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Immunohistochemical localization of clock proteins (DBT and PER), and [His^7]- and [Arg^7]-corazonins in the cerebral ganglia of *Bombyx mori:* Are corazonins downstream regulators of circadian clocks?

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Abstract. The brain and subesophageal ganglion (BR-SG) of the commercial silk worm, *Bombyx mori*, were stained immunohistochemically at the larval stage for circadian clock neurons with antibodies against Doubletime (DBT) of *B. mori* and Period (PER) of *Periplaneta americana*. The BR-SGs were also stained with antisera against [Arg^7]-corazonin, which has been known to be present in *B. mori* and co-localized with PER in *Manduca sexta,* and against [His^7]-corazonin, a homolog identified in other species. From co-localization of [Arg^7]-corazonin and PER-like reactivities in the pars lateralis, [Arg^7]-corazonin is suspected to be a downstream regulator of the circadian clock in *M. sexta.* DBT- and corazonin-like immunohistochemical reactivities were found in both the neurosecretory cells of the pars intercerebralis (PIC) and pars lateralis (PL) in *B. mori*. Small numbers of neurons shared both reactivities against anti-DBT and anti-corazonin. The majority of the immunopositive cells were common to both corazonins, but some cells were unique in expressing either reactivity against [His^7]-corazonin or [Arg^7]-corazonin only. The results suggest that there is a diversity in the clock output pathway among lepidopterans and that [His^7]-corazonin may be present in *B. mori*, as well as [Arg^7]-corazonin, although the former has not been chemically identified in this species. Corazonin may be a downstream regulator of circadian clocks in *B. mori* because of the co-localization of [His^7]-corazonin at PIC and [Arg^7]-corazonin at PL with anti-DBT.

INTRODUCTION

The molecular biology of circadian systems has advanced recently and provided many molecular tools to investigate the anatomy of circadian/photoperiod clocks in various organisms (Williams & Sehgal, 2001). Also demonstrated was the co-localization of some peptides with circadian clock markers such as Period (PER), Timeless (TIM) and Doubletime (DBT). Pigment dispersing factor (PDF), one such peptide, has been demonstrated in the PER-expressing lateral neurons in *Drosophila melanogaster* (Helfrich-Forster, 1995), and is thus suspected to be a candidate as an output peptide messenger of circadian clock neurons (Renn et al., 1999). In *Manduca sexta*, however, pigment dispersing hormone (PDH), a homolog of PDF, was found in the pars intercerebralis (PIC) (type IIb) which lacked PER-expressing cells (Homberg et al., 1991; Wise et al., 2002). PER-expressing cells were instead found in neurosecretory cells of group Ia at the pars lateralis (PL), which expressed immunohistochemical (IHC) reactivity against [Arg^7]-corazonin (Wise et al., 2002). Thus, corazonin may function as an output messenger of the circadian clock in this species.

DBT (a homolog of casein kinase I δ) clone was found in the EST data base of the commercial silkworm, *Bombyx mori*, for which we determined an entire nucleotide sequence and produced a GST-DBT fusion protein in *E. coli*. We then immunized rabbits with this fusion protein, obtained an antibody against this antigen in several insects, and found that the reactivity shown with this antiserum was the same as that of anti-*Periplaneta* PER antiserum in *P. americana* and *Antheraea pernyi* (Ichihara et al., in preparation). In the present investigation, we mapped DBT-like immunohistochemical reactivity in the larval brain of *B. mori*. We used this antibody to locate circadian clock neurons and co-stained with two anti-corazonin sera against [Arg^7]-corazonin (Veenstra, 1989) and [His^7]-corazonin (Veenstra, 1991). In *M. sexta* only lateral neurosecretory cells of group Ia express both PER- and [Arg^7]-corazonin-like antigens and extend their processes to the ipsilateral cerebral hemisphere and to the CC and CA. PER in this group of neurosecretory cells resides both in the nucleus and cytoplasm (Wise et al., 2002). In *A. pernyi* three groups of neurosecretory cells show PER-like IHC reactivity in the dorsal protocerebrum, including PIC (Sauman & Reppert, 1996).

MATERIALS AND METHODS

Insects

Larvae of the Daizo p50 strain of *B. mori* were reared on artificial diet (Silkmate^{R,} for early instar larvae of original strains, Nihon Nohsan Kogyo, Co.) under 12L : 12D at 25°C and used for this investigation. Brains of larvae were dissected at the mid point of daytime and night time. The brains were fixed shortly thereafter in Bouin for 2 hours, dehydrated with a series of ethanol treatments and cleared with xylene, then incubated with paraffin at 59°C overnight.

