

Neurohormones as putative circadian clock output signals in the central nervous system of two cricket species

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Abstract Antisera to the neuropeptides corazonin (Crz) and crustacean cardioactive peptide (CCAP) and to the diapause hormone (DH) react with small sets of neurones in the cephalic ganglia of the crickets *Dianemobius nigrofasciatus* and *Allonemobius allardi*. The distribution of their immunoreactivities is similar in the two species and overlaps with the locations of presumed circadian clock components in the optic lobes, protocerebrum, tritocerebrum, suboesophageal ganglion (SOG) and frontal ganglion. *D. nigrofasciatus* contains two Crz-immunoreactive (Crz-ir) cells in each optic lobe, six cell groups in the protocerebrum, four in the tritocerebrum, and one in SOG, whereas *A. allardi* harbours only five Crz-ir groups in the protocerebrum and four in the tritocerebrum. CCAP immunoreactivity occurs in both species in four protocerebrum cell clusters, four tritocerebrum cell clusters, four SOG cell clusters, one frontal ganglion cell cluster, and two optic lobe cell clusters; *D. nigrofasciatus* possesses two additional cells with unique links to the lamina in the optic

lobe. DH-related antigens are present in four cell clusters in the optic lobe, six (*D. nigrofasciatus*) or eight (*A. allardi*) in the protocerebrum, four in the tritocerebrum, and three (*A. allardi*) or five (*D. nigrofasciatus*) in the SOG. Some of the detected cells also react with antibody to the clock protein Period (PER) or lie close to PER-ir cells. Crickets reared at two different photoperiods do not differ in the distribution and intensity of immunoreactivities. No changes have been detected during the course of diurnal light/dark cycles, possibly because the antisera react with persistent prohormones, whereas circadian fluctuations may occur at the level of their processing or of hormone release. The projection of immunoreactive fibres to several brain regions, the stomatogastric nervous system and the neurohaemal organs indicates multiple functions of the respective hormones.

Keywords Circadian rhythm · Corazonin · CCAP · Diapause hormone · FXPRLamide · Cricket, *Dianemobius nigrofasciatus*, *Allonemobius allardi* (Insecta)

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Introduction

Within the circadian timing systems, input pathways transmit information concerning diurnal environmental changes to the endogenous core oscillator, which generates signals conveyed to the body periphery by the output pathways. We have explored the localisation of neurones producing three putative output messengers from the circadian clock of insects: corazonin (Crz), crustacean cardioactive peptide (CCAP) and the diapause hormone (DH). This study has been performed in two cricket species that differ in the distribution of proteins associated with the central clock oscillator (Shao et al. 2006).

Corazonin (Crz) is an insect-specific undecapeptide, pGlu-Thr-Phe-Gln-Tyr-Ser-Arg(His)-Gly-Trp-Thr-Asn-amide, that was discovered as a cardioaccelerator in a cockroach (Veenstra 1989) and a pigmentation modulator in locusts (Tawfik et al. 1999). Kim et al. (2004) have suggested that Crz is used as a signal transmitting the circadian timing command for eclosion. The Crz relationship to the circadian clock has been deduced from immunocytochemical studies. For example, in a cockroach (Petri et al. 1995), a locust and a grasshopper (Roller et al. 2003), Crz-immunoreactive (Crz-ir) fibres ramify in the accessory medulla of optic lobes (OL) in which the clock protein Period (PER) is expressed (Lupien et al. 2003). Crz immunoreactivity in the brain of *Manduca sexta* is restricted to four pairs of Ia₁ cells that co-express PER (Wise et al. 2002). Immunoreactivities to Crz (Hansen et al. 2001; Roller et al. 2003) and several clock proteins (Sauman and Reppert 1996; Sehadová et al. 2004) have been found in homologous cells in other moth species but no co-localisation has been demonstrated. Interaction between the circadian clock and Crz has also been proposed to occur in *Drosophila melanogaster* (Choi et al. 2005).

CCAP (Pro-Phe-Cys-Asn-Ala-Phe-Thr-Gly-Cys-amide) was originally isolated as a myotropic factor from a crab and later found, without any amino acid sequence variation, in a number of crustaceans and insects (for a review, see Dirksen 1998). It is involved in the control of ecdysis, which often occurs at a specific diurnal time. Immunocytochemical investigations have shown that a region of the accessory medulla of the locust *Locusta migratoria* (Dirksen and Homberg 1995) and the cockroach *Periplaneta americana* (H. Sehadová, unpublished) contains CCAP immunoreactivity. CCAP expressed in a series of neurones in the ventral nerve cord of several Holometabola is involved in the regulation of ecdysial behaviour (Truman 2005). CCAP-ir neurones in the ventral nerve cord of *D. melanogaster* contain the RNA-binding protein LARK, which has been proposed to function as a regulatory element in the circadian clock output pathway (McNeil et al. 1998).

DH (TDMKDES DRGAHSER GALC(W)FGPRLamide) belongs to the FXPRLamide (X=Ser, Thr, Val or Gly) neuropeptide family named after the consensus C-terminus. Moreover, DH, the pheromone biosynthesis activating neuropeptide (PBAN) and three other members of the family are derived in the silkworm *Bombyx mori* from a single prohormone by differential peptide cleavage (Kawano et al. 1992; Sato et al. 1993). DH is secreted in the pupal stage of *B. mori* after development under a long-day photoperiod and induces embryonic diapause in the progeny (Yamashita and Hasegawa 1985). PBAN is produced immediately before adult emergence and every night afterwards, independently of the length of the photoperiod but synchronously with the circadian rhythm

(Ichikawa et al. 1996). In situ hybridisation (Sato et al. 1994) and immunohistochemistry (Ichikawa et al. 1995; Sato et al. 1998) have demonstrated that the *dh-pban* gene is expressed in four neurones of the mandibular, six of the maxillary, and two of the labial neuromeres of the suboesophageal ganglion (SOG). Identically located neurones in the mandibular and maxillary neuromeres express the clock proteins DBT and CYC (H. Sehadová, unpublished).

We have chosen to examine the distribution of immunoreactivity to Crz, CCAP and DH in two cricket species that (1) exhibit distinct circadian rhythms in their behaviour, (2) respond similarly to the photoperiod (including the induction of embryonic diapause) but (3) differ in the distribution of circadian clock proteins (Shao et al. 2006). Our reasoning has been that corresponding differences in the patterns of neuropeptide immunoreactivities might reveal the existence of links between the clock components and the secreted peptides. Co-localisation of neuropeptides in presumed clock cells has been examined by means of double-labelling with an anti-PER antiserum. Changes in the intensity of immunoreactivity during two types (long and short photoperiods) of the circadian light/dark cycles have been sought in an attempt to find circadian or seasonal rhythms in peptide secretion. Circadian changes in CCAP immunoreactivity have also been probed on the Western blots.

Materials and methods

Animals

Immunocytochemical investigations were performed on adult males and females of the Japanese cricket *Dianemobius nigrofasciatus* and the North-American cricket *Allonemobius allardi* (Orthoptera, Gryllidae, subfamily Nemobiinae). In *D. nigrofasciatus*, the day-length within a 24-h photoperiod cycle determines the length of wings, the duration of larval development and the diapause versus non-diapause embryogenesis in the deposited eggs (Masaki 1972; Kidokoro and Masaki 1978). A similar dependence of the duration of larval development and egg diapause on the photoperiod was found in *A. allardi* (Q.-M. Shao, unpublished; the control of wing length has not been examined). Our insects were kept at 25°C, under either a 16:8-h (long-day conditions) or a 12:12-h (short-day conditions) light-dark cycle. Dim light at a wavelength of 660–670 nm was used to collect and dissect the insects during the dark phase.

Antibodies

For the detection of Crz, we used a rabbit polyclonal antibody raised against [His⁷]-Crz (Wako, Nagano, Japan). Its speci-

ficity was established by the loss of immunoreactivity in six diverse insect species, including the cricket *Gryllus bimaculatus*, when the antiserum was pre-absorbed with synthetic [Arg⁷]-Crz (Roller et al. 2003). Furthermore, a rabbit polyclonal antibody to [Arg⁷]-Crz (provided by Dr. Seiji Tanaka, Japan) recognised, in our preparations, the same neurones (data not shown) as the anti-[His⁷]-Crz antibody. The specificity of the rabbit polyclonal anti-CCAP antibody (Genemed, California, USA) was deduced from its strong reaction on Western blots with a protein corresponding in size to the CCAP prohormone (see Results). The polyclonal rabbit antibody aDH#1 was generated against the synthetic [Cys¹⁹]-DH of *B. mori* and its specificity was determined by dot-immunoblotting assay with synthetic fragments of *B. mori* DH and several other members of the FXPRLamide family (Sato et al. 1998). aDH#1 reacted with the FXPRLamide peptides and, in the SOG of *B. mori*, detected three clusters of neurones (Sato et al. 1998) that were apparently identical with the cells expressing the *dh-pban* gene (Sato et al. 1994). No immunoreactivity with these cells occurred after aDH#1 pre-absorption with DH. Since no DH homologue is known from crickets, we refer to reactions with the aDH#1 antibody as FXPRLamide-like immunoreactivity. The last antiserum in our tests, viz. the anti-PER polyclonal rabbit serum, was directed against a region of PER of *Periplaneta americana*; this region is largely conserved in other insects (Reppert et al. 1994). Its specificity is discussed in Sehadová et al. (2004).

Immunocytochemistry

Immunocytochemistry was performed as described by Shao et al. (2006). Whole cricket heads or only the cephalic ganglia were fixed in Bouin or modified Bouin-Hollande solution overnight at 4°C. Standard techniques were employed to prepare tissue sections (8 µm) in paraplast and to remove heavy metals after the Bouin-Hollande fixative. The sections were washed in TRIS-buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM TRIS-HCl, pH 7.6) at room temperature (rT), blocked with 1.5% normal serum in TBS for 30 min at rT and incubated with a primary antibody at 4°C overnight. The antisera were diluted as follows: anti-CCAP antiserum, 1:500; anti-Crz and aDH#1 antisera, 1:1,000. Normal goat serum was used to verify the absence of non-specific binding. Bound antibody was detected with the rabbit IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.). Horseradish peroxidase (HRP) conjugated to the secondary antibody was visualised with 0.005% H₂O₂ and 0.25 mM 3,3'-diaminobenzidine tetrahydrochloride (DAB; in 0.1 M TRIS-HCl, pH 7.5). Stained sections were mounted in Bioleit medium (Kouken Rika, Osaka, Japan). The results of this DAB staining were confirmed with independent

immunofluorescence labelling. In this case, TBS was replaced with phosphate-buffered saline (145 mM NaCl, 8.55 mM Na₂HPO₄, 1.45 mM NaH₂PO₄, pH 7.5) supplemented with 0.3% Tween 20 (PBS-Tw). Following incubations with 10% normal serum and the primary antibody, the sections were rinsed in PBS-Tw and incubated for 1 h at rT with Cy2-conjugated goat antibody to rabbit IgG (Jackson Immuno-Research, West Grove, Pa.) diluted 1:500 with PBS-Tw. The sections were then rinsed three times for 10 min with PBS-Tw and once for 5 min with distilled water. All preparations were examined under a BX50 microscope (Olympus, Tokyo, Japan) equipped with Nomarski contrast, epifluorescence optics and a charge-coupled device camera.

