

University of South Bohemia in České Budějovice

Faculty of Biological Sciences



Bc. Thesis

**IMMUNOHISTOCHEMICAL LOCALIZATION OF NEUROPEPTIDES IN THE
CENTRAL NERVOUS SYSTEM OF THE MEDITERRANEAN FLOUR MOTH
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Annotation:

The aim of this study was to determine, with the aid of immunoaffinity techniques, both the spatial and temporal pattern of production of several insect neuropeptides (allatostatin, allatotropin, diuretic hormone, eclosion hormone, prothoracicotropic hormone, corazonin and pigment-dispersing factor) and thus explore their possible connection to the circadian clock system.

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I hereby declare that I have written this thesis by myself, on the basis of my own results and with the help of the literature resources listed.

I declare that in accordance with the § 47b of the Act No. 111/1998 Sb., as ammended, I agree with publishing of the electronic version of this Bc. thesis in its full form. I agree that this electronic version will be placed in the STAG database administred by the University of South Bohemia in České Budějovice at its websites. I am aware of the fact that this part of the STAG database is open to public.

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1. Introduction

The research summarized in this thesis examines seven insect neurohormones (allatostatin, allatotropin, diuretic hormone, eclosion hormone, corazonin, prothoracicotropic hormone and pigment-dispersing factor). The major aim of this work was to determine their cellular localization within the brain ganglion and especially the temporal pattern of their production. In other words, the goal of this study is to explore their possible connection to the circadian clock system. For this reason, the distribution of the neurohormones listed above has been determined at two timepoints – ZT (Zeitgeber time) 4 and 16, that is, four hours after the “lights on” and “lights off”, respectively. The preparations were then screened for any differences in either the intensity of staining of the immunoreactive material or its distribution within the cell compartments (e.g. translocations between nucleus and cytoplasm). Such differences would indicate that the protein production of the particular neurohormone oscillates and might therefore be under direct circadian control.

As a model organism was chosen the white-eyed mutant of the Mediterranean flour moth, *Ephestia kuehniella* which belongs to the relatively less advanced group of lepidopterans, the family *Pyralidae*. This choice was made for several reasons: *Ephestia* is a very convenient model species, because it has quite a short reproductive cycle and its breeding in laboratory is relatively simple and well established at the Institute of Entomology. *Ephestia* also ranks to the major insect pest species and thus the research on this animal could also have some practical applications in the pest control.

1. 1. Circadian clocks

Although we perceive the unidirectional flow of time from past to future our life is largely organized into a 24-hrs sections of day and night. This inescapable rhythm driven by the rotation of Earth around the Sun rules every living thing from the very beginning of its existence and thus creates not only spatial but also temporal organization of nature. Since the sun is the primary source of energy for every organism, the living creatures could not but adapt to its rhythm. This rhythmic organization of time forms new niches to exploit and also new borders not to be crossed; it truly adds another dimension to our lives: you have to know where to go but also when. Perfect timing is absolutely essential in a world where living organisms have to compete for almost everything from food to mate. A self-sustaining timekeeping device would therefore provide the animal not only with important information but also with a significant head start. It would give it a chance to prepare before actual need in order to fully exploit all the possibilities to feed or mate. It would also enable the organism to maintain synchrony even when the weather conditions are unfavorable and force the organism to seek shelter where it receives little or no information about the phase of the day of the outside world.

Essentially all organisms we have ever looked at truly do display daily rhythms in behavior and physiology. This obvious rhythmicity in animal or plant behavior and/or physiology though, was long considered being just a direct response to certain environmental cues such as the solar phase or temperature. The first one who thought otherwise was a French astronomer Jean Jacques d'Ortous de Mairan. In 1729 he carried out several experiments with the plant *Mimosa* that displayed daily leaf movement. He observed that the leaves continued to open and close at about the same time even though the plant had been moved to constant darkness having no information about the phase of the day of the outside world. This led him to a belief that the observed rhythm was not just passively driven by the rhythmic environment but that it was an innate property of the plant itself. From these humble beginnings a whole new scientific field arose – the chronobiology. (Edery, 2000; Dunlap *et al.*, 2004)

It was not until the 20th century though the phenomenon of biological timekeeping received a proper attention. At least two names have to be mentioned when talking about chronobiology – J. Aschoff and C. S. Pittendrigh, the true founders of the modern-day research of biological rhythms.

The phenomenon of circadian (circa – about, dies – day) clocks is rather loosely defined by its three most outstanding properties. First, they are endogenous and able to “free-run” with a period close to (but not exactly) 24 hrs even in the absence of environmental cues (e.g. in constant darkness); second, they can be entrained or reset by changes in environmental conditions to maintain synchrony with local time. Under normal conditions such entrainment occurs daily via master oscillations in the environment, particularly the solar or temperature cycle. Third, they possess a property of so called temperature compensation, which means that the circadian clock system keeps the same period length across a wide range of physiologically relevant temperatures. This is undoubtedly very important especially in poikilothermic organisms because no matter if it is a cold day or a warm one, it still lasts 24 hours. Yet this property is extraordinary given the fact, that biochemical interactions dramatically change their rate with decreasing or increasing temperature. (Saunders *et al.*, 2002; Stanewsky, 2003; Dunlap *et al.*, 2004)

Although everybody felt that keeping the pace with the environment brings many advantages and that it must be an evolutionary selected trait, for long there was no direct evidence to support this belief. Not until the experiment of Ouyang *et al.* (1998). The authors tested the adaptive significance of circadian clock system by measuring relative fitness under competition between various strains of cyanobacteria *Synechococcus* sp. The strains used displayed different free-running period (FRP) lengths. When they were grown in pure cultures, no differences in growth rates were seen, regardless of the light conditions. When the different strains were competed against each other though, the outcome of such competition was dependent on the free-running periods (FRP) of the particular strains and the

light conditions. The strain whose FRP was closer to the actual light-dark cycle length inevitably out-competed the rival strain with not that close match.

The pioneering studies focused on the problem of circadian rhythms mainly from the behavioral point of view and it was much later when the molecular biology took the lead. The burst of new discoveries concerning the molecular basis underlying the rhythmic behavior was so massive that almost overnight we find ourselves having quite a detailed knowledge of factors participating in the clock system in several model species. Nevertheless, there is still much to be elucidated, especially how is the information from the core clock system passed on and processed to finally trigger an appropriate behavior at appropriate time. Undoubtedly, this huge task will occupy yet many generations of chronobiologists.

Nowadays, we have quite a good notion about how the time is measured at the cellular level. We know the core clock components and their interactions. Of course, this information comes from a very restricted number of model species and thus we can experience yet great surprises. As always, probably the most studied insect model species is the fruitfly *Drosophila melanogaster*. Here, I will only briefly summarize the key features and mention only the very basic regulatory factors of its timekeeping system.

Generally, the *Drosophila* clock genes generate a self-sustained circadian oscillation via interconnected positive and negative feedback loops on transcriptional and translational level. In *Drosophila*, the core players in the negative feedback loops are proteins CLOCK (CLK), CYCLE (CYC), PERIOD (PER) and TIMELESS (TIM). CLK and CYC are transcription factors containing a PAS¹ protein dimerization domain and a basic helix-loop-helix (bHLH) DNA-binding domain. *Cyc* is constitutively expressed and CYC protein is therefore abundant, on the other hand *clk* is circadianly regulated (its expression is reduced by the action of another factor – VRILLE - VRI) and thus its mRNA and protein levels oscillate. CYC and CLK heterodimerize and bind to the E-box enhancer elements in the upstream regulating sequences of the *per*, *tim* and *vri* genes thus activating their expression. The levels of mRNAs of these genes peak early at night and they are translated into their respective proteins in the cytoplasm. While in the cytoplasm, PER is phosphorylated by DOUBLETIME (DBT) kinase unless it is protected by association with TIM protein. It takes some time though until enough TIM accumulates in the cytoplasm, which causes a delay in the accumulation of PER contributing to the unbelievably long (about 24 hrs!) cycle length. In addition, another kinase, SHAGGY (SGG) influences the temporal pattern of TIM phosphorylation, which promotes the access of TIM into the nucleus, thereby determining

¹ PAS – PER/ARNT/SIM domain, called after the founding members of the group of proteins containing this dimerization domain: Period protein, human Aryl hydrocarbon receptor nuclear translocator protein and *Drosophila*'s single-minded protein.

the time of its (and consequently also PER's) nuclear entry. When enough TIM and PER proteins finally accumulate, they form a heterodimer and together with (at least) DBT translocate into the nucleus (this happens in the middle of the night). Once in the nucleus, the PER/TIM heterodimer or possibly the PER by itself brings about a repression of their own expression by interfering with the CLK/CYC complex. TIM probably gradually disappears from the heterodimer and thus the unprotected PER is phosphorylated, perhaps through the action of DBT and eventually degraded. There is yet another role for PER (or PER/TIM): they somehow positively influence the expression of *clk* gene so that its protein level begins to rise even as the phosphorylation-induced turnover of PER proceed. By morning the PER

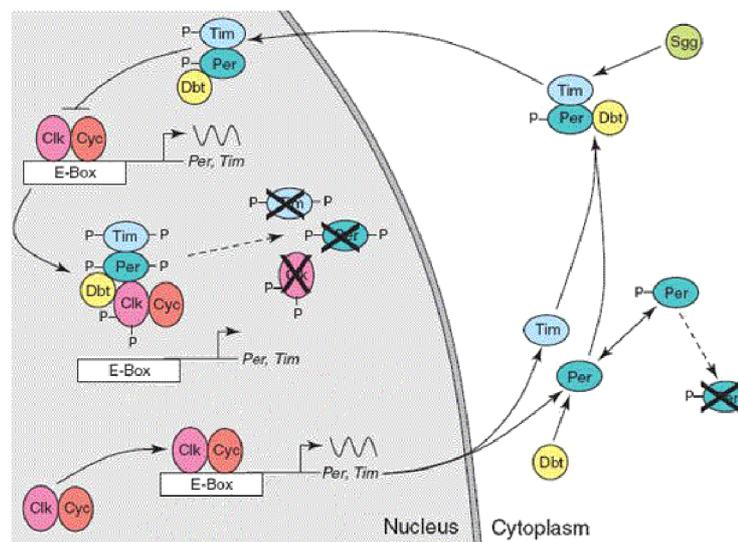


Fig. 1. A schematic picture depicting the basic regulatory loops of the *Drosophila*-like model of the circadian clock system. For description of the regulatory actions see the text. Hardin, P., 2005. The circadian timekeeping system of *Drosophila*. Current Biology 15: R714-R722, modified.

