiaca (CC), corpora allata (CA), optic lobe (OL), pars intercerebralis (PI), pheromone gland (PG), protocerebral bridge (PB), central body (CB), suboesophageal ganglion (SOG), tritocerebrum (Tr), *nervi corporis cardiaci* (NCC), *nervi corporis allati* (NCA), protocerebrum (Pr), mushroom body (MB), frontal ganglion (FG), lamina (la), medulla (me) and lobula (lo). Scale bar 100 μm.

based on the female scotophase samples, but no obvious differences between the sexes were detected.

3. Results

3.1. PBAN-ir in the hemolymph

Lower amounts of PBAN were found in female hemolymph collected before and after the scotophase than during the latter part of the night in an L:D cycle (Fig. 2A). The amounts of PBAN per 10 μl hemolymph varied from ca. 2–3 fmol during the daytime (80 pM) to 6.6 fmol (264 pM) at the late scotophase when there were detected approximately three times higher amounts of PBAN-ir than during the daytime. These differences were persistent after 1 day in constant darkness, with a delay of the peak by 2 h (Fig. 2B). Phase-shifted females, exposed to a scotophase advanced by 4 h for 3 days, showed a less distinct peak in hemolymph PBAN than L:D and D:D females, but the highest average value (135 pM; 3.4 fmol per 10 μl of hemolymph) was obtained at the end of the shifted scotophase, 4 h earlier than in unshifted females after the same treatment (though this amount was not significantly higher than the values obtained 2 h before and after) (Fig. 2D). Males showed no daily changes of amounts of PBAN-ir in the hemolymph, with average values of 1.2–2.6 fmol (46–106 pM) per 10 μl hemolymph. There was a trend towards higher values during the scotophase but generally the male values were similar to the light phase values of females (Fig. 2C). A biological rhythm may be reliably described as circadian if it shows cycles of approximately 24 h, is persistent in a constant environment and is phase-shifted in time by some temporally reliable external cue. We can therefore draw the conclusion that female *A. segetum* show a circadian rhythm in PBAN-ir in the hemolymph.

3.2. PBAN-ir in the brain and the pheromone gland

Immunocytochemistry with Hez-PBAN antiserum revealed 10 strongly stained cells in the *pars intercerebralis* (PI) in each hemisphere (Fig. 3A and B). Clear cytoplasmic staining occurred in an additional pair of cells located in the superior lateral region of the protocerebrum (Fig. 3A and C). Axonal projections of these cells ramified in the frontal protocerebrum forming a meshwork above the calyx and peduncles of the mushroom body, and connected the opposite hemisphere through the protocerebral bridge and the central body (Fig. 3A and E). This bundle of fibres included projections of the PI cells passed across to the base of the protocerebrum sending branches to ipsilateral cardiacal nerves (Fig. 3A and K). A single small cell located in the proximal fronto-ventral part of the optic lobe seemed to be conjoined to stained axons in the ventro-lateral protocerebrum (Fig. 3A and D). PBAN-ir occurred in a single cell located fronto-ventrally at the proximal edge of the medulla in each optic lobe.

Ten to 12 clearly stained cells were detected in the tritocerebrum. Their axons headed towards the base of the protocerebrum via deutocerebrum while other fibres continued to the suboesophageal ganglion (SOG) that harboured ca12 large PBAN-ir positive neurones located in the labial neuromere ventrally while a small cell in each hemisphere was located more laterally (Fig. 3A, G and H). An additional pair of perikarya was detected in the region of maxillary neuromere (Fig. 3A and I). Their strongly stained processes (Fig. 3A and J) continued via circumesophageal connectives to the tritocerebrum. The corpora cardiaca (CC) showed very strong immunoreactivity (Fig. 3K). Slightly stained axons followed via the *nervi corporis allati* the dorsal surface of the corpora allata (CA, Fig. 3L). The corpora allata themselves demonstrated no immunoreactivity.

No staining in the ovipositor was obtained with the PBAN antiserum.

4. Discussion

PBAN is a member of the multifunctional pyrokinin/myotropin family of peptides (Altstein, 2004) and has been identified with highly conserved sequences in several moth species. The high degree of conservation suggests a vital function in moth physiology and multiple studies indicate that PBAN stimulates sex pheromone biosynthesis in female moths (reviewed in Rafaeli, 2002). The study presented here evolved from the hypothesis that PBAN functions as a humoral factor and that the previously reported circadian rhythm in pheromone biosynthesis in *A. segetum* (Rosén, 2002) is regulated by controlled release of PBAN into the hemolymph. In support of this, we here show that the hemolymph of female *A. segetum* displays a daily rhythm in PBAN-ir. The rhythm was persistent after 1 day in constant darkness, and a L:D cycle advanced by 4 h shifted the highest amounts of hemolymph PBAN-ir by the corresponding number of hours. This strongly indicates that the daily rhythm in hemolymph PBAN-ir is regulated by a circadian clock mechanism. Males, which do not produce any known sex pheromone, showed no higher amounts of PBAN-ir in hemolymph, though slightly higher average amounts were detected during the scotophase than the preceding light phase. We also confirm, as has been previously shown in other moth species, that neurons immunostained with the antibody targeting PBAN project to the corpora cardiaca in *A. segetum*.