Some preparations were double-labelled to check for the possible co-localisation of the examined antigens with the PER-like protein. They were treated with the primary rabbit antiserum to Crz, CCAP or DH, incubated with the secondary goat anti-rabbit IgG biotinylated antibody and stained with DAB. After three washes in PBS-Tw, the same sections were incubated overnight in the polyclonal rabbit antiserum to PER (1:1,000), followed by rinses in PBS-Tw and reaction with the goat Cy2-conjugated antibody to rabbit IgG for 1 h at rT. The reverse procedure, i.e. anti-PER fluorostaining followed by the DAB staining of another antigen, proved equally successful. Possible cross-reactivity of the anti-rabbit IgG-Cy2 or biotinylated IgG with the primary antibody of the first staining step was checked by comparing the numbers of cells stained by the single- and double-labelling methods. The

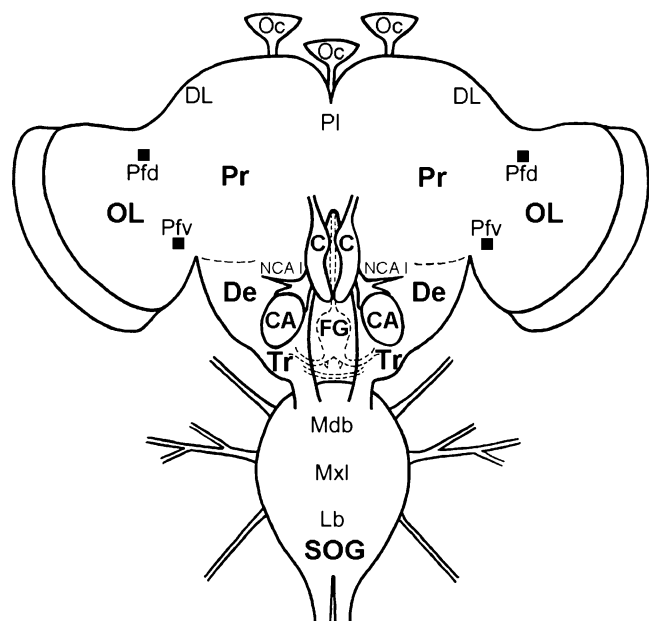


Fig. 1 Representation of the cephalic neural complex of adult crickets (*CA* corpora allata, *CC* corpora cardiaca, *De* deutocerebrum, *DL* dorsolateral region of protocerebrum, *FG* frontal ganglion, *Lb* labial neuromere, *Mdb* mandibular neuromere, *Mxl* maxillary neuromere, *NCA I* nervus corporis allati I, *Oc* ocellus, *OL* optic lobe, *Pfd* proximal frontodorsal cluster, *Pfv* proximal frontoventral cluster, *PI* pars intercerebralis, *Pr* protocerebrum, *SOG* suboesophageal ganglion, *Tr* tritocerebrum)

double-labelled slides were first photographed under regular light (DAB staining), then after fluorescence emission with ~488-nm blue light (Cy2 staining) and finally under both (but dim) types of illumination.

Assessment of immunostaining intensity

The intensity of staining was scored subjectively on the DAB-stained preparations with a scale ranging from 0 (no reaction) to 4 (maximal response). Tissues were prepared at *Zeitgeber* time 0 (ZT0), ZT4, ZT8, ZT12, ZT16 and ZT20 (h after lights-on) of two light/dark cycles in three separate

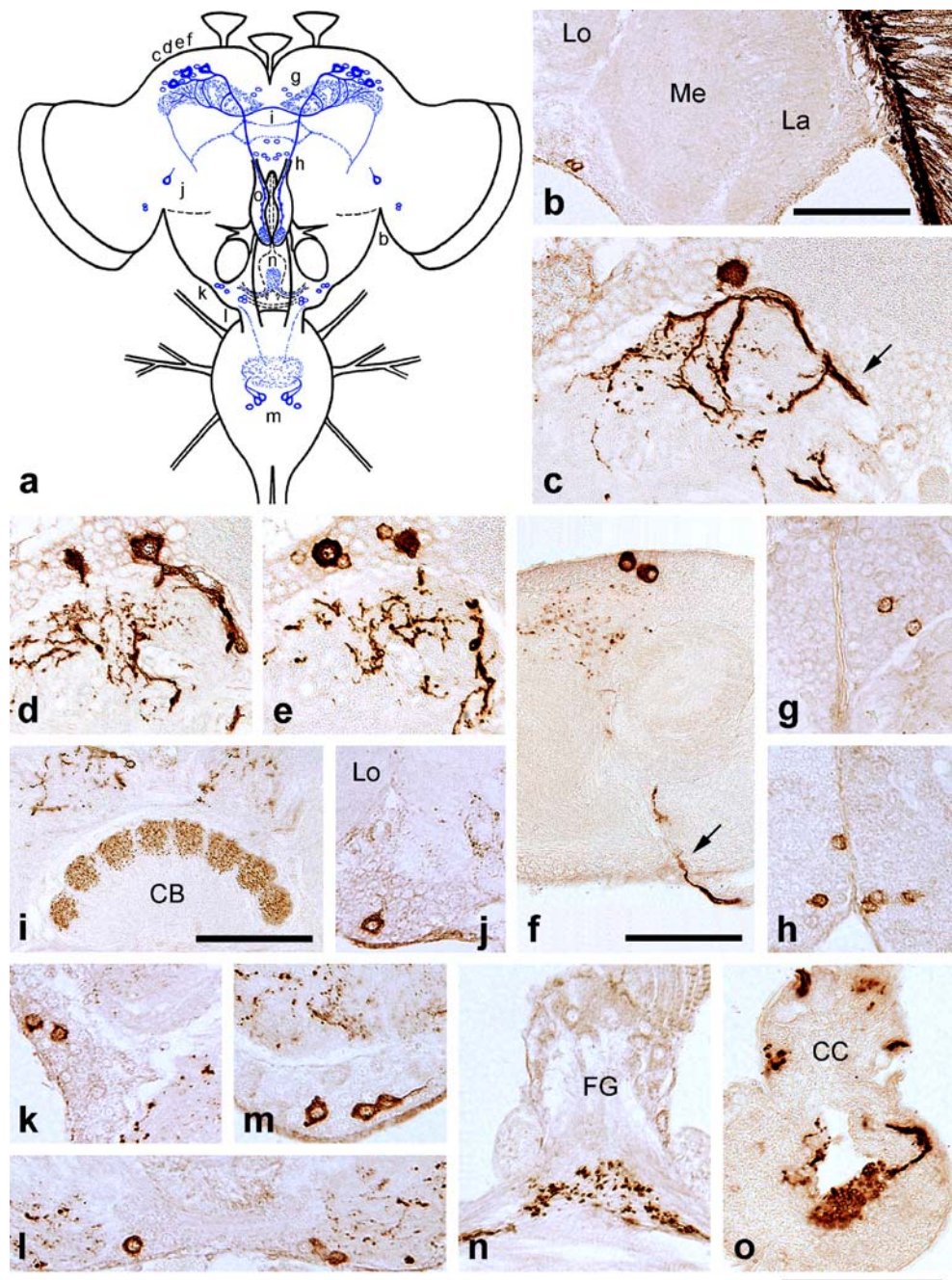
generations of crickets. In every generation, each of the six time groups included two males and two females; three animals were stained with DAB and used for antigen quantification, with the remaining one being taken for a check with Cy2-labelling. The entire immunocytochemical procedure, from tissue dissection to staining evaluation, was standardised with respect to time, temperature and other conditions. The length of exposure to DAB was set in preliminary runs with preparations from animals sacrificed between ZT4 and ZT10. Exposures for 10 min, 15 min and 10 min were optimal for the antisera to Crz, CCAP and DH, respectively. The preparations of every time series

Fig. 2 Corazonin (Crz) immunoreactivity in the cephalic ganglia of *D. nigrofasciatus*.

a Representation of the numbers and topography of Crz-ir cells and the pathways of their projections (*a–o* positions of the respective micrographs in this figure). **b** Two cells in the proximal frontoventral part of the optic lobe (*La* lamina, *Lo* lobula, *Me* medulla). *Bar* 100 μ m. **c–e** Consecutive sections through the dorsolateral protocerebrum tracking a nerve bundle (*arrow*) projecting into the ipsilateral corpora cardiaca.

f Ipsilateral axonal projections arising from cells in the dorsolateral protocerebrum (*top*) and passing through the nervus corporis cardiaci I (*arrow*) into the corpora cardiaca. *Bar* 100 μ m. **g** Weakly stained neurones in the anterior pars intercerebralis. **h** Cells in the posterior pars intercerebralis. **i** Varicose fibres in the medial protocerebrum and within layer I in the upper division of the central body (*CB*). *Bar* 100 μ m. **j** Neurones at the base of the optic stalk below the lobula (*Lo*). **k** Neurones in the lateral and fine fibres in the ventral tritocerebrum.

l Bilateral cluster of about four cells in the ventral tritocerebrum, near the postoesophageal tritocerebral commissure. **m** Neurones of the *MxI-Vm* cluster in the SOG. **n** Extensive fibre ramification in the ventral region of the frontal ganglion (*FG*). **o** Nerve bundle arborising in the distal division of the corpus cardiacum (*CC*). *Bar* (*bottom right*) 100 μ m (applies to all micrographs without an internal bar)