protein is degraded and this releases the CLK/CYC protein complex to activate the expression of clock genes again. CLK/CYC heterodimer seems to provide a link between the core clock mechanism and its output, as the expression of the clock-controlled genes is probably also activated by this dimer. (Stanewsky, 2003; Dunlap *et al.*, 2004; Helfrich-Förster, 2005)

I mentioned before that one of the defining properties of the circadian clock system is its ability to get entrained by the current environmental conditions, and thereby to adapt to the rhythm of the environment. The most prominent entraining agents are undoubtedly light and temperature, here I will only focus on the first one, not only because it is best understood but also because in the study presented here, it is the only factor that differs between the night and day conditions; temperature was held constant. It is known that light resets the clock through a mechanism involving the light-dependent turnover of TIM protein. In *Drosophila*, the principal and cell-autonomous photoreceptor for the clock

appears to be a CRYPTOCHROME (CRY). CRY uses a flavin-based photoreceptor that is independent of the light sensors used for vision. It seems though, that the information about the ambient light conditions is probably mediated through the rhodopsin-based photoreceptors in the compound eye as well. Animals lacking CRY can still be entrained to light-dark cycles but do not react to short-duration light signals (Stanewsky *et al.*, 1998). Light acts acutely through CRY to block PER/TIM activity and leads to the degradation of TIM. As TIM serves as a protector of PER from phosphorylation by DBT the turnover of TIM leads inevitably to the loss of PER as well. (Stanewsky *et al.*, 2003; Dunlap *et al.*, 2004)

The nice and clear theory, derived from the experiments on *Drosophila*, on how the clock mechanism works in insects was seriously shaken by the findings that in the silkworm *Antheraea pernyi*, a homolog of *Drosophila*-PER does not ever translocate into the nucleus of the presumptive clock-neurons. As was mentioned above this translocation is a crucial step in the model of circadian timing as we know it from *Drosophila*. The levels of PER protein do oscillate in these cells though, similarly to those of TIM which is also expressed in the very same group of neurons. Yet neither PER nor TIM was ever detected in the nucleus (Šauman and Reppert, 1996a). Even more surprising was the finding that the clock neurons of *A. pernyi* also express antisense *per* RNA that also undergoes striking circadian oscillation but with an opposite phase to that of sense *per* mRNA, PER or TIM proteins (Šauman and Reppert, 1996a). Certainly the *Drosophila*-like model of the circadian network cannot explain the mechanism of *Antherea*-like timekeeping. Question remains, whether *Antherea* represents just an exceptional case or whether it can be a more widely distributed pattern. Immunocytochemical studies (Závodská *et al.*, 2003) indicate that the latter might be true after all, since the PER-like immunoreactivity was never detected in nuclei of none of the investigated insect species. It is even possible that if there is any exceptional case it is rather that of *Drosophila* than the *Antherea*, because even in the housefly *Musca domestica*, a close relative of *Drosophila* – both belong to the *Cyclorrhapha* group of higher dipterans, no indication of nuclear translocation of PER was found (Šauman, personal communication).

1. 2. Neurohormones

1. 2. 1. Allatostatin

Juvenile hormones are sesquiterpenoids synthesized by corpora allata (CA) and play a key role in both metamorphosis and reproduction in insects. A great deal of *in vivo* studies implicated that the regulation of juvenile hormone biosynthesis occurs via inhibitory (allatostatic) and/or stimulatory (allatotropic) signals from the brain. Shrarrer, for example, demonstrated that severing the nerves projecting from the brain to the corpora allata in the cockroach *Leucophaea maderae* resulted in supernumerary larval stages (Scharrer, 1952). Later, long postulated neurohormones with inhibitory

effect on CA, the allatostatins, were identified in a variety of species (Woodhead *et al.*, 1989; Kramer *et al.*, 1991).

Allatostatins, at least those so far discovered can be divided into three structurally unrelated groups: AST-A group, AST-B group and AST-C group. It seems quite likely that all three types of allatostatins may be present in each insect species, but only one type being allatostatic in the particular one. The type of the active allatostatin varies among species (reviewed by Nässel, 2002).

Allatostatins belonging to the largest (over 70 peptides) **AST-A group** (“cockroach type”) are characterized by a highly conserved core C-terminal sequence, Y/F-X-F-G-L-amide, but otherwise can be quite varied. These allatostatins were first identified by Woodhead *et al.* (1989) in the cockroach *Diploptera punctata* and since that time they have been found in many other insect species – other cockroaches – *Periplaneta americana*, *Blattella germanica* (Bellés *et al.*, 1994), the blowfly *Calliphora vomitoria* (Duve *et al.* 1993), the fruitfly *Drosophila melanogaster* (Yoon and Stay, 1995), locust *Schistocerca gregaria* (Veelaert *et al.*, 1996) and others. These peptides are expressed in the form of a prohormone precursor encoding several putative peptides clustered into domains separated by acidic spacers, e.g. from the cockroach precursor 13 or 14 peptides can be derived, depending on the species (Bellés *et al.*, 1999). Although Lepidopterans have their own family of allatostatins, Davis *et al.* (1997) isolated the cockroach-type allatostatin from *Manduca sexta* as well and named it lepidostatin-1. The authors have proposed rather a neuromodulatory, myomodulatory and myotropic function for lepidostatin in this species as the immunoreactivity to YXFGLamides occurred not only in the brain, abdominal ganglia and their respective neurohemal organs but in many thoracic interneurons as well. It may also function in regulation of fluid transport as it was found to be co-localized and maybe co-released with the *M. sexta* diuretic hormone (Davis *et al.*, 1997). Cockroach-type of AST has been isolated from the codling moth *Cydia pomonella* and the cotton bollworm *Helicoverpa armigera* as well (Duve *et al.*, 1997). For many species where the cockroach-type allatostatins have been actually identified it was shown though that they are not the functional allatostatins and probably have some other function - neuromodulatory and/or myoinhibitory perhaps (reviewed by Nässel, 2002). For example, Yoon and Stay (1995) localized immunopositive neurons using monoclonal antibody against *Diploptera* allatostatin I in *Drosophila melanogaster*. Reactive neurons were found throughout the central nervous system, mostly interneurons, but some motoneurons and endocrine cells in the midgut epithelium too. Neurons projecting to the corpora allata were not immunopositive though, neither in larvae nor adults, suggesting a different role for AST-A in *Drosophila* than JH synthesis inhibitor.

AST-A allatostatins were even found in crustacean (Skiebe, 1999; Duve *et al.* 2002) or mollusks (Rudolph and Stay, 1997).

The second group of allatostatins, the **AST-B** or “cricket type” **allatostatins** has been identified in crickets, locusts and stick insects. In fact, they were first discovered in the *Locusta migratoria* by their myoinhibitory function and were not associated with the regulation of JH biosynthesis at that time (Schoofs *et al.*, 1991). Some time later, Lorenz *et al.* (1995) identified allatostatic peptides of similar structure in the cricket *Gryllus bimaculatus*. (That explains why these peptides are called cricket type allatostatins – they were first found to be allatostatic in crickets despite the fact that similar peptides had been already known from other species, but for their myoinhibitory function.) AST-B allatostatins have a common sequence of W₂W₉-amide – tryptophan residues at the positions 2 and 9 of the N-terminus. Until present, several peptides of this family have been identified in *G. bimaculatus*, stick insect *Carausius morosus* (but it seems they are not allatostatic in this species), *L. migratoria* (where, on the contrary, they were suggested to be truly involved in inhibiting juvenile hormone biosynthesis) and in many other orders as diverse as the Dictyoptera (*P.americana*), Diptera (*Drosophila melanogaster* – drostatins) and Lepidoptera (*M. sexta* and *Bombyx mori*). In many of these species AST-B peptides show no functional relationship to the regulation of JH synthesis and they were discovered on the basis of amino acid sequence (reviewed by Nässel, 2002).

The first peptide to be identified of the **AST-C family** of allatostatins was isolated from pharate adults' heads of *M. sexta* (Kramer *et al.*, 1991). It's a 15 residue, non-amidated peptide with a free C-terminus and a disulphide bridge between the cysteine residues at positions 7 and 14. This so called Mas-AST was found to inhibit completely juvenile hormone synthesis in *Manduca* and to some degree in other moths as well. In the *in vitro* studies, Mas-AST inhibits JH biosynthesis by CA of adults by 100% in *M. sexta* and by 77% in *Heliothis virescens* (Mas-AST dosage: 10 and 50 pmol, respectively), but is not active in other species (the American cockroach *P. americana*, the beetle *Tenebrio molitor* and the grasshopper *Melanoplus sanguinipes*) outside the Lepidoptera order (Kramer *et al.*, 1991). Mas-AST also causes an inhibition of juvenile hormone synthesis in other Lepidopterans – the tomato moth *Lacanobia oleracea* (Audsley *et al.*, 1999) and the true army worm *Pseudaletia unipuncta* (Jansons *et al.*, 1996). It was shown too, that Mas-AST (together with Mas-allatotropin and cydiastatins, helicostatins) has an effect on contractions of the foregut in larvae of *L. oleracea*. Foregut peristaltis is stimulated by Mas-AT whereas both types of allatostatins are myoinhibitory; the inhibitory effect cannot be reversed by application of Mas-AT (Matthews *et al.*, 2007). It seems likely that the AST-C allatostatins inhibit the JH biosynthesis only in Lepidopterans, though they very likely have a pleiotropic function in this order.