The highest amount of PBAN-ir in the hemolymph appears 3–5 h into the scotophase, which corresponds well with previous studies of sexual rhythms in *A. segetum*. Rosén (2002) showed that pheromone glands of female *A. segetum* contain highest amounts of the main sex pheromone component during the mid-scotophase (4 h after lights-off), while Löfstedt et al. (1982) found that highest calling activity occurred 4.5 h into the scotophase. This mid-scotophase peak in female sexual activity also corresponds very well with the daily rhythm in male pheromone response (Rosén et al., 2003), though the peak in male response is temporally wider than the female pheromone release. This temporal concordance between studies of several interrelated behaviours and processes supports our assumption that the peak in hemolymph IR is indeed related to sex pheromone biosynthesis in the pheromone gland. This is the first study that demonstrates a circadian rhythm in PBAN content in the hemolymph.

To support the hypothesis of circadian release of PBAN from the CC, we performed immunocytochemical studies of the distribution of PBAN-like peptides in the brain of *A. segetum* (see Fig. 3). The distributions of this protein have previously been studied in other moths but never in the same species. The circadian activity of the PBAN-producing cells, which project to the CC, is further supported by Tawata and Ichikawa (2001). In their study of *Bombyx mori*, these cells showed distinctly circadian firing patterns, which were strongly affected by light conditions.

Previous studies revealed that three clusters of neurosecretory cells in the midventral SOG show strong immunoreactivity towards PBAN in *H. zea*, *B. mori* and *A. ipsilon* (Kingan et al., 1992; Ma et al., 1996; Ichikawa, 2002; Duportets et al., 1998; refs in Rafaeli, 2002). In *H. zea*, the two anterior clusters feature projections to the maxillary nerve and VNC, while the posterior cluster projects to the CC (Kingan et al., 1992; Ma et al., 1996). Cells producing PBAN in *B. mori* also project axons to the CC via the maxillary nerve (Ichikawa, 2002). Three groups of PBAN-like positive cells were distributed in the mandibular, maxillary and labial neuromeres in *Samia cynthia ricini* (Wei et al., 2004) and the tortrix *Adoxophyes* sp. (Choi et al., 2004).

The two clusters of strongly stained neurons we observed in the *A. segetum* SOG correspond to the medio-ventral position in the maxillary/labial neromeres. A lateral pair of small immunopositive cells was located at the level of the labial neuromere.

The anti-PBAN serum stained cell group (10 somata) in the *pars intercerebralis* and a pair of cells in the superior lateral protocerebrum in each hemispheres. Choi et al. (2004) detected three pairs of PBAN-ir positive neurons in the dorso-lateral protocerebrum. Duportets et al. (1998) observed weak staining in the brain near the mushroom bodies. A pair of somata in the dorso-lateral part of the protocerebrum was revealed by the Hez-PBAN antiserum in *Drosphila melanogaster* (Choi et al., 2001). Immunopositive fibres from the pars intercerebralis most probably lead into the *nervi corporis cardiaci* (NCC 1 + 2) and ramify in the CC. The immunostained cells in the tritocerebrum send their processes via the NCC 1 + 2 into the CC. These results can suggest that PBAN-like peptides synthesised in the neurosecretory cells of the SOG may be released into the hemolymph by the CC. The understanding of regulation of PBAN release is required to understand the role of circadian clocks in moth reproductive physiology.

Acknowledgements

BSA-linked PBAN and primary PBAN antibodies were gratefully received from Dr. Gemma Fabriàs, C.I.D.-C.S.I.C., Barcelona, Spain. The authors would also like to thank Erling Jirle and Annika Söderman for help in rearing the insects and Einar Everitt and Dennis Hasselquist for advice on how to run ELISAs. This study was financially supported by grants from Crafoordska stiftelsen, Kungl. Fysiografiska Sällskapet I Lund and the Swedish research council VR to W.Q. Rosén and C. Löfstedt. This study was also supported by grants from MSMT LC07032 and 6007665801 (I. Sauman, R. Závodská).

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