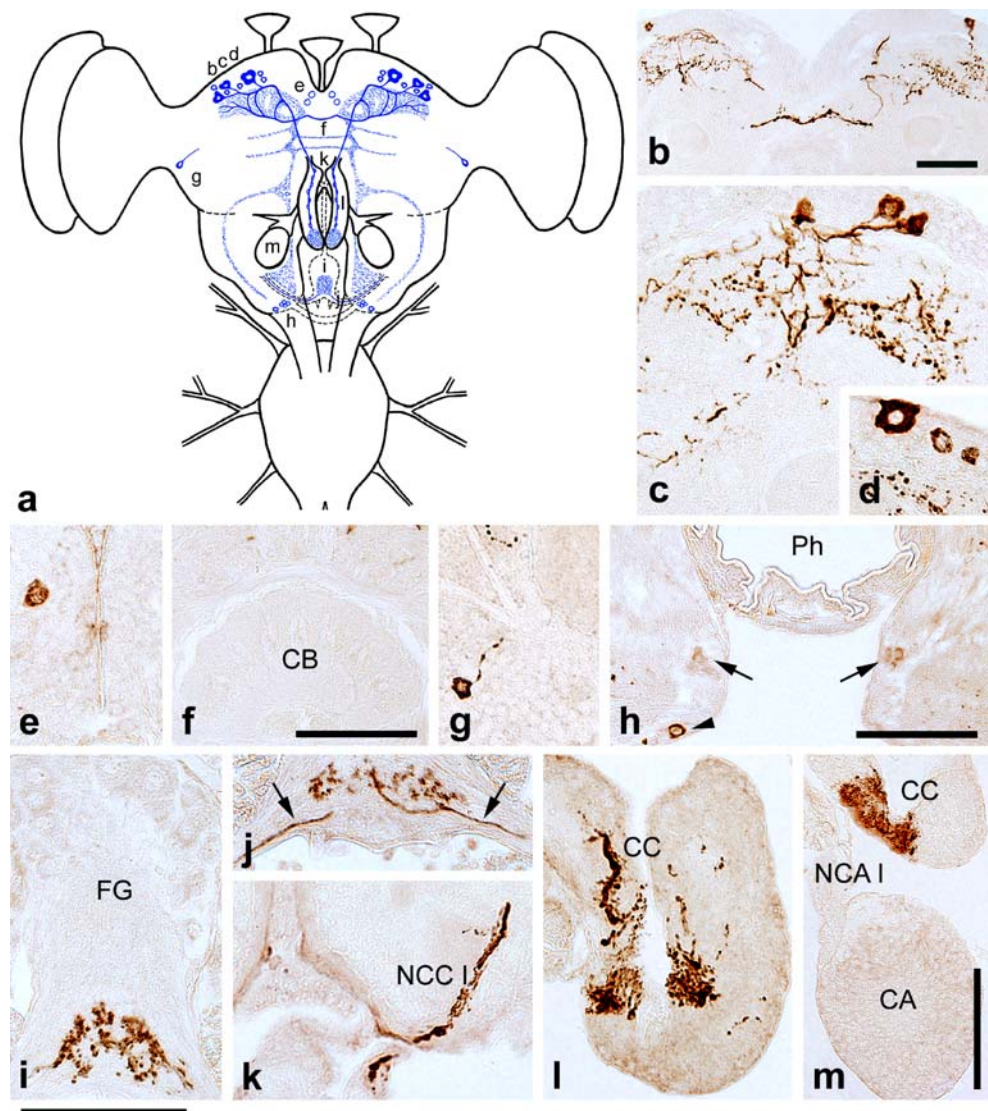
were processed simultaneously but separately from the corresponding series based on a different cricket generation. The intensity of staining was scored by two independent observers who first compared different cells in each preparation, then different preparations of a given time series and finally preparations corresponding to the same circadian time in different insect generations. Staining intensities found in most of the 18 preparations examined for each antibody and time point were taken as a measure of antigen amount. Variability in the staining intensity at a given circadian time typically did not exceed ± 1 point of our scale.

Western analysis of CCAP-like antigen

Western analysis was performed with the extracts of four trimmed heads (the antennae and the mouth parts were removed) of *D. nigrofasciatus*. Samples were collected every 6 h through the long-day light/dark cycle. Heads were

homogenised in 200 μ l sample buffer (25% 0.5 M TRIS-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 1.3% SDS, 0.8 mg/ml bromophenol blue, 37% millipore water) and the homogenate was centrifuged (10,000g, 5 min at 4°C) to eliminate cellular debris. The supernatant was denatured at 95°C for 10 min and stored at -20°C. The samples were heated again to 95°C for 5 min just before being loaded onto 10% SDS-polyacrylamide gel (3 μ l per lane). Broad-range pre-stained protein marker 6–175 kDa (BioLabs, New England) was used to estimate the molecular weights of separated proteins that were transferred onto nitrocellulose membranes (Sigma-Aldrich) in the semidry blotting system AE-6675 (ATTO, Japan; 40 mV for 45 min). After three washes with PBS (each for 5 min at rT), the membrane was treated for 1 h at rT with 5% nonfat dry milk in PBS supplemented with 0.1% Tween 20 to block unspecific IgG-binding sites. Incubation with the anti-CCAP antibody in 5% bovine serum albumin in PBS-

Fig. 3 Crz immunoreactivity in *A. allardi*. **a** Representation of the immunostaining pattern (a–m positions of the respective micrographs in this figure). **b** Bilateral fibre networks in protocerebral hemispheres connected by a neurite bundle. Bar 100 μ m. **c, d** Dorsolateral neurones in the right hemisphere. **e** One of the faintly stained somata in the pars intercerebralis. **f** Absence of Crz immunoreactivity in the central body (CB). Bar 100 μ m. **g** Neurone at the base of the optic stalk sending axonal projection dorsally to the protocerebrum. **h** Bilateral clusters of weakly stained cells (arrows) and one strongly stained cell (arrowhead) in the ventral tritocerebrum adjacent to the oesophagus (Ph). Bar 100 μ m. **i** Extensive fibre network in the ventral region of the frontal ganglion (FG). **j** Fibres in the frontal connectives (arrows). **k** Varicose fibres in the nervus corporis cardiaci I (NCC I). **l** Nerve bundle arborising in the distal region of the corpus cardiacum (CC). **m** Fibre arborisation in the corpus cardiacum (CC) and absence of immunoreactivity in the nervus corporis allati I (NCA I) and the corpus allatum (CA). Bar 100 μ m. Bar (bottom left) 100 μ m (applies to all micrographs without an internal bar)



Tw (1:5,000 dilution) was carried out overnight at 4°C and under gentle agitation. The membrane was then washed three times (5 min each) in PBS-Tw and incubated for 1 h at rT with HRP-conjugated goat anti-rabbit IgG (Amersham Biosciences, UK) diluted 1:10,000 in PBS-Tw. After three washes in PBS-Tw (each for 5 min at rT), HRP-IgG bound to the membrane was revealed by the standard staining protocol employing H₂O₂ (0.005%) and DAB (0.25 mM in 0.05 M TRIS-HCl, pH 7.5).

Results

Distribution and nomenclature of the immunoreactive cells

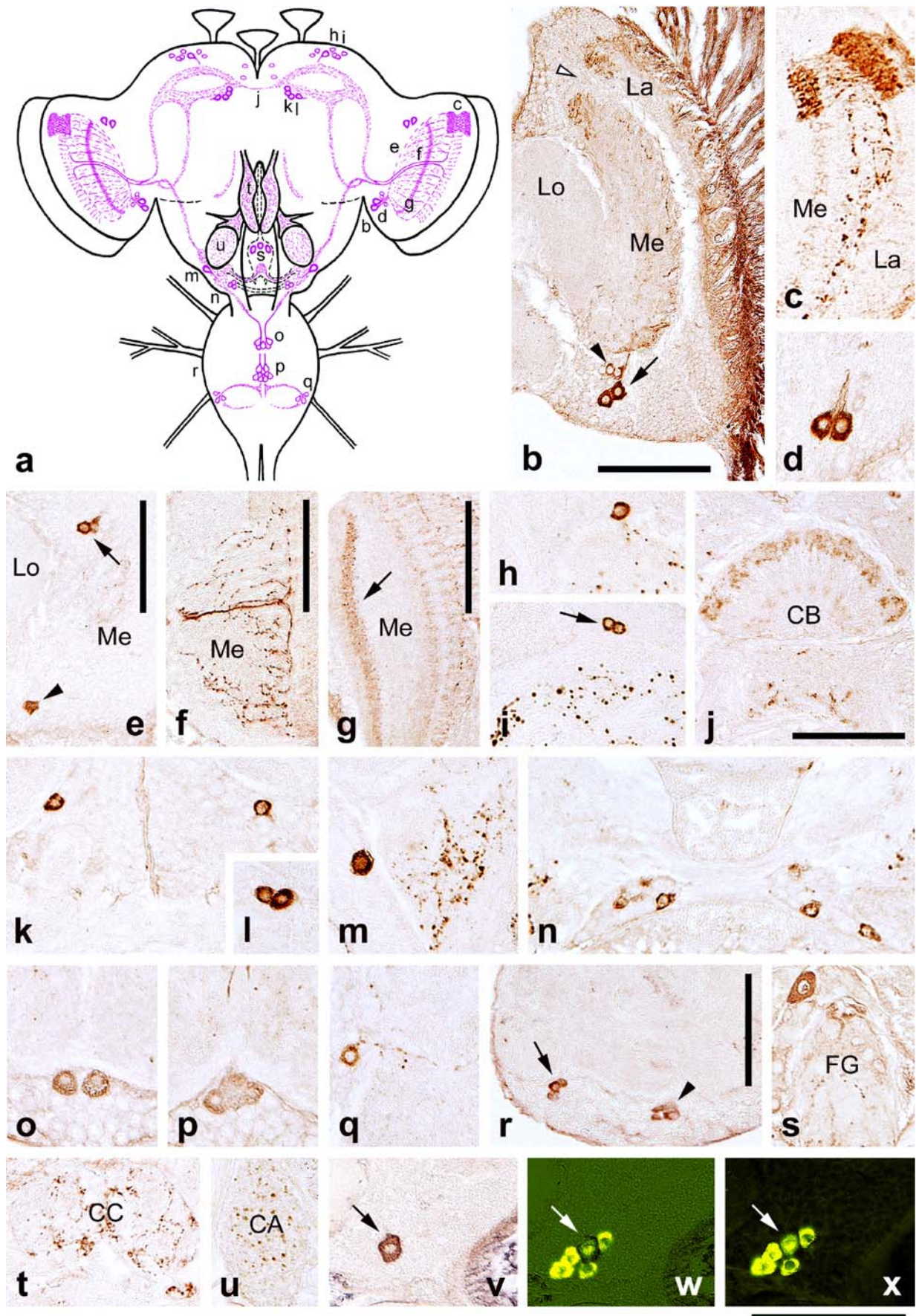
The antisera recognised a few singly distributed cells and several clusters of cells in specific regions of the cephalic ganglia (Fig. 1). Cells and clusters in similar positions were mapped with antisera against the clock proteins PER, DBT and CRY (Shao et al. 2006). They were named in reference to the region; we have adopted this nomenclature here. A proximal frontoventral (Pfv) cell cluster occurred close to the accessory medulla and a proximal frontodorsal (Pfd) cluster on the dorsal side of the OL. In the protocerebrum, we distinguished the pars intercerebralis (PI) and bilateral dorsolateral (DL) regions and, in the tritocerebrum, the bilateral medial and lateral regions. Immunoreactivity in the SOG was found in clusters located ventromedially in the mandibular (Mdb-Vm), maxillary (Mxl-Vm) and labial (Lb-Vm) neuromeres; the labial neuromere contained a pair of lateral clusters (Lb-L). However, the occurrence of different immunoreactivities in designated positions should not be taken to imply antigen co-localisation or expression in immediately adjacent cells.