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TABLE 1. A summary of pre-absorption tests for corazonin-like immunohistochemical reactivities in larval brain of *B. mori*.

Dilution		Concentration of antigens (μM)			
		[His^7]-corazonin		[Arg^7]-corazonin	
		100	20	100	20
Anti-[His^7]-corazonin	1:1000	+	nc	±	nc
Anti-[Arg^7]-corazonin	1:1000	±	nc	±	nc
	1:20000	nc	±	nc	±

+: absorbed, ±: partially absorbed, nc: not conducted.

Immunohistochemistry

Eight micrometer paraffin brain sections were prepared and adjacent sections were stained with two antibodies. The sections were first de-paraffinated with xylene twice and passed through a decreasing ethanol series containing phosphate-buffered saline (PBS). Based on the instruction manual of the Vecta-stain Elite kit (ABC), primary antibody was overlaid on sections and incubated overnight at 4°C. After rinsing with PBS twice, the secondary antibody, biotinylated anti-rabbit Ig, was overlaid. After rinsing with PBS twice, the tertiary layer, HRP-avidin, was overlaid. After rinsing with PBS twice, the sections underwent chromogenic reaction with DAB.

Preabsorption tests

Preabsorption of the anti-[His^7]-corazonin overnight at 4°C with 100 μM [His^7]-corazonin in the working dilution of the antiserum abolished all immunoreactivity. During the overnight pre-absorption of the anti-[Arg^7]-corazonin at 4°C with 100 μM [Arg^7]-corazonin in the working dilution of the antiserum, the immunoreactivity was partly abolished. When anti-[Arg^7]-corazonin was used which had been diluted 20,000 times, most of the immunoreactivity was abolished (Table 1 and Fig. 1).

RESULTS

Preabsorption tests and antibody specificity

Preabsorption tests conducted at two dilutions in combination with two antigen concentrations are summarized in the results of Table 1. The results indicated complex reactivities between antigens and antibodies. The antibodies recognized two types of antigens, but at the individual neuron level pre-absorption was clearly demonstrated. Based on these results, two types of antigens seem to be present in the larval brain of *B. mori*. Figure 1 shows the pre-absorption tests for the two corazonins. Fig. 2 shows that some neurons share DBT-like and PER-like IHC reactivities. It is thus likely that this DBT-like IHC reactivity represents DBT, rather than general casein kinases. Namely, this DBT-like IHC reactivity represents the site of circadian clock neurons.

IHC reactivity in adjacent sections of larval brain stained alternately with anti-DBT and anti-[His^7]-corazonin sera

Nine pairs of cell bodies of the brains dissected at midday stained with [His^7]-corazonin, 3 in the PIC and 6 in the PL of the larvae sampled at midday. In the PIC, the neuronal processes also strongly stained with anti-[His^7]-corazonin. Out of the nine neurons 3 pairs co-stained with anti-DBT, 2 in the PIC and 1 in the PL of the brain of larvae (Fig. 3).

Nine pairs of cell bodies of the brain dissected at midnight from larvae stained with anti-[His^7]-corazonin, 4 in the PIC and 5 in the PL. Neuronal processes in the PIC were strongly stained with anti-[His^7]-corazonin. Two pairs in the PIC and 2 pairs in the PL were co-stained with anti-DBT (Fig. 4).

IHC reactivity in adjacent sections of larval brain stained alternately with anti- DBT and anti-[Arg^7]-corazonin sera

Five pairs of cell bodies in the PIC and 5 pairs of cell bodies in the PL of the brain of larvae dissected at midday stained with anti-[Arg^7]-corazonin serum. Of these, only 1 pair in the PL co-stained with anti-DBT serum (Fig. 5).

Five pairs of cell bodies in the PIC and 4 pairs in the PL of the brain of larvae dissected at midnight stained with anti-[Arg^7]-corazonin. Of these, 2 pairs in the PL were co-stained with anti-DBT (Fig. 6).

Fig. 7 illustrates the staining patterns of larval brains sampled at midday and midnight with 3 antibodies–DBT, [His^7]-corazonin and [Arg^7]-corazonin. The first prominent features were the number and sites of DBT, [His^7]-corazonin and [Arg^7]-corazonin. In *M. sexta* PER and [Arg^7]-corazonin-like IHC reactivities were restricted to the PL, while a substantial number of cells which were IHC reactive to anti-DBT and anti-corazonin were also recognized in the PIC in *B. mori*. Secondly, some cells showed day/night fluctuations in the IHC reactivity.