Using immunocytochemical techniques the allatostatin-positive neurosecretory cells with axons projecting to the CA have been localized in the lateral protocerebrum. In addition to these cells, there are also interneurons in the brain producing AST (reviewed by Stay, 2000). Allatostatins though are not

localized only within the retrocerebral complex of the brain, they have been found in thoracic and abdominal ganglia, in peripheral nerves innervating visceral and skeletal muscles or in midgut cells too (reviewed by Nässel, 2002). Duve *et al.* (2000, 2005) localized two allatostatins (AST-A and AST-C type) and an allatotropin (Mas-AT) in the frontal ganglion of two lepidopteran species, *L.oleracea* and *Helicoverpa armigera*. This localization implies that these neuropeptides are myoactive on the foregut, since the frontal ganglion is the central part of the stomatogastric nervous system. It innervates parts of the stomodeum via the frontal nerve and posteriorly through the recurrent nerve.

From the diverse localization of the allatostatins we can assume that they have a rather pleiotropic effect. Apart from their name-giving property of inhibiting the juvenile hormone biosynthesis by the corpora allata, they seem to be involved in controlling the gut movement, the action of skeletal muscles and since they were localized also in interneurons of the brain ganglion, they also might have a neuromodulatory function.

1. 2. 2. Allatotropin

Allatotropin was named after its ability to positively influence the biosynthesis of juvenile hormone in several insect species. As we shall see, much more than this can be expected from this neuropeptide.

Allatotropin was first isolated from the tobacco hornworm *M. sexta* as a factor stimulating synthesis of JH in the corpora allata of adult females of not only *M. sexta* but also in *Heliothis virescens*. On the contrary, this peptide had no such effect on CA of *M. sexta* larvae and of the adult females of the beetle *Tenebrio molitor*, the grasshopper *Schistocerca nitens* or the cockroach *Periplaneta americana* (Kataoka *et al.*, 1989). The absence of allatotropic activity of this peptide in larvae of *M. sexta* led the authors to the notion, that its function might be restricted to adult lepidopterans. Nevertheless, Mas-AT mRNA and immunoreactivity have been detected in both the larval and adult stages of various insects, not only lepidopterans (Veenstra *et al.*, 1994; Žitňan *et al.*, 1993), suggesting that Mas-AT may have a biological role in larval insects that is different from its role in the adults.

To date, primary structure of only two types of allatotropin is known: *Manduca sexta* – allatotropin (Mas-AT) is an amidated tridecapeptide and *Aedes aegypti* – allatotropin (Aedae-AT) is a tetradecapeptide which differs in its primary sequence from the Mas-AT at the N-terminus, whereas the C-terminal aminoacids are identical (reviewed by Gäde and Hoffmann, 2005).

Mas-AT gene was shown to express three mRNA isoforms differing through alternative splicing (Taylor *et al.*, 1996). The basis for differences in the spliced mRNAs is the inclusion of one or two additional exons within the open reading frame which contain region predicted to encode peptides with structural similarity to Mas-AT. These peptides have been named allatotropin-like peptides and

designated Manse-ATL (Lee *et al.*, 2002). The presence of the mRNA isoforms is regulated in a tissue-specific manner; the brain and the nerve cord contain primarily one isoform (Horodyski *et al.*, 2001).

Mas-AT like peptide has been isolated from and shown to be active in other lepidopterans (*in vitro* studies): adult *Spodoptera frugiperda* (Oeh *et al.*, 2000), both larval and adult *Lacanobia oleracea* (Audsley *et al.*, 2000), *Pseudaletia unipuncta* (Koladich *et al.*, 2002a) and the Eri silkmoth *Samia cynthia ricini* (Li *et al.*, 2002). Oeh *et al.* demonstrated that synthetic Mas-AT increases the synthesis of JH by isolated CA of adult *S. frugiperda* up to sevenfold and that Mas-AT inhibits the activity of CA only after their prior treating with Mas-AT. A gene encoding a peptide identical to Mas-AT has been cloned from *Bombyx mori* as well (Park *et al.*, 2002) and its expression has been located by Northern Blot to the head, gut and integument tissue.

Mas-AT has also been identified in several non-lepidopteran species, e.g. the blowfly *Phormia regina* (Tu *et al.* 2001), the cockroach *Leucophaea maderae* (Petri *et al.*, 1995), the earwig *Euborellia annulipes* (Dermaptera) (Rankin *et al.*, 2005) and others. Sort of problematic results were obtained from the experiments with the honeybee *Apis mellifera* (Glasscock *et al.*, 2005). Although Mas-AT was shown to be a potent activator of *in vitro* JH biosynthesis of honeybee worker larvae it could not be localized immunocytochemically in the CNS of respective developmental stages. Mas-AT immunoreactivity was only found in brains of early worker prepupae and no reactivity was seen in CA. The authors offer two possible explanations: First, the *A. mellifera* - allatotropin may be structurally different from the Mas-AT and it is therefore not recognized by the anti Mas-AT antiserum; second, Mas-AT peptide is indeed produced in the larval stages of workers, but it is rapidly secreted, which makes it difficult to detect it in cell bodies. In prepupae there is a greater amount of allatotropic factor (Rachinsky, 1996) which accumulates in cells and thus can be detected, the authors deduce.

Mas-AT has been shown to have a rather pleiotropic effect in insects. Veenstra *et al.* (1994) located Mas-AT immunoreactive neurons in abdominal ganglia of *Manduca sexta*, projecting to the transverse nerves which are known to be neurohemal organs in Lepidoptera. The peptide was shown to have strong excitatory effect on the heart of adult *M. sexta*. On the other hand, Lee *et al.* (1998) demonstrated that Mas-AT rapidly inhibits *in vitro* active ion transport across the midgut epithelium of the feeding larvae of *M. sexta* but it exerts little or no such inhibition in the silkworms *Hyalophora cecropia* and *Bombyx mori*. Yet another role for Mas-AT has been proposed by Koladich *et al.* (2002b) based on their experiments with *Pseudaletia unipuncta*. In this species, Mas-AT (together with serotonin) not only positively affects the rate of contraction of the dorsal vessel (insect heart) but also modulates the contractions of ventral diaphragm and thus enhances the hemolymph circulation. This may have an important role in effective mobilization on energy sources as well as in thermoregulation during sustained flight. Most interestingly, Petri *et al.* (2002) reported a role for Mas-AT in the photic

entrainment of the circadian pacemaker in the cockroach *Leucophaea maderae*. This neuropeptide has been localized immunocytochemically to the nodules of a small neuropil, so called accessory medulla, situated ventrally in the optic lobes. The lesion studies had located the circadian pacemaker to this brain region, so Mas-AT became a candidate for processing photic information received from the compound eye or from nearby of the eye. In addition, microinjections of Mas-AT into the vicinity of the accessory medulla resulted in phase-dependent phase shifts in the circadian locomotor activity.

1. 2. 3. Pigment-dispersing factor

The fact that certain hormones are involved in the control of pigment movements in crustacean chromatophores and distal pigment cells in the eye was discovered quite long ago (reviewed by Rao, 2001). The first pigment dispersing hormone to be characterized is an octadecapeptide called light-adapting distal retinal pigment hormone from the eyestalks of the shrimp *Pandalus borealis* (Ferlund, 1976). It triggers light-adapting pigment movements in the eye as well as pigment dispersion in the chromatophores and it is referred to as α -pigment-dispersing hormone (α -PDH) nowadays. Subsequently, an octadecapeptide differing from α -PDH in six positions was identified in eyestalks of the crab *Uca pugilator* (Rao *et al.*, 1985) and was designated as β -PDH. Since then, PDHs from many other crustacean species have been characterized.

In 1987, Rao *et al.* reported the discovery of an analog of the crustacean PDH in the lubber grasshopper *Romalea microptera* and it was even found that insect head extracts can elicit pigment dispersion in crustacean chromatophores. The peptide for which no function could be assigned at that time was therefore named pigment-dispersing factor (PDF) to distinguish it from the crustacean peptides. Pigment-dispersing factors or PDH-reactive material have been identified in many other insect species since that time: cockroaches, crickets, locusts, flies, beetles, earwigs, termites, walking sticks and cicadas (Park and Hall, 1998; Sato *et al.*, 2002; Závodská *et al.*, 2003a, Sehadová *et al.*, 2003). The insect pigment-dispersing factors are closely related to crustacean β -PDH and share several common features: conserved chain length (18 residues), conserved termini (N-terminal Asn, C-terminal Ala-NH₂) and other conserved residues.

PDFs have been shown to play an important role in the regulation of biological rhythms in insects. In *Drosophila melanogaster* PDF has been localized immunocytochemically to three discrete clusters of cells of which so called PDF-Me neurons are particularly interesting. These neurons are named after their localization in the accessory medulla (aMe), a presumptive circadian pacemaker center of *Drosophila* (Helfrich-Förster, 1997). The role of these neurons in the circadian clock system has been further supported by the fact that the same neurons but called as the lateral ventral neurons (LN_vs), had

been previously recognized as neurons containing well established components of the fruitfly pacemaker system (period, timeless, clock and cycle) (reviewed by Nässel, 2002). This group of neurons is heterogeneous and consists of large, strongly immunostained somata (4-6 in adult flies) and small, weakly stained somata (4 in adult flies) (Helfrich-Förster and Homberg, 1993). The adult small ventro-lateral neurons (s-LN_vs) send projections to the dorsal protocerebrum, whereas the large ventro-lateral neurons (l-LN_vs) have wide arborizations in the medulla and send axons via the posterior optic tract to the contralateral medulla, connecting the two bilaterally positioned centers (Helfrich-Förster and Homberg, 1993). Renn *et al.*, (1999) demonstrated that null mutation in the *pdf*-gene or selective ablation of the pdf-neurons results in largely arrhythmic behavior under constant conditions supporting the notion that PDF has indeed a role in the circadian clock system. Subsequently, Park *et al.* (2000) reported striking circadian oscillations of the PDF staining intensity in the cell termini of the s-LN_vs, no such oscillations occurred in the l-LN_vs or in the cell bodies of either type of neurons. The amount of PDF mRNA remains constant throughout the day though, so the cycling must occur on the posttranscriptional level, perhaps through regulated secretion (Park and Hall, 1998). It has been thus proposed that PDF is important in coordinating the circadian network within the fly brain. This notion is further supported by the work of Lin *et al.* (2004) demonstrating that in the absence of PDF the phases of individual clock-neurons (s-LN_vs) gradually disperse, the authors used the cytoplasmic accumulation of clock-protein PER (period) as a marker of this process.