Crz immunoreactivity

Crz immunoreactivity occurred in the OL of *D. nigrofasciatus* only in two small Pfv cells (Fig. 2a,b) and not at all in the OL of *A. allardi* (Fig. 3a). The pattern of staining in the central brain was similar in both species. About five weakly stained cells were present in the PI (Figs. 2g, 3e) and each DL hemisphere contained two large perikarya and one very large, strongly stained perikaryon (Figs. 2c–f and 3b–d), and five to six small, weakly stained perikarya (Figs. 2e, 3d). The projections of the large neurones arborised within the dorsolateral protocerebrum and formed two nerve bundles. One ran posteriorly into the nervus corporis cardiaci II (NCC II) and ramified in the distal division of the ipsilateral corpora cardiaca (Figs. 2c, f, o, 3k–m). This bundle sent collaterals to the ventrolateral protocerebrum and short branches to the opposite brain hemisphere. The second thinner bundle ran anteriorly into

the contralateral hemisphere (Fig. 3b). Varicose fibres also occurred within the upper division of the central body in *D. nigrofasciatus* (Fig. 2i), whereas the central body of *A. allardi* was devoid of staining (Fig. 3f). *D. nigrofasciatus* additionally contained six to eight somata in the posterior PI (Fig. 2h). Both species harboured a single neurone near the base of the optic stalk, with a neurite projecting into the dorsal protocerebrum (Figs. 2j, 3g). The tritocerebrum included two populations of Crz-ir neurones. A cluster of 3–4 Crz-ir cells without discernible projections occurred in the ventral tritocerebrum close to the base of the postoesophageal tritocerebral commissure in both species (Figs. 2l, 3h). In *A. allardi*, one to two larger and strongly stained perikarya were shifted slightly ventrally from this cluster (Fig. 3h), whereas in *D. nigrofasciatus*, three Crz-ir neurones lay laterally, in the border between the deuto- and tritocerebrum (Fig. 2k). The tritocerebrum was innervated by a network of Crz-ir processes that continued and extensively ramified in the frontal ganglion (FG; Figs. 2n, 3i,j). In the SOG, Crz immunoreactivity was detected only in *D. nigrofasciatus*. A population of three bilaterally paired neurones in the Mxl-Vm cluster (Fig. 2m) sent neurites in a lateral direction but they reversed their course and terminated in a prominent fibre arborisation in the medial area of SOG. Analysis of daily changes in the abundance of the Crz-ir antigen revealed no distinct fluctuations. Furthermore, no differences in the

Fig. 4 CCAP immunoreactivity in *D. nigrofasciatus*. **a** Representation of the immunostaining pattern (a–u positions of the respective micrographs in this figure; v–x are from region d). **b** Pfv cluster of two large (arrow) and several small cells (solid arrowhead) with fibres extending over the medulla (Me) surface (Lo lobula). These fibres pass through dense arborisation in the posteriodorsal region of the optic lobe (open arrowhead) and continue over the inner lamina (La). Bar 100 µm. **c** Detail of the posteriodorsal arborisation connecting the medulla (Me) and lamina (La). **d** Two large cells of the Pfv cluster with neurites entering the ventral medulla. **e** Proximal part of the optic lobe with cells of the Pfv (arrowhead) and Pfd clusters (arrow). Bar 100 µm. **f** Major fan of immunoreactive fibres embracing the surface of the frontal medulla (Me). Bar 100 µm. **g** Minor fan of fibres (arrow) over the edge of the rear medulla (Me). Bar 100 µm. **h** Large neurone in dorsolateral protocerebrum. **i** Small cells (arrow) in a similar location. **j** Fibres innervating the central body (CB). Bar 100 µm. **k** Bilateral distribution of neurones (three to four on each side) in the posterior pars intercerebralis. **l** Cells from a bilateral cluster in the pars intercerebralis. **m** A neurone in the lateral wedge between the deuto- and tritocerebrum gives rise to arborisation within the tritocerebrum. **n** Cluster of about four cells on each side of the ventral tritocerebrum, near the postoesophageal tritocerebral commissure. **o–q** CCAP-ir neurones of the Mdb-Vm, Mxl-Vm and Lb-L clusters, respectively. **r** Lateral view of the SOG with the Mdb-Vm (arrow) and Mxl-Vm (arrowhead) cell clusters. Bar 100 µm. **s** One of four perikarya detected in the frontal ganglion (FG). **t, u** Fibres in the corpora cardiaca (CC) and corpora allata (CA), respectively. **v–x** CCAP immunoreactivity, simultaneous detection of immunoreactivities for CCAP and PER, and PER immunoreactivity, respectively, in the Pfv cluster; one cell shares both immunoreactivities (arrow). Bar (bottom right) 100 µm (applies to all micrographs without an internal bar)



distribution, number or staining intensity of the Crz-ir neurones were detected between crickets kept under different photoperiodic conditions.

CCAP immunoreactivity

Intensive CCAP immunoreactivity was detected in a few somata and a rich fibre network in the OL of both cricket species. A group of CCAP-ir cells in a Pfv cluster near the accessory medulla contained two large and three to four small neurones in *D. nigrofasciatus* (Fig. 4a,b,d) and two large and five to six small cells in *A. allardi* (Fig. 5a,c,d). Unique to *D. nigrofasciatus* were two large perikarya in the Pfd region (Fig. 4e). In both species, the fibres of the Pfv CCAP-ir cells embraced, in a fan-like fashion, the frontal face of the medulla (Fig. 4f). Upon reaching the distal medulla edge, the fibres followed its lateral face and eventually turned back and ran anteriorly over the inner lamina surface (Figs. 4c, 5b). *D. nigrofasciatus* contained a small region of dense fibre network in the posteriodorsal region of the lobe, where numerous neurites linked the distal medulla with the inner lamina surface (Fig. 4b,c). In this species, we also found a fan of fibres extending over the posterior medulla face (Fig. 4g) without reaching its edge. Both species possessed two nerve bundles that ran from the OL centripetally, bypassed the lobula and split into at least three branches in the lateral protocerebrum. Two of them ramified in the DL region and included a weak anastomosis to the opposite brain hemispheres (Fig. 5f); the third one innervated the deuto- and tritocerebrum.

Prominent CCAP immunoreactivity was consistently observed in a bilateral set of three to four neurones packed in the posterior PI (Figs. 4k,l, 5h). In *A. allardi*, one more pair of bilaterally symmetrical neurones was located slightly dorsomedially from this group (Fig. 5g). The anterior PI region harboured four to six small weakly stained cells in both species (Fig. 5f) but fibres extending to the central body were detected only in *D. nigrofasciatus* (Fig. 4j). In DL, one large and six to seven small somata were strongly labelled in *D. nigrofasciatus* (Fig. 4h,i) but only two small cells were stained consistently in *A. allardi* (Fig. 5e). In both species, the ventral tritocerebrum contained a cluster of three to four small weakly stained neurones with neurites projecting through the postoesophageal commissure into the contralateral tritocerebrum (Figs. 4n, 5j). A single CCAP-ir neurone in the lateral tritocerebrum gave off a wide arborisation in the ventral tritocerebrum and projected through the contralateral frontal connectives into the FG, where it ramified (Figs. 4m, 5i). The dorsal region of the FG contained three to four large CCAP-ir perikarya (Figs. 4s, 5n). The SOG harboured, in both cricket species, four to five weakly stained CCAP-ir neurones in the Mdb-Vm, six to seven in the Mxl-Vm, and

two to three in the Lb-L cluster (Figs. 4o–r, 5k–m). Cells of the medial clusters sent projections into two fibre bundles that ran along the dorsal SOG midline into the circumoesophageal connectives. Short lateral projections from the medial clusters were also detected. The lateral somata sent neurites towards the SOG midline. CCAP immunoreactivity occurred in fibre arborisation within the corpora cardiaca, corpora allata, and nervi corporis allati I (Figs. 4t,u, 5o). Immunoreactive fibres permeated the corpora cardiaca in *D. nigrofasciatus* but were restricted to their cortex in *A. allardi*.

A detailed examination of crickets kept under short-day and long-day conditions did not disclose any differences in the distribution and numbers of CCAP-ir neurones or in their staining intensity. Around the clock analysis performed on samples collected in 4-h intervals through the short-day and long-day light-dark cycles also revealed no daily oscillation in CCAP immunoreactivity. This result was verified by Western blot analysis of proteins extracted from the head of *D. nigrofasciatus* (Fig. 6). CCAP immunoreactivity was confined to a strong band at a position of about 17 kDa and a very weak band at 6.5 kDa; their staining intensity was independent of the phase (*Zeitgeber* time) of the long-day light/dark cycle. The molecular weight 17 kDa is close to the size of the CCAP precursor protein in *D. melanogaster* (17.6 kDa; Adams et al. 2000), *Manduca sexta* (14 kDa; Loi et al. 2001) and *P. americana* (19.5 kDa; Sakai et al. 2004). We assume that the 6.5-kDa product is an intermediate of the precursor processing. The molecular weight of CCAP is less than 1 kDa (Furuya et al. 1993) and such a small peptide probably escaped detection with our method.

FXPRLamide immunoreactivity

The widespread distribution of FXPRLa immunoreactivity showed many similarities between the two cricket species (Figs. 7a, 8a). Prominent immunostaining occurred in the OL in the Pfv and Pfd cell clusters, each consisting of two to three large and about ten small neurones (Figs. 7a–c, 8a–e). Primary neurites arising from these cells entered the medulla individually and formed an extensive fibre arborisation embracing its frontal surface without reaching the edges (Figs. 7b,c, 8b,e). Two axonal fascicles ran through the optic stalk into the lateral protocerebrum where they separated into several branches. A thin fibre took a posterior direction to the ventral protocerebrum and two branches dispersed in a network within the DL in which they seemed to associate with the neurites of cells stained in this area and then continued centripetally close to the dorsal brain surface (Fig. 9a,d). Each hemisphere contained five to six small and three large neurones in the DL (Figs. 7e–g, 8f–h); one of the large cells was shifted ventrolaterally into

Fig. 5 CCAP immunoreactivity in *A. allardi*. **a** Representation of the immunostaining pattern (a–o positions of the respective micrographs in this figure). **b** Fibres embracing distal medulla (*Me*) and inner lamina (*La*) surfaces and fibres passing through lobula (*Lo*) to the lateral protocerebrum. Bar 100 μ m. **c** One large and four small cells of the Pfv cluster and connection of the cluster to the fibre network in the medulla. **d** Two large neurones of the Pfv cluster. **e** One of the two CCAP-ir cells in the dorsolateral protocerebrum. **f** Bilateral fibre arborisations in anterior protocerebrum linked with a nerve bundle and one of about five faintly stained neurones in the pars intercerebralis (arrow). **g** Bilateral pair of somata in the posterior pars intercerebralis. **h** Neurones in the posterior pars intercerebralis (*Ca* mushroom body calyx). **i** Neurone in the lateral wedge between the deuto- and tritocerebrum sending a fibre to an arborisation in the tritocerebrum. **j** About four pairs of cells in the ventral tritocerebrum send projections to the contralateral side through the postoesophageal tritocerebral commissure (arrow). **k–m** CCAP-ir cells of the Mdb-Vm, Mxl-Vm and Lb-L clusters, respectively. **n** Two of four perikarya and a fibre ramification detected in the frontal ganglion (*FG*). **o** Fibres dispersed through both corpora allata (*CA*). Bar (bottom right) 100 μ m (applies to all micrographs without an internal bar)