Fig. 1. Pre-absorption tests for two corazonins. A – anti-[His^7]-corazonin serum diluted at 1:1000 pre-absorbed with 100 μM [His^7]-corazonin peptide; B – anti-[His^7]-corazonin serum at 1:1000, with 100 μM [Arg^7]-corazonin peptide; C – anti-[Arg^7]-corazonin at 1:20000 times; D – anti-[Arg^7]-corazonin at 1:20000, with 20 μM [Arg^7]-corazonin peptide; E – anti-[Arg^7]-corazonin 1:20000, with 20 μM [His^7]-corazonin peptide; F – schematic representations of IHC reactivity in the brain of larva with the two anti-corazonin sera. [His^7]-like IHC reactivity was illustrated in the left hemisphere, while [Arg^7]- like IHC reactivity in the right. Scale = 100 μm.

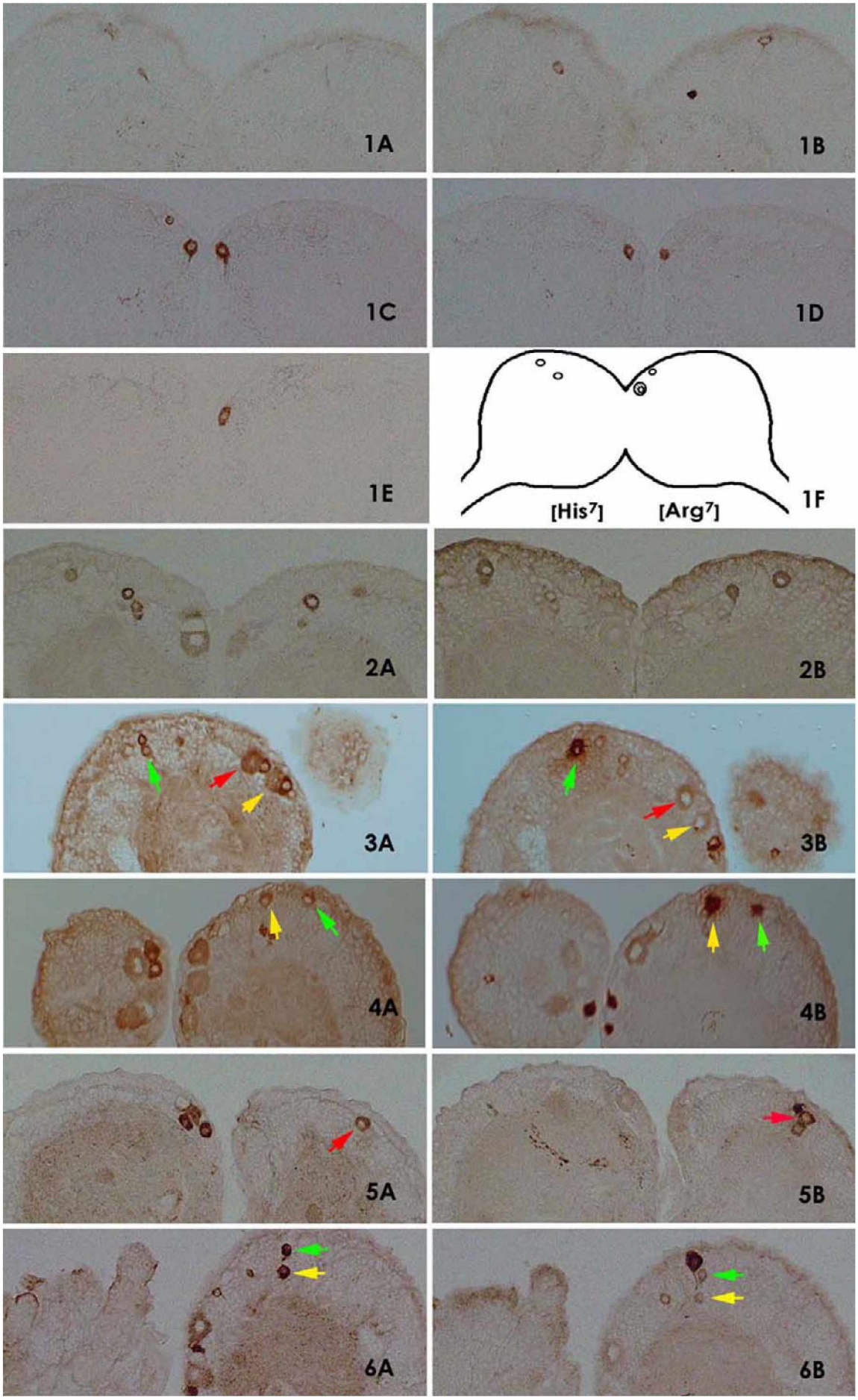
Fig. 2. Adjacent sections alternately stained with anti-DBT (A) and anti-Per (B) sera in larval brain dissected at midnight. Scale =100 μm.