Similar data have been obtained from the experiments on the cockroach *Leucophaea maderae*. Immunoreactive material to the PDF antisera was also localized to the aMe (Nässel *et al.*, 1991) and the PDF-immunoreactive neurons were shown to form a direct coupling pathway between the optic lobes in this species (Reishig *et al.*, 2004). Petri and Stengl (1997) demonstrated that microinjections of synthetic PDF into the vicinity of the accessory medulla result in significant phase shifts in the circadian locomotor activity rhythm. The phase response curve obtained for PDF was different from that obtained for light pulses though, and was similar rather to that for low temperature pulses. These results suggest a possible role for PDF in the nonphotic input into the clock system (Petri and Stengl, 1997). In *L. maderae*, in contrast to *Drosophila*, no significant daily changes in the amount of PDF-immunoreactivity in the brain were found indicating that Lem-PDF is not released in a circadian fashion (Hamasaka *et al.*, 2005).

In other insect species, unlike in *Drosophila*, the PDF does not seem to be co-localized with the products of the clock genes, as was clearly shown in the beetle *Pachymorpha sexguttata* (Frish *et al.*, 1996), the moths *Antherea pernyi* (Šauman and Reppert, 1996a) and *Manduca sexta* (Wise *et al.*, 2002) and suggested for a number of insects in the study of Závodská *et al.* (2003a).

Chuman *et al.* (2002) cloned a different isoform of PDF from the cricket *Gryllus bimaculatus*: It is extraordinarily short and contains nuclear localization signal (NLS) sequence, but it still exhibits high sequence identity and similarity (78-94% and 89-100%, respectively) with the normal-length insect PDFs and β -PDHs. The authors found the PDF-immunoreactive neurons in both medulla and lamina neuropils and observed immunoreactivity also in nuclei of some of the PDF-positive neurons in the lamina. Nevertheless, these results might represent just an exceptional case, since no other PDFs contain a NLS sequence and/or were reported to be present in the cell nuclei.

In summary, the connection of PDF to the circadian clock system appears to be well supported, but its exact role in this system is yet to be elucidated. It is likely though that PDH *per se* is not part of the central clocks and may function rather in synchronization of clock centers in both hemispheres and in modulation of information coming out of the core oscillator.

1. 2. 4. Corazonin

Corazonin was first identified from corpora cardiaca (CC) of the American cockroach *Periplaneta americana* and was found to have strong cardioacceleratory effects; that is why it has been called corazonin, from *corazon*, the Spanish expression for heart (Veenstra, 1989). The *P. americana*-corazonin or the [Arg⁷]-corazonin, is an undecapeptide blocked by pyroglutamate at the N-terminus. The same molecule was subsequently detected in the cockroach *Nauphoeta cinerea* (Veenstra, 1991), the cricket *Gryllus bimaculatus* (Hua *et al.*, 2000), the tobacco hornworm *Manduca sexta* (Veenstra, 1991) and the silkworm *Bombyx mori* (Hua *et al.*, 2000). In the greater wax moth *Galleria mellonella* (Hansen *et al.*, 2001) and in the fruitfly *Drosophila melanogaster* (Veenstra, 1994) a cDNA containing sequence coding for [Arg⁷]-corazonin has been cloned. In *Manduca*, where PER-expressing neurons (and thus the presumptive clock cells) reside not in the aMe, but in the dorsal lateral protocerebral neurons, the [Arg⁷]-corazonin immunoreactivity was localized to the very same group of cells (Wise *et al.*, 2002). Precisely the same situation is seen in another moth, the silkworm *Antheraea pernyi*: the PER expression is confined to four pairs of dorsal lateral cells in protocerebrum and only these neurons are also stained by the [Arg⁷]-corazonin antiserum (Šauman, unpublished data). Considering this, it has been suggested that corazonin may function as an output messenger of the circadian clock.

A slightly different peptide with histidine at the position seven, the [His⁷]-corazonin was initially isolated from the CC of the locust *Schistocerca americana* (Veenstra, 1991) and was then found in *S. gregaria*, *Locusta migratoria* (Tawfik *et al.*, 1999) and the stick insect *Carausius morosus* (Predel *et al.*, 1999) as well. Recently a third isoform of corazonin, the [Thr⁴; His⁷]-corazonin has been identified in *Apis mellifera* (Verleyen *et al.*, 2006).

Although the primary sequence of corazonin appears to be highly conserved throughout the class Insecta, the function of this peptide seems to be quite heterogenic. Certainly, its name-giving property of cardioacceleration is not its common function among insect species and it even seems to be restricted to *Periplaneta* alone (Predel *et al.*, 1994). Corazonin, though, is involved in the regulation of color polymorphism in certain locusts. Locusts, *Locusta migratoria* and *Schistocerca gregaria* being the most intensively studied species, display an interesting property of so called density-dependent phase polymorphism. This phenomenon involves graded changes in morphological, physiological and behavioral traits depending on the population density of the particular insect species. Two extreme forms can be seen, originally recognized as different species, the solitary form and the gregarious form. Solitary *L. migratoria* adult individuals show greenish coloration and no tendency to aggregation, gregarious individuals are brownish and tend to swarm and often migrate over long distances. Tanaka and his colleagues discovered that an albino mutant of *L. migratoria* lacks a dark-color inducing factor normally present in the brain, CC and thoracic ganglia and a similar factor was suggested to be present in the CC of *S. gregaria*. The factors isolated from these insect were subsequently shown to be a [His⁷]-corazonin (reviewed by Tanaka, 2006). The other two types of corazonin display similarly potent dark-color inducing activity in the albino mutants of *L. migratoria* (Hua *et al.*, 2000; Roller *et al.*, 2006). All three types also reduce (with a similar efficiency) the spinning rate in the prepupae of *Bombyx mori* (Tanaka *et al.*, 2002, Roller *et al.*, 2006).

Injections of corazonin into pharate larvae of *M. sexta* elicit release of preecdysis- and ecdysis-triggering hormones (PETH and ETH, respectively) from Inka cells of the tracheal glands, which induce precocious preecdysis and ecdysis behavior. A different peptide, the eclosion hormone also acts directly on Inka cells to cause release of PETH and ETH, but whereas eclosion hormone induces “all-or-nothing” secretory response and peptide depletion, corazonin causes a graded and rarely maximal secretion of these peptides from the Inka cells (Kim *et al.*, 2004).

Immunoreactivity to corazonin typically occurs in lateral protocerebrum, forming a cluster of 4-5 cells in each hemisphere. The bilateral longitudinal tracts extending from the abdominal neuromeres to the brain are also stained in all examined species too. The bilateral interneurons distributed in the thoracic and abdominal ganglia that contribute to these tracts are also known from several species. Only in beetles no immunoreactivity was observed so far. (Veenstra and Davis 1993; Cantera *et al.*, 1994; Roller *et al.*, 2003 and 2006)

Corazonin is a highly conserved neuropeptide and its occurrence in insects is widespread, however no universally recognized function can be assigned to it at present.

1. 2. 5. *Ecdysis hormone*

As insects have a rigid exoskeleton, they have to shed it periodically to enable further growth. This process called ecdysis or eclosion² take place at the end of each developmental stage and represents the terminal phase of the most fascinating event in the life cycle of holometabolous insects – the metamorphosis.

The process of ecdysis is driven through a complicated and precisely timed behavioral sequence that can be divided in two phases: 1) the pre-ecdysis phase which includes the motor patterns believed to separate the old and new cuticle and 2) the ecdysis phase during which the insect frees itself from the old cuticle. A number of peptides have been implicated in the control of ecdysis behavior, eclosion hormone being the first in the list.

Eclosion hormone is structurally known from *Manduca sexta* (Kataoka *et al.*, 1987), *Bombyx mori* (Kono *et al.*, 1987), *Helicoverpa armigera* (Zhang *et al.*, 2006) and *Drosophila melanogaster* (Horodyski *et al.*, 1993). It has (at least in lepidopterans) 62 residues and six characteristically positioned cysteines that form three internal disulphide bonds (Kataoka *et al.*, 1992). The EH-like immunoreactive material was detected in a number of species from various insect orders (Závodská *et al.*, 2003a,b). The EH typically occurs in two pairs of ventro-medial (VM) cells in the protocerebrum (Horodyski *et al.*, 1989; Závodská *et al.*, 2003a,b; Zhang and Xu, 2006). A vast majority of experiments trying to elucidate the hormonal network regulating the ecdysis was carried out using various life stages of *Manduca sexta*.

Eclosion hormone was long believed to be the only factor involved in insect ecdysis as injections of this hormone resulted in the premature pre-ecdysis and ecdysis behaviors (Copenhaver and Truman, 1982). To bring about this result however, the presence of the tracheal system was necessary, which was explained by a need for high oxygen supply at that time. In 1996 however, Žitňan *et al.* reported the discovery of a new peptide involved in regulation of ecdysis behavior, the ecdysis-triggering hormone (ETH). ETH injections also induce ecdysis, even more rapidly than the injections of EH. The ETH peptide is produced by the Inka cells of the small epitracheal glands (situated on the trachea), which explains why the tracheal system was crucial for the successful initiation of premature (pre-) ecdysis behavior in the early experiments.