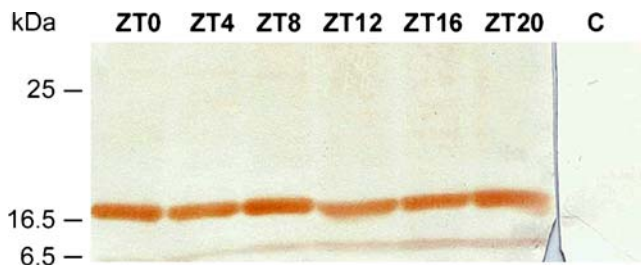
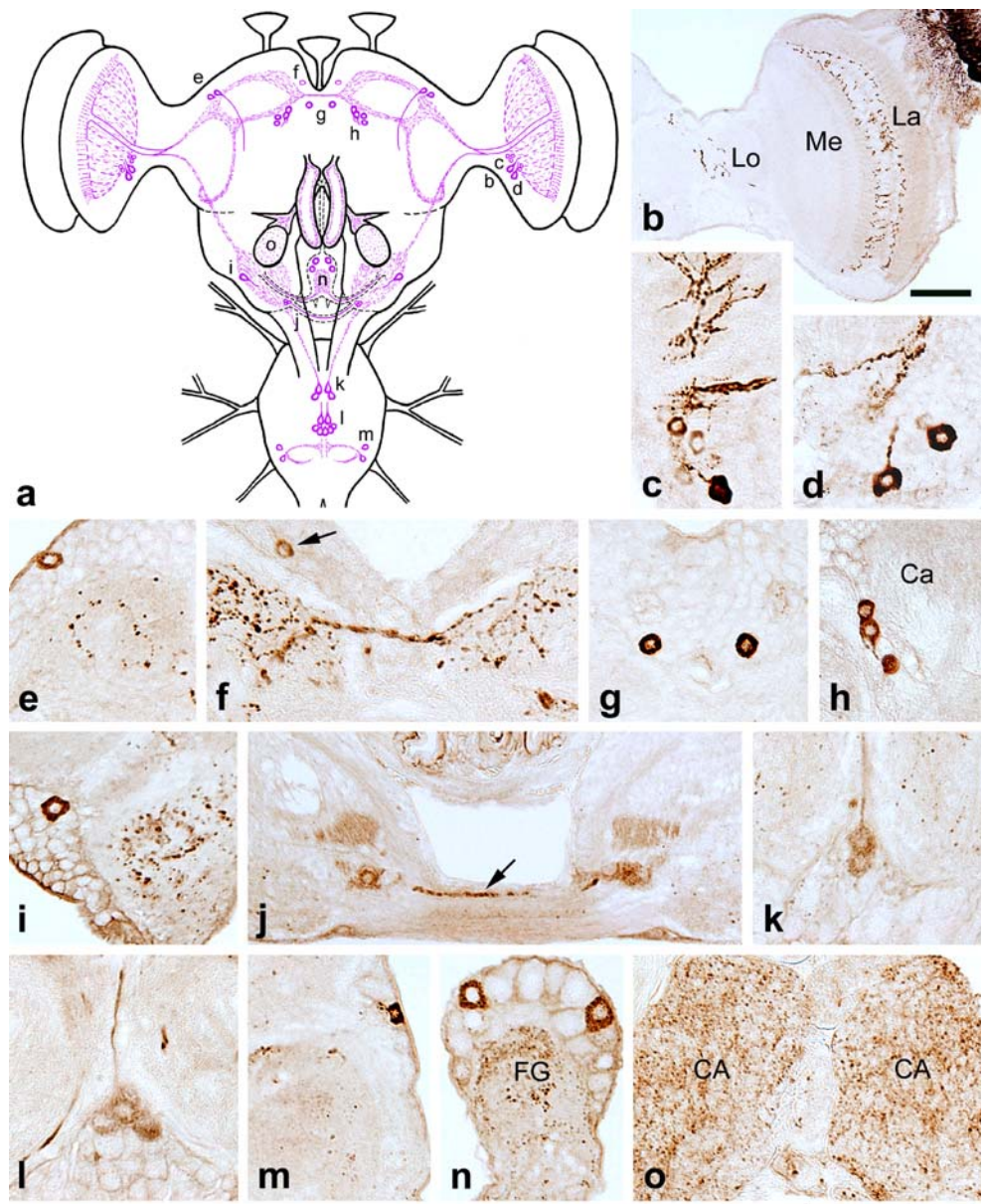
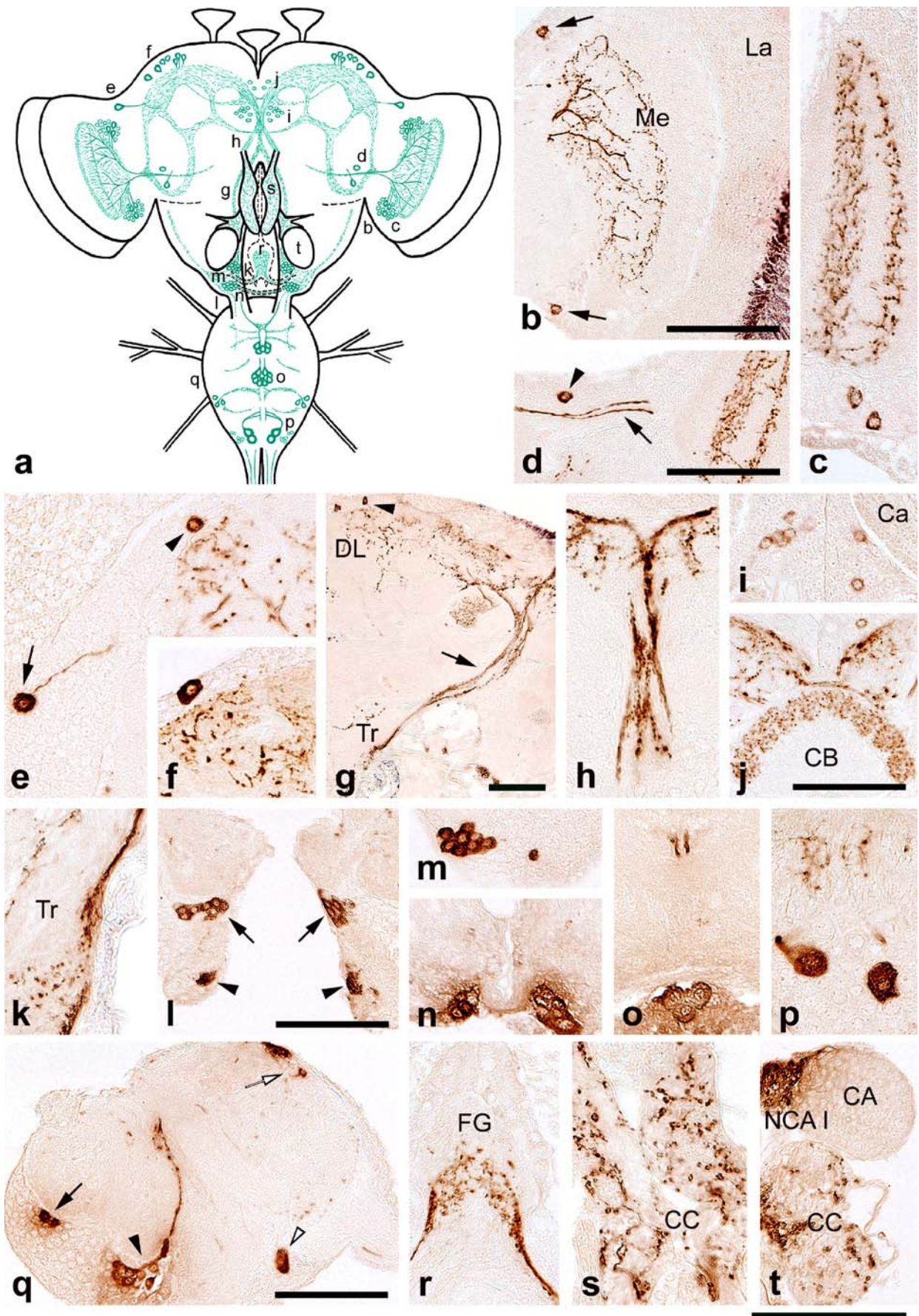


Fig. 6 CCAP immunoreactivity on the Western blot of proteins extracted from the heads of *D. nigrofasciatus*. Samples were collected at 4-h intervals throughout the long-day light/dark cycle beginning with lights on at the *Zeitgeber* time 0 (*ZT* lanes). Normal goat serum was used in place of the anti-CCAP antiserum in the control (lane *C*). The position of 25-kDa, 16.5-kDa and 6.5-kDa markers are shown (left)

the base of optic stalk (Figs. 7e, 8h). All these cells seemed to supply fibres to a rich arborisation in the dorsal protocerebrum. One group of smaller somata occurred in the anterior region of the DL (Fig. 8f) and another was located in the close vicinity and posteriorly to the large cells. The dorsal fibre arborisation frontally condensed into two thin nerve tracts linking the opposite hemisphere. One bypassed the dorsal edge of the central body (Fig. 9b) and the second one ran over its ventral surface, giving rise to thin fibres reaching dorsally into the central body (Fig. 9a). A prominent arborisation of immunoreactive fibres innervated layer I in the upper division of the central body (Figs. 7j, 8l, 9b). In the posterior protocerebrum, the varicose fibres medially split into several main branches crossing the brain midline and continuing to the contralat-