Fig. 3. Successive frontal sections of midday larval brain alternately stained with anti-DBT 1:1000 (A) and anti-[His^7]-corazonin at 1:1000 (B). The arrows with different colors show different cells that were co-stained. Scale = 100μm.

Fig. 4. Successive frontal sections alternately stained with anti-DBT (A) and anti-[His^7]-corazonin (B) in larval brain dissected at midnight. The arrows with different colors show different cells that were co-stained. Scale =100 μm.

Fig. 5. Adjacent sections alternately stained with anti-DBT (A) and anti-[Arg^7]-corazonin (B) sera in larval brain dissected at midday. The arrows show the cells that were co-stained. Scale =100 μm.

Fig. 6. Adjacent sections alternately stained with anti-DBT (A) and anti-[Arg^7]-corazonin (B) sera in larval brain dissected at midnight. The arrows show the cells that were co-stained. Scale =100 μm.


1A
1B
1C
1D
1E
[His7]
[Arg7]
1F
2A
2B
3A
3B
4A
4B
5A
5B
6A
6B

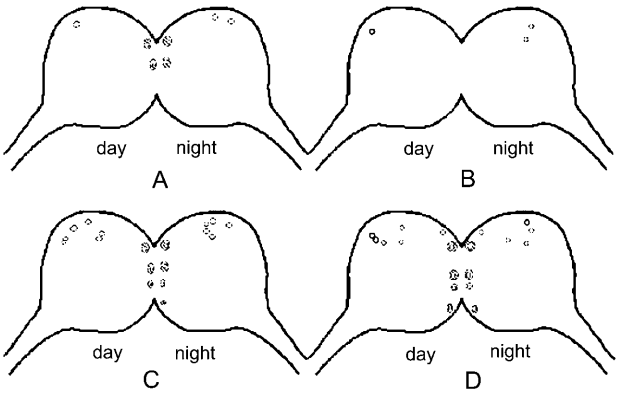


Fig. 7. Schematic representations of IHC reactive cell bodies in larval brains. A – cell bodies co-stained with anti-DBT and anti-[His^7]-corazonin sera; B – cell bodies that co-stained with anti-DBT and anti-[Arg^7]-corazonin; C – cell bodies stained uniquely with anti-[His^7]-corazonin; D – cell bodies stained uniquely with anti-[Arg^7]-corazonin. Left hemisphere represent the larval brain dissected at midday. Right hemisphere the larval brain dissected at midnight.

Thirdly, unique [His^7]-corazonin-like IHC reactivity was observed.

DISCUSSION

[Arg^7]-corazonin was first isolated from *P. americana* as a potent cardioaccelerator (Veenstra, 1989) and was chemically identified from *B. mori* (Hua et al., 2000). It induced melanization of body color in *Locusta migratoria*, but failed to induce the same effect in *B. mori* (Hua et al., 2000). The true function of this peptide in this species remains unknown, although it has recently been demonstrated to affect spinning activity when injected into spinning larvae (Tanaka et al., 2002). The present study suggests that its secretion may be under circadian control, as it is co-localized with PER, as in *M. sexta.*

Not only [Arg^7]-corazonin, but also [His^7]-corazonin-like IHC reactivities, were present in the BR-SG complex of *B. mori.* The reactivities against both corazonins partially overlapped but not always (Fig. 7). The shared reactivities against both DBT and [His^7]-corazonin occurred in some neurons, but these neurons were not the same ones that showed shared reactivities against DBT and [Arg^7]-corazonin (Fig. 7A, B). This may show that circadian output pathways are not singular, but that there are two distinct corazonin output pathways, even in the same species. Day/night variation in IHC reactivity was apparent in different neurons.

Distinct patterns of distribution of the two corazonins, although partially overlapping, were obtained in the present investigation. Together with the cross-reactivity shown in Table 1, both [His^7]- and [Arg^7]-corazonin-like antigens are co-localized in some clock neurons, though antisera may cross-react with different corazonin antigens. The results indicate at least a diversity in downstream peptide pathways among lepidopterans (cf. Wise et al., 2002). Although a previous attempt failed to isolate [His^7]-corazonin from *B. mori* (Hua et al., 2000), functional [His^7]-corazonin may be hidden in *Bombyx* brain. This peptide may have some role in neurotransmission between the circadian pacemaker neurons and peripheral target, as the IHC reactivity showed a dynamic day/night fluctuation in some neurons and some reactive neurons share IHC reactivity against DBT.

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