A regulation by a positive feedback loop is not probably very common but there is a good evidence for such a regulation functioning in the process of ecdysis (Ewer *et al.*, 1997). The current model is following: The drop in the amount of ecdysteroids at the end of each life stage causes a release of eclosion hormone (and also make the CNS responsive to it), EH triggers release of ecdysis-triggering hormone from the Inka cells and ETH in turn acts on the brain to release more EH from the

² The term eclosion originally referred only to the adult ecdysis but these two terms seem to blend nowadays and they are used as synonyms in this study.

ventro-medial cells; ETH (together with PETH, pre-ecdysis triggering hormone, also released from the Inka cells) triggers the pre-ecdysis behavior. From this point on the situation becomes a bit confusing and opinions on how the process goes on varies. Žitňan and Adams (2000) believe that ETH is responsible for both the pre-ecdysis and ecdysis behavior and that the delay of onset of ecdysis behavior is mediated by an unknown inhibitory factor from cephalic and thoracic ganglia. According to this model, ETH achieves the initiation of ecdysis motor program by acting on the cephalic ganglia (tritocerebrum and/or suboesophageal ganglion) and these in turn activate the CCAP (crustacean cardioactive peptide) neurons in the abdominal ganglia to release their peptide content, thereby starting the ecdysis behavior. EH is not necessary in the process, though it may increase (via elevated cGMP levels) the excitability of the network. This contrasts with the earlier model of Ewer *et al.* (1997): It has been shown that ETH typically causes only pre-ecdysis behavior unless the brain is also included in the preparation. Consequently the authors proposed that the ability of ETH to produce ecdysis behavior was performed through its action on releasing the EH which in turn activates the CCAP. In the studies of Gammie and Truman (1999) and of Žitňan and Adams (2000) though, the EH application lead to the onset of ecdysis program in a rather smaller portion of experiments and it never produced any action until the ganglionic sheath is removed. This and other lines of evidence, including those coming from the studies on *Drosophila* (see below) support rather the first model.

To my knowledge, the role of eclosion hormone in activating the ecdysis motor program has not yet been fully resolved and according to the recent study of Clark *et al.* (2004) on *Drosophila* the whole situation may get even more complicated. Park *et al.* (2002) confirmed the essential role of ETH using a genetic approach: 98% of *Drosophila eth* mutants do not survive the first ecdysis; animals rescued by the injection of *Drosophila* ETH shed the cuticle normally. Consistent with this are the results obtained earlier by McNabb *et al.* (1997): about a third of the flies that lacked EH cells were able to complete development and eclose. In view of this fact it is especially puzzling that exogenous *Drosophila*-ETH cannot cause premature pre-ecdysis and ecdysis behavior in EH-knockout larvae (Clark *et al.*, 2004). Clark and colleagues therefore propose involvement of other regulatory neuropeptides that would also be released in response to EH. This seems reasonable as Kim *et al.* (2004) have already reported a role of corazonin in the process of ecdysis of *M. sexta* larvae; other such reports may soon follow.

Time of eclosion is carefully gated because insects are during or soon after ecdysis especially vulnerable. Double layer of cuticle before ecdysis not only impairs locomotion but also breathing and thus the insects aim for the ecdysis to happen at convenient time and on a secluded place to minimize the risk of being devoured. The need for eclosion to occur at a time of day when the predators are inactive is one of the main reasons why the circadian control of molting is necessary. Another reason is that many insect species mate soon after eclosion; it is known that closely related species may have

eclosion gates separated by many hours. Thus the possibility of inter-species cross is minimized and the timing of eclosion may therefore represent one of the mechanisms for reproductive isolation. (reviewed by Vafopoulou and Steel, 2005)

1. 2. 6. Diuretic hormone

Terrestrial insects are susceptible to desiccation and thus the water loss through excretion is under a strict endocrine and possibly neural control. The endocrine factors affecting the regulation of diuresis are referred to as diuretic and antidiuretic hormones. Generally, diuretic factors stimulate secretion of primary urine by the Malpighian tubules (MTs), whereas antidiuretic factors increase the water reabsorption from the hindgut. Since the insect hemolymph does not function in gas transport (which is ensured by the tracheal system) there is no need for it to circulate rapidly and/or regularly. That is why insects have normally little or no “blood” pressure; diuresis must therefore be driven by active ion transport rather than by blood pressure as is the case in vertebrates. The Malpighian tubules secrete a cation-rich (usually predominantly K^+) solution that is essentially isoosmotic with the hemolymph, water and other solutes follow passively, but a number of toxic substances (e.g. plant alkaloids) are secreted actively. Water is then reabsorbed in the hindgut, but especially after feeding its excretion through feces is increased as the excess of water and toxic waste must be voided. At such times, the diuretic hormones are released into the hemolymph and stimulate the production of primary urine by MTs. Not surprisingly, their effect is most pronounced in hematophagous insects as they intake liquid food. However, the action of diuretic factors does not have to be associated with higher water loss from the insect body, because it can be counteracted by the enhanced fluid reabsorption in the hindgut. The high urine flow rate increases the rate of elimination of the toxic compounds though. (Schooley *et al.*, 2005; Gäde *et al.*, 1997; Coast, 1996 – reviews; Coast and Kay, 1994)

A number of compounds have been demonstrated to possess a diuretic activity; they include biogenic amines (serotonin, tyramine) and at least five families of neuropeptides (CRF-like peptides, CT-like peptides, CAP_{2b}-like peptides, tachykinin-related peptides and kinins)³ (Coast and Garside, 2005). Here I will focus only on the CRF-like family of peptides as the diuretic hormone of our interest belongs to this group.

As the name of the family indicates, these peptides show quite high sequence similarity to the corticotropin-releasing factor (urotensin I, sauvagine and urocortin) of vertebrates. The first insect peptide belonging to this family to be identified was isolated from the heads of larval *Manduca sexta* by Kataoka *et al.* (1989) and was called *M. sexta*-diuretic hormone (Manse-DH). Manse-DH consists of 41

³ CRF – corticotropin-releasing factor; CT – calcitonin; CAP – cardioactive peptide

amino acids and has an amidated C-terminus. It was shown to stimulate posteclosion diuresis by decapitated newly emerged adult butterflies *Pieris rapae* (Kataoka *et al.*, 1989). Later, a smaller diuretic peptide (30 aa) was isolated from *M. sexta* and called Manse-DPII (Blackburn *et al.*, 1991). Many other CRF-like diuretic hormones were isolated from a variety of insect species (reviewed by Schooley *et al.*, 2005), sometimes two peptides differing in length were found in the same species (e.g. the beetle *Tenebrio molitor*: DH₃₇ and DH₄₇ or the moth *Hyles lineata*: DH₃₀ and DH₄₁). All these peptides except for those of *Tenebrio* have amidated C-terminus, only Tenmo-DHs exist as a free acid, which have serious functional consequences: Tenmo-DH₃₇ has no detectable effect on fluid secretion by *M. sexta* Malpighian tubules *in vitro* (unlike the CRF-related DHs of other insect species – Audsley *et al.*, 1995), although it functions, of course, on *T. molitor* MTs (Furuya *et al.*, 1995). That is not very surprising as the free acid form of Manse-DH is about 1000-fold less potent than its natural amidated form (Kataoka *et al.*, 1989) in the same assay.

All CRF-like diuretic hormones studied to date act through increasing intracellular levels of cAMP to stimulate fluid secretion by MTs. The increase in cAMP is believed to activate Na⁺/K⁺/2Cl⁻ cotransporter (Schooley *et al.*, 2005).

The cellular localization of CRF-like DHs has been studied immunocytochemically in several insect species. Using two antisera raised against the N- and C-terminal halves of Manse-DH, respectively, Veenstra and Hagedorn (1991) identified about 40 relatively small median neurosecretory cells in each hemisphere. These cells send their axons to the contralateral nervus corporis cardiaci and terminate in the corpora cardiaca. No other cells in the brain or ventral nerve cord reacted with either of the antisera. On the other hand, Chen *et al.* (1994) reported Manse-DH immunoreactivity to be present in the lateral neurosecretory cells of the abdominal ganglia as well. These were shown to co-localize the DH immunoreactivity and the material recognized by the antiserum against the *Manduca sexta*-leucikinin IV. In *Tenebrio molitor*, immunoreactivity to anti-Tenmo-DH₃₇ antiserum was seen not only in the protocerebral median cells in the brain (24-30 cells) but also in the neurosecretory cells in the abdominal ganglia and in the midgut (Wiehart *et al.*, 2002). Iaboni *et al.* (1998) identified neurons reactive to the *Locusta*-DH-antiserum in both larval and adult housefly *M. domestica*. In adults, 10-12 immunoreactive neurosecretory neurons are located in the median protocerebrum, other two clusters of cells are in the lateral protocerebrum and consist of four and two cells, respectively. In addition, there are two pairs of immunoreactive neurons alongside the oesophageal foramen. Stained neurons were also found in the fused thoracic-abdominal ganglia. In the blood-feeding bug, *Rhodnius prolixus*, anti-*Locusta*-DH antiserum has showed immunoreactivity throughout the CNS and gut. In the brain, about 450 cells were stained, some of them very heavily; the immunoreactivity was found mostly in the

protocerebrum and at the base of the optic lobes and also in the suboesophageal ganglion (Te Brugge *et al.*, 1999).

Several studies have reported co-localization of CRF-like diuretic peptides with kinins (TeBrugge *et al.*, 2001; Chen *et al.*, 1994; Thompson *et al.*, 1995) and it has been suggested that these peptides may act synergistically in the control of fluid secretion. The study of Patel and colleagues (1995) on *Locusta migratoria* highlights the advantages such a system could have: quite a high amount of Locusta-DP is needed to stimulate maximal tubule secretion, but in the presence of low concentration of locustakinin the required amount of Locusta-DP is significantly lower. Tubule secretion could therefore be sustained for many hours by the release of Locusta-DP from the stores in brain and corpora cardiaca instead of metabolically very costly synthesis of large amounts of new peptides. Coast (1995) further supports this notion by his more detailed study of the connection of these two peptides and shows that low concentrations of *Locusta*-DP and locustakinin act co-operatively producing stimulating effect on the MTs that is greater than the sum of their separate responses.