◀ **Fig. 7** FXPRLamide immunoreactivity in *D. nigrofasciatus*. **a** Representation of the immunostaining pattern (*a–t* positions of the respective micrographs in this figure). **b** Pfv and Pfd cell clusters (*arrows*) and extensive fibre network in the central medulla (*Me*), contrasting with the absence of FXPRLa immunoreactivity in medulla edges and in the lamina (*La*). *Bar* 100 μ m. **c** Two large Pfv cells and fibres in the medulla. **d** Two nerves (*arrow*) from the optic lobe to the central brain bypassing a neurone (*arrowhead*) in the cortex of posterior protocerebrum. *Bar* 100 μ m. **e** Protocerebral neurones at the base of optic stalk (*arrow*) and in the DL region (*arrowhead*). **f** A cell and fibres in the dorsolateral protocerebrum. **g** Two large cells (*arrowhead*) and the nerve bundles (*arrow*) connecting the dorsolateral protocerebrum (*DL*) with ramifications in the tritocerebrum (*Tr*). *Bar* 100 μ m. **h** Nerve bundles crossing the brain midline. **i** Faintly stained neurones in the posterior pars intercerebralis located medially to the mushroom body calyx (*Ca*). **j** Varicose fibres in layer I of the upper division of the central body (*CB*) and in the anterior pars intercerebralis, which also contains weakly stained somata. *Bar* 100 μ m. **k** Nerve bundle arising from the protocerebrum and ramifying in the tritocerebrum (*Tr*). **l** Two pairs of clusters, each composed of about 10 cells, located between the deuto- and tritocerebrum (*arrows*) and near the postoesophageal commissure egress (*arrowheads*), respectively. *Bar* 100 μ m. **m** Cell cluster between the deuto- and tritocerebrum. **n** Cluster at the commissure egress. **o** Cells of the Mxl-Vm cluster in the SOG. **p** Cells of the Lb-Vm cluster. **q** The Mdb-Vm (*solid arrow*), Mxl-Vm (*solid arrowhead*), Lb-Vm (*open arrowhead*) and Lb-L (*open arrow*) cell clusters in a lateral SOG view. *Bar* 100 μ m. **r–t** Fibre ramifications in the frontal ganglion (*FG*), corpora cardiaca (*CC*) and nervus corporis allati I (*NCA I*) and absence of staining in the corpora allata (*CA*). *Bar* (*bottom right*) 100 μ m (applies to all micrographs without an internal bar)

eral corpora cardiaca (Figs. 7s,t, 9j). The extensive system of immunoreactive fibres in the nervi corporis allati I and II contrasted with the lack of FXPRLa immunoreactivity in the corpora allata (Figs. 7s,t, 9j–l). Some of the FXPRLa-ir fibres from the protocerebrum adjoined within a distinct cord that ran into the tritocerebrum where it extensively ramified (Figs. 7g,h,k, 8i, 9c,e). The central part of the tritocerebrum contained two pairs of bilateral FXPRLa-ir cell clusters, each of about ten cells. One cluster was located medially in the wedge between the deuto- and tritocerebrum (Figs. 7l,m, 8o) and the other lay near the egress of the postoesophageal commissure that contained fibre links to the contralateral cluster (Figs. 7l,n, 8p). FXPRLa-ir fibres running from the tritocerebrum through the contralateral frontal connective into the FG, and the fibres continuing through the circumoesophageal connectives into the SOG might originate in the tritocerebral FXPRLa-ir neurones but could equally likely arise in the protocerebral FXPRLa-ir neurones. The existence of axonal pathways extending from the SOG to the tritocerebrum and further into the protocerebrum also could not be ruled out. In the FG, the fibres dispersed into a rich network (Figs. 7r, 9i) and some continued through the recurrent nerve (Fig. 9l).

The faintly stained cells in the PI appeared to be separate from the FXPRLa-ir neuronal architecture described above. The PI of *D. nigrofasciatus* contained four to six somata in

the anterior region and approximately 20 in the posterior region (Fig. 7i,j), whereas in *A. allardi*, these regions harboured about 15 small and up to 10 large perikarya, respectively (Fig. 8j,k). Two bilaterally symmetrical cells occurred in the lateral cortex of the protocerebrum close to the posteroventral nerve bundle arising from the OL (Figs. 7d, 8n). These cells sent axons dorsally into the protocerebrum but we were unable to trace their endings. One bilaterally paired neurone with soma at the base of the optic stalk and neuronal projection to the dorsal protocerebrum was detected in *A. allardi* (Fig. 8m). Prominent FXPRLa immunoreactivity was consistently found in three cells clusters located along the ventral midline of SOG. The Mdb-Vm cluster harboured four to six somata (Figs. 7q, 8q), the Mxl-Vm about 10 bodies (Figs. 7o,q, 8r) and the Lb-Vm cluster four distinctly larger cells (Figs. 7p,q, 8s). The Lb-Vm cluster was always present, whereas the Mdb-Vm and Mxl-Vm clusters were missing in some preparations. Their absence had no relationship to circadian time or cricket physiology. The neurites extending from all medial SOG clusters formed two parallel fibre bundles projecting into the dorsal SOG midline. The collaterals arising from the Mdb-Vm cells arborised in the dorsal SOG region and formed several lateral branches (Figs. 8q, 9f). The processes of the Mxl-Vm cells reaching the dorsal SOG surface turned laterally and seemed to provide the fibre network encircling ventrally the maxillary neuromere (Fig. 9g,h). In *D. nigrofasciatus*, about 15 small weakly stained cells occurred close to the Lb-Vm cluster and an additional bilateral cluster comprising two to three neurones was detected the Lb-L region (Fig. 7q). These somata sent neurites towards the midline where they adjoined fibres from the Lb-Vm neurones. A pair of longitudinal lateral and medial nerves originating in SOG extended to the ventral nerve cord.

Adult females and males reared under long-day (non-diapause egg producers) and short-day conditions (diapause eggs producers) were compared with respect to the distribution and staining intensity of FXPRLa immunoreactivity. No differences in the staining pattern were found, either between sexes or between the non-diapause and diapause egg producers. Furthermore, no regular variation in immunostaining was detected among samples collected at 4-h intervals throughout the light/dark cycle.

Co-localisation of PER immunoreactivity

Co-expression of the examined antigens with PER immunoreactivity was examined in *D. nigrofasciatus*. Reliable results were obtained only in cells with strong immunoreactivities. Double-staining showed that the Crz-ir perikarya and axons in the dorsolateral brain were located in close vicinity to the PER-ir cells and their neurites

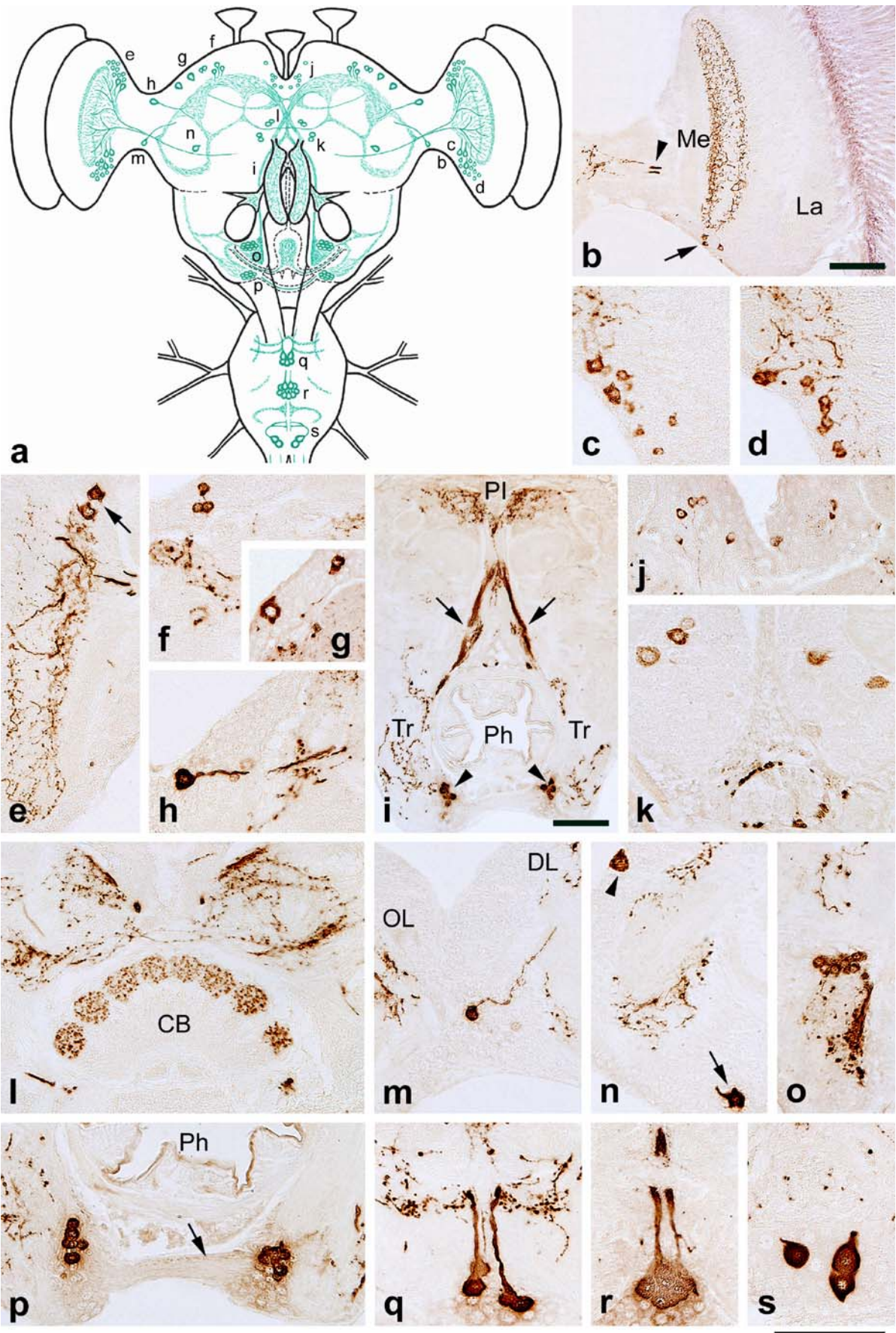


Fig. 8 FXPRLamide immunoreactivity in *A. allardi*. **a** Representation of the immunostaining pattern (*a–s* positions of the respective micrographs in this figure). **b** Pfv cell cluster (*arrow*), two nerve bundles running into the lateral protocerebrum (*arrowhead*), network of fibres in the medulla (*Me*) and absence of fibres in the distal medulla edge and in the lamina (*La*). *Bar* 100 μm . **c, d** Cells of the Pfv cluster with primary neurites extending individually into the medulla. **e** Two neurones of the Pfd cluster (*arrow*) and fibre network in the medulla. **f** Group of three small cells (*top*) in the anterior region of the dorsolateral protocerebrum. **g** Two of the large neurones in the dorsolateral protocerebrum. **h** Neurone at the base of the optic stalk projecting into the protocerebrum. **i** Nerve bundles (*arrows*) extending from the fibre networks in the pars intercerebralis (*PI*) and dorsolateral protocerebrum to the contralateral tritocerebrum (*Tr*) where they extensively ramify, and clusters of about 10 cells (*arrowheads*) in the ventral tritocerebrum close to the egress of the postoesophageal commissure that circumvents the oesophagus (*Ph*). *Bar* 100 μm . **j, k** Weakly stained cells in the anterior and posterior pars intercerebralis, respectively. **l** Varicose fibres in the medial protocerebrum and within the layer I in the upper division of the central body (*CB*). **m** Neurone at the base of the optic lobe (*OL*), with axon projecting into the dorsolateral protocerebrum (*DL*). **n** Cell in the posterior cortex of the protocerebrum sending its axonal projection dorsally (*arrow*) and one of the neurones in the dorsolateral protocerebrum (*arrowhead*). **o** About 10 neurones clustered in the medial wedge between the deuto- and tritocerebrum. **p** Bilateral cluster of about 10 cells in the ventral tritocerebrum sending fibres to the contralateral tritocerebrum through the suboesophageal commissure (*arrow*) and passing around the oesophagus (*Ph*). **q–s** Neurones in the Mdb-Vm, Mxl-Vm and Lb-L clusters of the SOG. *Bar* (*bottom right*) 100 μm (applies to all micrographs without an internal bar)

(Fig. 10a–d). The positions and morphology of two Crz-ir cells in the OL and a single neurone at the lobe base strongly resembled that of some of the PER-ir cells but proof of co-localisation was technically impossible. The presence of CCAP immunoreactivity was established in two of the six large PER-ir cells in the Pfv cluster (Fig. 4v–x). None of the FXPRLa-ir cells in the OL was identical with the PER-ir somata but the FXPRL-ir and PER-ir fibres within the medulla and in the bundles projecting to the lateral protocerebrum were in close contact (Fig. 10e–h).