Precise control of water loss is absolutely crucial for all terrestrial insects and it is regulated by a number of compounds to fine-tune the whole excretory system. It is obvious that diuretic hormone is one of the key players in this system since it has been identified in many insect species from very diverse orders.

1. 2. 7. Prothoracicotropic hormone

Vertebrates and invertebrates differ greatly in their postembryonic development. Vertebrate newborn individuals are not generally that very different from the adults and their development proceeds through relatively small and gradual changes. In invertebrates, it is often a very different story. Insects, for instance, undergo striking and periodic changes in the rate of growth and development that (in holometabolous species) culminates in the process of metamorphosis – an event for which we have absolutely no comparison among vertebrates. Such a dramatic change in appearance which occurs when an adult butterfly comes out of its pupal case could not have possibly escaped notice of the early entomologists and it is therefore not surprising that it has been studied for centuries.

The major factor that regulates molting and metamorphosis is a steroid hormone, the 20-hydroxyecdysone produced by an endocrine organ called prothoracic glands. The physiology of these glands is under hormonal control of the brain neuropeptide prothoracicotropic hormone (PTTH) which stimulates the glands to produce ecdysone. This peptide was discovered in 1922 by Kopec who referred to it as a “brain factor”. It was the first evidence that neural tissue may have an endocrine function too.

PTTH has been studied primarily in lepidopteran species, the most prominent being *Bombyx mori* and *Manduca sexta*. The early transplantation experiments performed by Wigglesworth (1940) localized

the PTTH activity into the dorsal brain region containing large neurosecretory cells and this finding was further refined by Agui and colleagues (1979) who identified two pairs of dorsolateral neurosecretory cells as the source of PTTH in the brain of *M. sexta* and corpora allata (not cardiaca) as the site of release of PTTH into the hemolymph.

Several attempts to isolate and sequence PTTH were undertaken; some of them were truly heroic. Kataoka and colleagues (1991) were almost completely successful in determining the sequence of *B. mori* PTTH yet the work that had to be done was gigantic. Extract from 1.8 million heads of adult *Bombyx* (about 1.5 tons of moths!) was used and yet a small C-terminal fragment could not be determined. This probably discouraged other scientists from similar attempts with other species and most of the PTTH sequences known today were obtained using molecular probes derived from the *Bombyx* PTTH sequence and subsequent cDNA isolation. The complete PTTH sequence is now known from lepidopterans *B. mori* (Kawakami *et al.*, 1990), *M. sexta* (Shinoya *et al.*, 2003), *Antheraea pernyi* (Šauman and Reppert, 1996b), *Samia cynthia ricini* (reviewed by Ishizaki and Suzuki, 1994), *Hyalophora cecropia* (Sehna *et al.*, 2002), *Helicoverpa zea* (Xu *et al.*, 2003), *Heliothis virescens* (Xu and Denlinger, 2003) and *Helicoverpa armigera* (Wei *et al.*, 2005) and others. Relatively little work has been done on characterizing the PTTH from non-lepidopteran species: only the lepidopteran-like PTTH of the blood-feeding bug *Rhodnius prolixus* has been partially identified so far (Vafopoulou and Steel, 2002) and a *ptth*-like gene was found in *Drosophila* genome using BLAST search (Liu *et al.*, 2006).

Lepidopteran PTTHs have molecular weight of about 30 kDa and exist as homodimers linked by disulfide bonds (Kataoka *et al.*, 1991). The monomeric proteins are glycosylated, though this modification does not appear to be necessary for protein function, and have seven characteristically positioned cysteines (Ishibashi *et al.*, 1994). Especially the conserved location of intramonomeric disulfide bonds led Noguti *et al.* (1995) to the suggestion that PTTH is a member of the vertebrate growth factor superfamily. Otherwise the amino acid sequence of all known PTTHs is not very conserved – the sequence identity and similarity averages around 50% and 80%, respectively – the peptides from closely related species display, of course, higher similarity (reviewed by Rybzynski, 2005).

The cross-species bioactivity tests have shown that *Bombyx* PTTH does not activate ecdysteroidogenesis in *Manduca* and *vice versa*; similarly, *Bombyx* PTTH does not function in *Samia* larvae and *Antheraea* PTTH does not stimulate *Manduca* prothoracicotropic glands *in vitro* (reviewed by Rybzynski, 2005). On the other hand, recombinant *Bombyx* PTTH elicited a statistically significant increase in production of ecdysteroids by *Rhodnius* prothoracicotropic glands *in vitro*, which is very surprising given the evolutionary distance between these two species (Vafopoulou and Steel, 1997). Besides, antisera raised against a particular PTTH have been successfully used to detect

immunoreactive material even in very evolutionary remote species (Žitňan *et al.*, 1993; Závodská *et al.*, 2003a,b) and anti-*Bombyx* PTTH antibody blocks the PTTH-activity in *Manduca* brain extracts (Rybzynski *et al.*, 1996). The question of species-specificity of PTTHs thus remains open.

The original findings of Agui (1979) on *M. sexta* were later confirmed by both *in situ* hybridization and immunocytochemistry (Gray *et al.*, 1994; Shionoya *et al.*, 2003). Šauman and Reppert (1996b) located both the *ptth*-mRNA and protein to the same pair of dorsolateral neurosecretory cells in each brain hemisphere of various developmental stages of *A. pernyi*. These cells send their axonal projections to the contralateral nervi corporis cardiaci. The same staining pattern was obtained by Wei *et al.* (2005) using whole mount immunocytochemistry in *H. armigera*. Yagi *et al.* (1995) learned the same by performing this technique on brains of *S. cynthia ricini*. On the other hand, Závodská *et al.* (2003b) demonstrated that anti-*Antherea* PTTH antibody stains a number of perikarya in various regions of the firebrat *Thermobia domestica* brain; although we cannot possibly exclude cross-reactivity with some other antigen, the very unambiguous results of Western blot analysis support the credibility of these findings. Five or even more pairs of PTTH-positive neurons were also found in the stonefly *Perla burmeisteriana*, the blowflies *Neobellaria bullata* and *Phormia regina*, the beetle *Pachnoda marignata* and the caddis fly *Hydropsyche contubernalis*. Most of the immunoreactive neurons were located in the protocerebrum, though in the mayfly *Siphonurus armatus* and damselfly *Ishnura elegans* these neurons were found in suboesophageal ganglion and not in the protocerebrum (Závodská *et al.*, 2003a).

Taking into account its important role in development, it is not surprising that the titer of prothoracicotrophic hormone in hemolymph is precisely controlled. The release of PTTH into hemolymph is under *circadian* control, as was long thought but first clearly shown by the experiments of Vafopoulou and Steel (1996a,b) on the blood-feeding bug *Rhodnius prolixus*. These studies revealed that release of PTTH occurs as a daily rhythm with maximum during the night throughout most of larval-adult development. This rhythm free-runs in DD with a period close to 24 hrs and is temperature compensated.

2. Materials and methods

2.1. Animals

Adult individuals of the Mediterranean flour moth *Ephestia kuehniella* of both sexes were used in this study. Animals were reared under a LD cycle 12:12 (12 hrs of dark and 12 hrs of light). To ensure an absolute entrainment to these light conditions the animals selected for analyses were moved from the common stock room into a separate compartment thus eliminating any unintentional disruption of the light regime. The temperature was held constant throughout the day at 25 °C. The animals were moved into the separate compartment as larvae and kept under these conditions until use, which usually took about a month but never less than three weeks, depending on how early were the animals moved. A general rule says that for a successful entrainment at least seven days under non-disrupted conditions are needed so this recommendation was more than fulfilled.

2.2. Immunocytochemistry on paraplasm sections

2.2.1. Sample preparation

Whole heads or brains were dissected from CO₂-anesthetized animals in chilled Ringer's solution at ZT 4 or ZT 16, ± 1 hr. The tissue was immediately submerged into a modified Bouin-Hollande fixing solution (0.7% mercuric chloride, no acetic acid) and incubated for 12 to 24 hrs at 4 °C (Levine et al., 1995). The fixed tissues were then brought through a dehydrating ethanol series and chloroform (70% EtOH – 96% EtOH – 100% EtOH – chloroform) into paraplasm. To ensure its complete penetration into the tissue the samples were incubated in a melted paraplasm in a vacuum oven overnight at 58 °C. After positioning and cooling at room temperature (RT) the samples were cut into 7-10 µm thick sections and attached onto microscopic slides. The sections were then dried on a hot plate (45°C) for at least 48 hrs and used immediately or stored refrigerated until use.

2.2.2. Procedure

The slide were first deparaffinized in xylene and then brought through a rehydrating ethanol series (96% EtOH – 70% EtOH) into distilled water. To remove residual heavy metal ions from the fixing solution the slides were treated with Lugol's iodine and subsequently with 7.5% sodium thiosulfate. Next, the slides were washed in distilled water and phosphate-buffered saline supplemented with 0.3% Tween 20 (PBST). The slides were then blocked in 10% goat normal serum in PBST for 30 min at RT in order to prevent unspecific antibody binding. After this, the samples were incubated with a rabbit primary antibody (for detailed information on dilution and origin of individual antibodies see Table 1.) in

a humidified chamber overnight at 4°C. Following day, the slides were rinsed thoroughly with PBST and then incubated with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch, diluted 1:1,000 in PBST) for 1 hr at RT. Subsequently the slides were washed in PBST again and after treating with 0.05 M Tris-HCl (pH 7.4) the HRP enzymatic activity was finally visualized with DAB staining solution (3,3'-diaminobenzidine tetrahydrochloride – 0.25 mM in 0.05 M Tris-HCl, pH 7.4 plus 0.005% of hydrogen peroxide). Stained sections were dehydrated again through an ethanol series (70% EtOH – 96% EtOH – 100% EtOH), treated with xylene and then mounted in DPX mounting medium (Fluka). Samples were examined and photographed using the Zeiss Axioplane 2 microscope equipped with Nomarski (DIC) optics and a CCD camera.

2.3. Whole mount immunocytochemistry

Brains dissected from CO₂-anesthetized animals at appropriate timepoints were fixed in 4% formaldehyde (diluted in PBS, pH 7.4) for 12 – 24 hrs. After fixation, the tissues were rinsed thoroughly with phosphate buffered saline supplemented with 1% Triton X (PBSTrX) and then treated with collagenase (0.5 mg/ml; Sigma) to permeabilize the neurolemma for 1 hr at RT. Subsequently, the brains were washed with methanol (50% MetOH – 70% MetOH – 100% MetOH – 70% MetOH – 50% MetOH) and PBSTrX and then bathed in 10% normal goat serum for 2 hrs at RT to block unspecific binding sites. After blocking, the samples were incubated with particular primary antibody (same dilutions as used for ICC on paraplant sections, see Table 1.) for 3 days at 4 °C. On the fourth day, the tissues were washed in PBSTrX and then incubated with goat anti-rabbit HRP- conjugated secondary antibody (Jackson ImmunoResearch, 1:1,000 in PBSTrX) for one day at 4 °C. Next, the samples were subjected to washing in PBSTrX and 0.05 Tris-HCl (pH 7.4) and then to staining in DAB solution as described above for ICC on paraplant sections. Stained tissues were mounted in 80% glycerol and stored refrigerated. The immunoreactivity was examined under microscope (Zeiss Axioplane 2 microscope equipped with Nomarski -DIC- optics and a CCD camera).

antibody	antigen	dilution	source
anti - PTTH	<i>Antherea pernyi</i> PTTH	1:1,000 in PBST	Šauman and Reppert, 1996
anti - CRZ	<i>Periplaneta americana</i> CRZ	1:1,000 in PBST	S. Tanaka; Wako Co., Nagano, Japan
anti - PDH	<i>Uca pugilator</i> PDH	1:1,000 in PBST	Dr. Dircksen; University of Bonn, Germany
anti - EH	<i>Manduca sexta</i> EH	1:2,000 in PBST	Dr. Truman; University of Seattle, USA
anti - ATH	<i>Manduca sexta</i> ATH	1:1,500 in PBST	Dr.Kramer; Sandoz Agro Inc., Palo Alto, California, USA
anti - ASH	<i>Manduca sexta</i> ASH	1:1,500 in PBST	
anti - DH	<i>Manduca sexta</i> DH	1:2,000 in PBST	

Table 1. Detailed information on antibodies employed in this study.

2.4. Western blot analysis

2.4.1. Protein extraction

Whole heads were dissected from CO₂-anesthetized animals on dry ice at appropriate timepoints and placed immediately into an ultra-low-temperatures freezer and stored at -70 °C. Upon use, the tissues were homogenized in a triple-detergent lysis buffer (50 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.1% SDS; 1.0% Nonidet P- 40; 0.5% sodium deoxycholate and 0,02% sodium azide) supplemented with Roche cocktail of protease inhibitors (sample/buffer volume ratio: 1:1. At each round of extraction samples of both sexes from both timepoints obtained at the same day were used and the number of heads from each category was the same). After adding appropriate volume of 5x (2x) dyed gel loading buffer, the samples were boiled for 10 min, sonicated (1.5 min at full power) and centrifuged for 5 min at 14 000g and 4°C. The sample was loaded on the gel (5 µl/well if not stated otherwise) or stored at - 70 °C.

2.4.2. Tris-glycine gel running conditions

A standard SDS/PAGE procedure and buffers were employed in this study: 12% SDS/polyacrylamide gel was run using Tris-Glycine-SDS buffer under constant current of about 15mA. Each round, samples extracted on the same day and under precisely the same conditions were run on one gel to enable reliable comparison. To estimate the protein molecular weights either the Chemiluminiscent BlueRanger marker (Pierce) or the Dual color marker (Biorad) was run on the same gel.

2.4.3. Tris-tricine gel running conditions

Ready-made Tris-tricine Ready Gel (Biorad) was run under constant current of about 65mA using two running buffers – anode buffer (0.2 M Tris, pH 8.9) and cathode buffer (0.1 M Tris; 0.1 M tricine; 0.1% SDS, pH about 8.25 – no adjustment). Kaleidoscope Marker or DualColor (both Biorad) was employed as a molecular weight marker.

2.4.4. Semi-dry electroblotting

After electrophoresis, the proteins were transferred by semi-dry electroblotting onto a nitrocellulose membrane. The solutions used during the procedure differed for tris-tricine and tris glycine gels: *tris-glycine*: anode buffer #1 (0.3 M Tris-Cl, pH 10.4; 20% MetOH), anode buffer #2 (25 mM Tris-Cl, pH 10.4; 20% MetOH) and cathode buffer (25 mM Tris-Cl, pH 9.4; 40 mM Glycine; 20% MetOH);

tris-tricine (taken from the manufacturer's website): anode buffer (60 mM Tris; 40 mM CAPS; 15% MetOH; pH 9.6) and cathode buffer (60 mM Tris; 40 mM CAPS; 0.1% (w/v) SDS; pH 9.6).

2.4.5. Immunostaining

After immunoblotting, the membrane was blocked with 10% non-fat dry milk diluted in PBS (phosphate buffered saline) for 2 hrs at RT followed by incubation with primary antibody diluted in 10% non-fat dry milk in PBS, overnight at 4 °C. After washing in PBST (5 times for 10 min) the membrane was incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch, diluted 1:50,000 or Pierce Stabilized secondary antibody diluted 1:1,000) for 2 hrs at RT. Subsequently, the membrane was washed in PBST again (5 times for 10 min) and then treated with the SuperSignal West Dura chemiluminescence substrate (Pierce); the reaction was then visualized and photographed using Fujifilm LAS 3000 Intelligent Dark Box luminoimager.

3. Results

Cellular localization of the seven neurohormones has been determined using antibodies listed in Table 1. All these antibodies were previously successfully employed in similar studies on insect species from very diverse orders and they always reacted very specifically. For this reason we are confident that results coming from this study should be reliable, although cross-reactivity with other antigens can never be absolutely ruled out. Western blot analysis has also been performed; it would enable rough evaluation of the relative amount of the particular neuropeptide present at each timepoint and would further support the specificity of the antibodies used.

3.1. Immunocytochemistry on paraplast sections and whole mounts

Generally no significant differences in the staining pattern have been found between the two timepoints at which the samples were collected, for any of the antibodies. Also the samples from males and females show no differences worth mentioning. The immunoreactive material has always been restricted to the cytoplasm so when talking about immunoreactive cells or neurons only the perikarya are actually meant by that expressions.

3.1.1. *Corazonin*

The antibody raised against *Periplaneta americana*-corazonin produced probably the most consistent staining pattern of all the antibodies used in this study. At both timepoints and in both sexes, two pairs of dorso-lateral protocerebral neurons located rather posteriorly in each hemisphere are stained very heavily (Fig. 2. B,H,I,M). These four cells are interconnected in a pair-wise manner and send axons to the ipsilateral corpus cardiacum as was nicely demonstrated in the whole mount

► **Fig. 2.** (A) a schematic picture of Peram-corazonin-like immunoreactivity in the cephalic ganglion – retrocerebral complex of *Ephesia*; (B) dorso-lateral immunoreactive cells in the protocerebrum (female, ZT 4, whole mount); (C) axons projecting from the dorso-lateral clusters of cells (female, ZT 4, whole mount); (D) nerve projecting from the ipsilateral cluster of dorso-lateral protocerebral neurons entering the corpus cardiacum (male, ZT 16, whole mount); (E) immunoreactivity in the corpus cardiacum, two stained neurons are indicated by the arrows (male, ZT 4); (F) corpus cardiacum and allatum: absence of immunoreactivity in the corpus allatum, meshwork of stained axons and cells in the corpus cardiacum (female, ZT 4); (G) nerves from the bilaterally positioned dorso-lateral clusters of protocerebral neurons heading to each other in the pars intercerebralis (female, ZT 4, whole mount); (H)&(I) axonal connections of pairs of cytoplasmically stained dorso-lateral cells in the protocerebrum (H: male, ZT 4; I: female, ZT 16); (J)&(K) immunoreactivity in the optic lobes (J: female, ZT 16; K: female, ZT 4); (L) two additional cells in the lateral protocerebrum (male, ZT 16); (M) dorso-lateral protocerebral neurons: all four cells from the main neurosecretory cluster and one of the smaller additive cells, dot-like staining (indicated by the arrow) suggests connection with the network of axons in the optic lobe (male, ZT 16); (N)&(O) immunoreactive cells in the lateral protocerebrum (N: female, ZT 4; O: female, ZT 4); (P) meshwork of axons in the pars intercerebralis (female, ZT 4). Scale bars: C, E, F, H, I, K-P – 50 µm; B, J – 100 µm; D, G – 20 µm.