Discussion

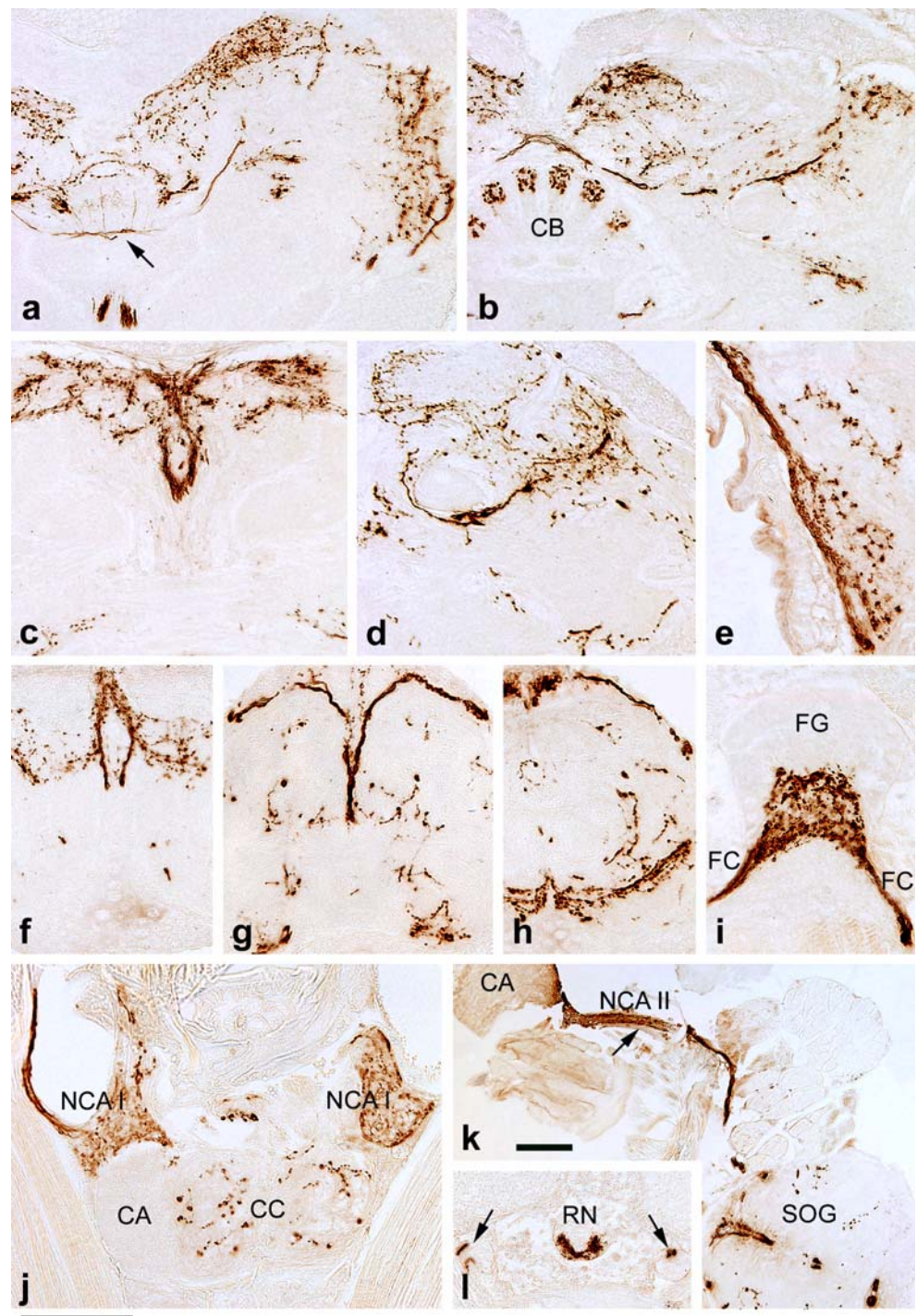
Our previous investigation of *D. nigrofasciatus* and *A. allardi* has shown that antigens related to the clock proteins PER, DBT and CRY are confined to specific cells, some of which co-express PER immunoreactivity with either DBT immunoreactivity or CRY immunoreactivity and may actually contain all three antigens (Shao et al. 2006). The reacting cells occur in clusters that are in similar positions in the two species but that differ considerably in numbers and in their antigen profiles. In contrast to these differences, both cricket species show similar distribution patterns of immunoreactivity for Crz, CCAP and FXPRLa (Table 1), which are always in regions in which the PER-ir, DBT-ir and CRY-ir cells are found, at least in one of the cricket

species. Some of the cells expressing immunoreactivity for Crz, CCAP or FXPRLa are actually either identical or lie in close proximity to the cells stained for a clock protein. Double-staining performed in *D. nigrofasciatus* has established the co-localisation of immunoreactivity for CCAP and PER in two large neurons of the Pfv cluster and the close proximity of the Crz-ir and PER-ir neurones in the medial part of the DL but has excluded the identity of the FXPRLa-ir and PER-ir perikarya in the OL. The low staining intensity has precluded co-localisation in several cases in which two antisera react with cells of identical morphology and position. One cell at the base of the OL and two small Pfv cells most probably contain immunoreactivity for both Crz and PER in *D. nigrofasciatus*, and two cells in the posterior protocerebrum cortex probably contain immunoreactivity for FXPRLa and PER in both cricket species. The Crz-ir, CCAP-ir, and FXPRLa-ir cells in the medial tritocerebrum might be identical and are probably components of the clusters of eight PER-ir and five to six DBT-ir cells found in the tritocerebrum of *A. allardi* (Shao et al. 2006). The CCAP-ir cells in FG are localised in a similar position to that of the DBT-ir cells present in *A. allardi* (Shao et al. 2006). The CCAP-ir and FXPRLa-ir cells of the Mdb-Vm and Mxl-Vm clusters and the FXPRLa-ir cells of the Lb-Vm cluster, which occur in the SOG of both species, apparently overlap in *A. allardi* with the DBT-ir and CRY-ir cells and with the DBT-ir cells, respectively.

The course of Crz-ir, CCAP-ir and FXPRLa-ir fibers often parallels the distribution pattern of processes containing the clock-related proteins. The CCAP-ir and FXPRLa-ir perikarya in the Pfv and Pfd clusters send fibres to the medulla and lamina, which are also innervated by the PER-ir neurones and, in *D. nigrofasciatus*, also by the DBT-ir neurones (Shao et al. 2006). The OLs are connected with the protocerebrum and tritocerebrum by several CCAP-ir and FXPRLa-ir nerve bundles that in part follow similar trajectories as the PER-ir and DBT-ir fibres in *D. nigrofasciatus*. Fibres carrying the hormone-like antigens to the corpora cardiaca, nervi corporis allati and FG apparently intermingle with the PER-ir, DBT-ir (absent in FG of *A. allardi*) and CRY-ir fibres.

The similarities between the distribution of the clock-related and the hormone-like antigens in the cephalic ganglia of crickets indicate some relationship but do not prove an interaction. The data from other insects also do not specify links between the circadian clock and hormone secretion. In Lepidoptera such as *M. sexta*, a direct dependence on the clock is likely in the case of Crz, which is produced by the PER-expressing Ia₁ neurones (Wise et al. 2002). Crz, which initiates ecdysis by stimulating hormone secretion from the Inka cells, is released in a diurnal fashion (Kim et al. 2004). CCAP

Fig. 9 FXPRLamide-ir fibres in *A. allardi*. **a** Frontal view of the protocerebrum with a fibre bundle (arrow) running under and sending numerous thin branches into the central body. **b** Medial section of the protocerebrum with fibres passing dorsally over the central body (CB). **c** Posterior pars intercerebralis with varicose fibres merging into several fascicles. **d** Fibre arborisation in the dorsolateral protocerebrum. **e** Nerve bundle from the protocerebrum ramifying in the tritocerebrum. **f** Fibres arising from the Mdb-Vm SOG cluster. **g, h** Fibres arising from the Mxl-Vm SOG cluster. **i** Fibres running through frontal connectives (FC) and dispersing in the frontal ganglion (FG). **j** Varicose fibres in the corpora cardiaca (CC) and nervi corporis allati I (NCA I) but an absence of FXPRLa immunoreactivity in the corpora allata (CA). **k** FXPRLa immunoreactivity in the nervus corporis allati II (NCA II, arrow) connecting the corpora allata (CA) and SOG. Bar 100 μ m. **l** Fibre varicosities in the recurrent nerve (RN) and nervi corporis cardiaci I (arrows). Bar (bottom right) 100 μ m (applies to all micrographs without an internal bar)



from the segmentally arranged neurones in the ventral nerve cord is another important regulator of ecdysial events in moths (Gammie and Truman 1999) and to a lesser extent also in *D. melanogaster* (Clark et al. 2004). CCAP secretion is under the control of an endocrine cascade (Truman 2005) that may have more than one link to the circadian clock. Among the FXPRLamides, PBAN is released every night with a precise diurnal rhythm (Ichikawa et al. 1996) but the control of this periodicity

has not been examined. PBAN is derived from a common precursor together with the diapause hormone (DH), which induces embryonic diapause in *B. mori* (Yamashita and Hasegawa 1985) and regulates pupal diapause in *Helicoverpa armigera* (Sun et al. 2005). DH production is determined by the photoperiod. Diurnal changes in the PBAN concentration have been reported to reflect fluctuations in the corresponding mRNA (Wei et al. 2004), suggesting regulation at the transcription level. However,

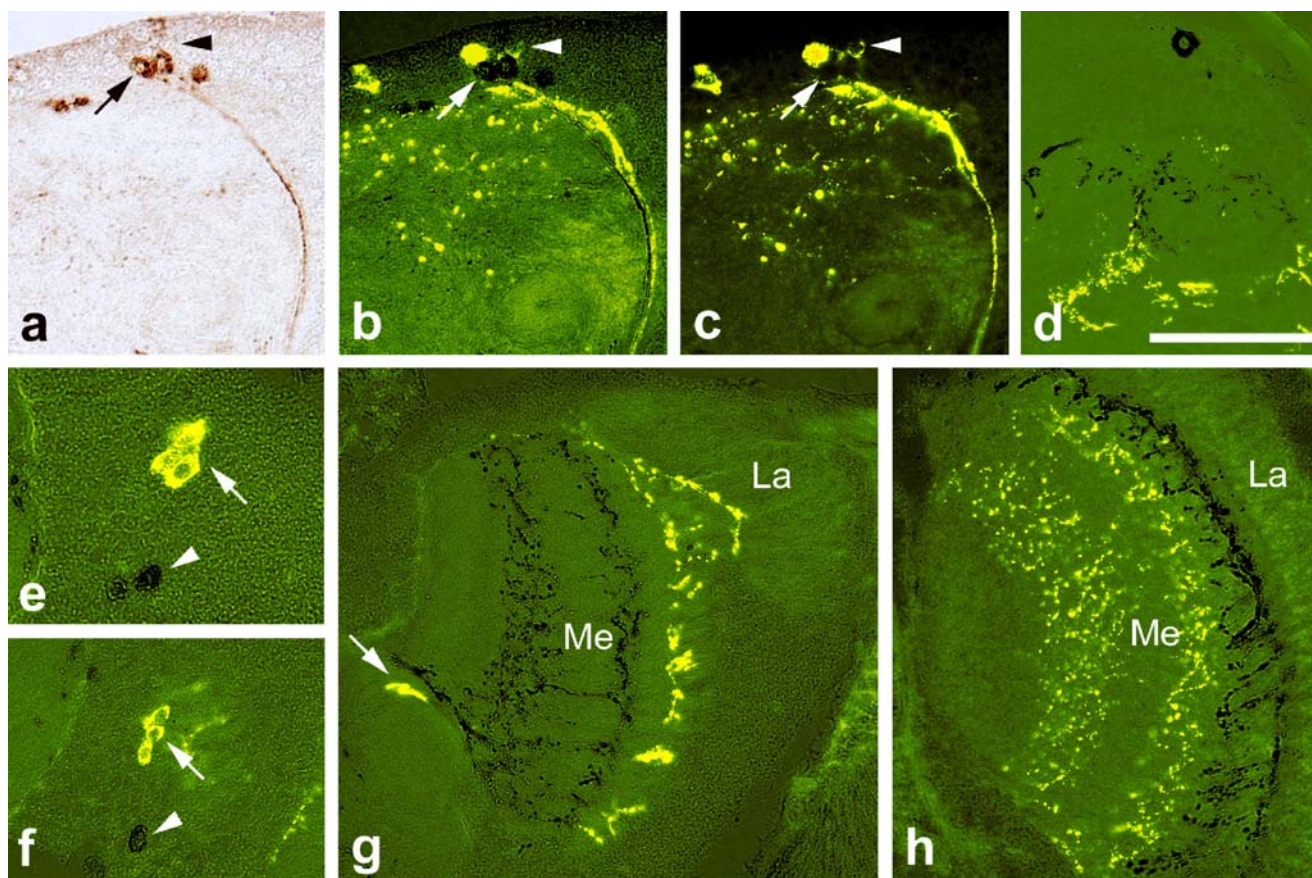


Fig. 10 Double labelling in *D. nigrofasciatus*. **a–d** Simultaneous detection of immunoreactivity for PER (*arrows*) and Crz (*arrowheads*) in the dorsolateral protocerebrum. **a** PER immunoreactivity detected by DAB staining. **b** Overlay of immunoreactivity for PER (DAB staining) and Crz (fluorescent labelling). **c** Immunofluorescent Crz immunoreactivity. **d** Overlay of immunoreactivity for Crz (DAB staining) and PER (fluorescent labelling). *Bar* 100 μ m. **e–h** Simultaneous detection of immunoreactivity for PER and FXPRLa in the optic lobe. **e**, **f** large and small PER-ir cells (*arrows*), respectively,

comprising the Pfv cluster are separated from the FXPRLa-ir neurones (*arrowheads*). **g** Immunofluorescent PER-ir fibres reaching the inner lamina (*La*) surface are near to the DAB-stained network of FXPRLa-ir fibres in the medulla (*Me*) and the fibre bundle (*arrow*) that projects into the lateral protocerebrum. **h** Enzymatic detection of immunoreactivity for PER and fluorescent detection of FXPRLa immunoreactivity in the outer medulla (*Me*). *Bar* (*bottom right*) 100 μ m (applies to all micrographs without an internal bar)

the different timing of the DH and PBAN secretions reveals that there is some control of the posttranslational processing of their common precursor.

We have not detected diurnal fluctuations in the staining intensity of any antigen in either perikarya or fibres. The photoperiod also has no effect on the distribution and the intensity of immunoreactivities. However, our data do not exclude fluctuations in the amounts of functional hormones. The presence of the apparent CCAP precursor on Western blots suggests that the antiserum readily detects the prohormone. Its content might be stable and the diurnal cycles of the hormone release might be regulated at the level of proteolytic prohormone processing. Moreover, oscillations in the concentration of circulating neurohormones might be controlled at the sites of their release into the haemolymph. Neither of these mechanisms can be detected by immunocytochemistry.

The extensive ramification of immunopositive fibres in several regions of the brain and SOG is consistent with the

notion that most insect neuropeptides act as neurotransmitters or modulators (Nässel 2002). Immunopositive fibres in the tritocerebrum and FG resemble the distribution of CCAP immunoreactivity in *L. migratoria*, a distribution that has been interpreted as an indication of neurohormonal regulation of feeding (Dircksen and Homberg 1995). Crz-ir, CCAP-ir and FXPRLa-ir fibres from the protocerebral, tritocerebral and possibly SOG neurones ramify in the corpora cardiaca and the nervi corporis allati; some neurites containing FXPRLa-ir also terminate in the corpora allata. All these organs are probable sites of hormone discharge into the haemolymph. The fibres entering the FG might release their contents in the recurrent nerve adjacent to the digestive tract. Circadian regulation of hormone release might be executed at any of these sites.

Taken as a whole, our results demonstrate a spatial relationship between the distribution of immunoreactivity to Crz, CCAP and FXPRLa and the expression of the

Table 1 Numbers of immunopositive cells and intensity of immuno-staining. A summary of the immunoreactivities to three antibodies, anti-Corazonin, -CCAP and -FXPLa antisera in two species of Nemobiinae, *D. nigrofasciatus* and *A. allardi*

Site	<i>D. nigrofasciatus</i>			<i>A. allardi</i>				
	Crz immunoreactivity	CCAP immunoreactivity	FXPRLa immunoreactivity	Crz immunoreactivity	CCAP immunoreactivity	FXPRLa immunoreactivity		
OL	Pfv Large cells	–	2 ++++	2–3 ++++	–	2 ++++	2–3 ++++	
		2 ++	3–4 +	10 ++ ^b	–	5–6 ++	10 ++++ ^b	
	Pfd Large cells	–	2 ++++	2–3 ++++	–	–	2–3 ++++	
		–	–	10 ++ ^b	–	–	10 ++++ ^b	
	Pr	Base of optic stalk	1 +++	–	–	1 ++++	–	1 ++++
		Posterior cortex	–	–	2 ++++	–	–	1–2 ++++
PI - anterior ^a		4–6 ++	4–6 +	4–6 ++	4–6 ++	4–6 +	15 ++ ^b	
PI - posterior ^a		6–8 ++	6–8 ++++	20 ++ ^b	–	6–8 and 2 ++++	10 ++ ^b	
DL Large cells		3 ++++	1 ++++	2 and 1 ++++	3 ++++	–	2 and 1 ++++	
	Small cells	5–6 +++	6–7 ++++	5–6 ++++	5–6 +++	2 +++	5–6 ++++	
Tr	Lateral region	3 +++	1 ++++	–	–	1 ++++	–	
	Medial region	3–4 ++	3–4 +++	10 ++++ ^b 10 ++++ ^b	3–4 ++ 1–2 +++	3–4 ++	10 ++++ ^b 10 ++++ ^b	
SOG	Mdb-Vm ^a	–	4–5 ++	4–5 ++++	–	4–5 ++	4–5 ++++	
	Mxl-Vm ^a	6 +++	6–7 ++	10 ++++ ^b	–	6–7 ++	10 ++++ ^b	
	Lb-L	–	2–3 ++	2–3 ++++	–	2–3 +++	–	
	Lb-Vm ^a	–	–	4 ++++ and 15 +	–	–	4 ++++	
FG	Cells	–	3–4 ++++	–	–	3–4 ++++	–	
	Arborisation	++++	++	++++	++++	++++	++++	
CC	Cortex arborisation	–	++++	++++	–	++++	++++	
	Central arborisation	–	++++	++++	–	–	++++	
	Fibre bundle	++++	–	–	++++	–	–	
NCA I	–	++++	++++	–	++++	++++		
CA	–	++	–	–	++	–		

Immunoreactivity was quantified as absent (–), weak (+), moderate (++) and strong (++++). Cell numbers are mostly given for one of the bilateral clusters (*OL* optic lobe, *Pfv* proximal frontoventral cluster, *Pfd* proximal frontodorsal cluster, *Pr* protocerebrum, *PI* pars intercerebralis, *DL* dorsolateral region of the protocerebrum, *Tr* tritocerebrum, *SOG* suboesophageal ganglion, *Mdb-Vm*, *Mxl-Vm*, *Lb-Vm* ventromedial cell cluster in the mandibular, maxillary and labial neuromere, respectively, *Lb-L* lateral cells in the labial neuromere, *FG* frontal ganglion, *CC* corpora cardiaca, *NCA I* nervus corporis allati I, *CA* corpora allata).

^a In the case of medial clusters, the numbers concern the whole ganglion

^b Numbers of cells are only approximate

clock-like proteins in the cephalic ganglia of two related cricket species. This distribution is consistent with the suggestion that the crickets possess two clocks overlapping in the central brain: one clock is associated with the OL and the other with the tritocerebrum and SOG (Shao et al. 2006). The occurrence of the clock-related proteins indicates that the OL system prevails in *D. nigrofasciatus* and the tritocerebral and SOG system in *A. allardi*; neverthe-

less, the distribution of the hormone-like antigens is similar in both species.

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