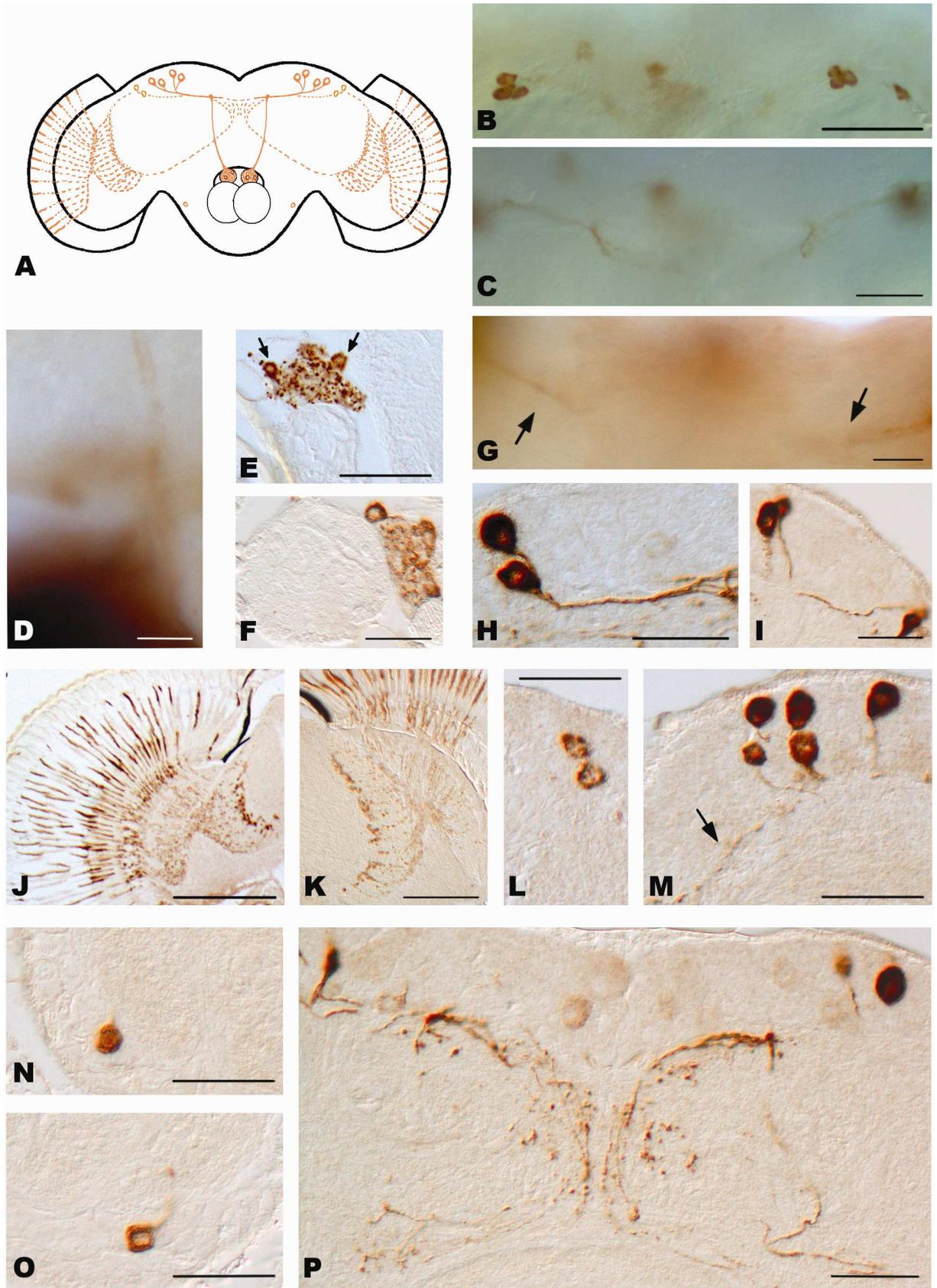


Fig. 2.

preparations (Fig. 2. D). The second main nervous branch coming from this cell cluster projects to the pars intercerebralis. It cannot be said, whether the cell clusters from both hemispheres are interconnected via these nerves or whether they only arborize in the central region without forming a direct nervous connection. The whole mount preparations have only shown that nerves from the bilaterally positioned centers are heading to each other but the track was lost in pars intercerebralis (Fig. 2. G). In the brain sections many axons can be seen in this region but none that would confirm direct connection of both the clusters (Fig. 2. P). Two additional cells smaller and less intensively stained are located more frontally in the lateral protocerebrum. It seems that they might also be linked with the main cluster of dorsal protocerebral neurons (Fig. 2. L,M).

A number of axons have been stained also in the optic lobes, they run on the surface of both medulla and lamina and project into the compound eye where many immunoreactive cells are found as well (Fig. 2. J,K). A dispersed dot-like staining suggest connection to the lateral-dorsal cluster of neurons.

Two additional cells have been stained in the suboesophageal ganglion, one in each half. They are located centrally, rather in the dorsal portion of the SOG, near to the deutocerebrum and close to the surface of the ganglion (Fig. 2. N,O). No connections to any other part of the brain were observed for these cells.

As to the neurohemal organs, only corpora cardiaca not allata contain immunoreactive material. In CC, a dense meshwork of axons is stained though in the sections. It seemed that also some immunoreactive cells are present. It is difficult to estimate the number of these cells; it probably ranges from two to four per one corpus cardiacum.

3.1.2. Pigment dispersing factor

The most prominent staining occurred postero-centrally in the lateral dorsal protocerebrum where four immunoreactive neurons were found in each hemisphere (Fig. 3. E,F,H,I). There is another group of

➡ **Fig. 3.** (A) a schematic picture of *Uca*-PDH-like immunoreactivity in the cephalic ganglion – retrocerebral complex of *Ephestia*; (B)&(C) nerves from the cluster of cells in the pars intercerebralis running towards the oesophageal foramen (B: male, ZT 4, whole mount; C: female, ZT 16, whole mount); (D) a group of neurons in the pars intercerebralis (male, ZT 4); (E) a foursome of stained cells in the dorso-lateral protocerebrum (female, ZT 4, whole mount); (F) a pair of perikarya from the bilaterally positioned clusters in the dorso-lateral protocerebrum (male, ZT 4); (G) the most posteriorly located immunoreactive neurons in the protocerebrum (male, ZT 4); (H)&(I) dorso-lateral protocerebral perikarya (H: male, ZT 4; female, ZT 16); (J)&(L) a pair of large immunolabeled neurons located centrally in the SOG (J: male, ZT 4; L: male, ZT 16); (K) a cell positioned centro-frontally in the SOG (male, ZT 16); (M)& (N)&(O) small perikarya placed at the margin of the optic lobe (M: male, ZT 16; N: female, ZT 4; female, ZT 4, whole mount); (P) corpora cardiaca and allata (female, ZT 4, whole mount). Scale bars: D, E, G-K, M-O – 50 µm; B, C, F, P – 100 µm; L – 20 µm.

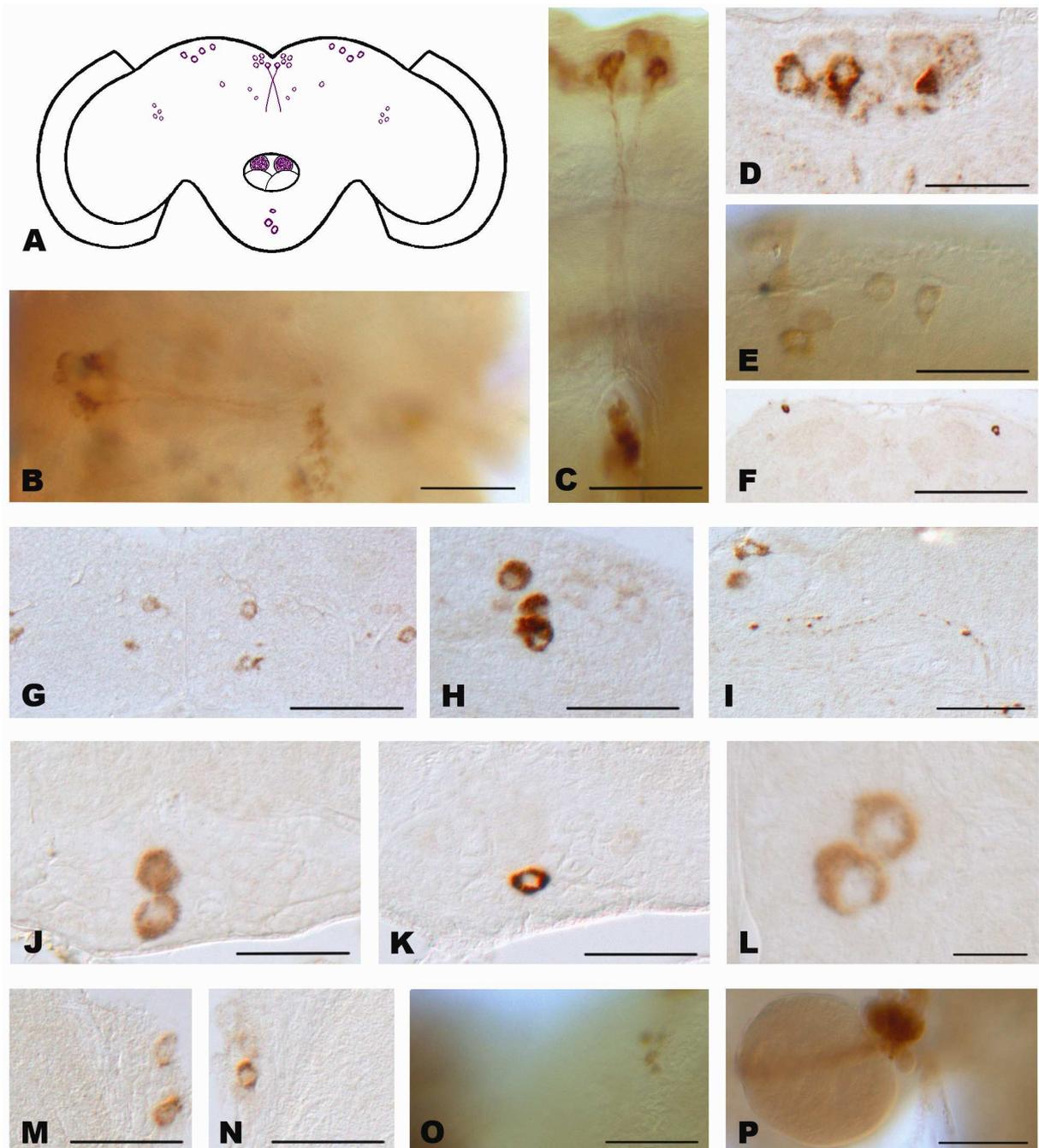


Fig. 3.

quite heavily stained neurons which begins at about the same depth in the pars intercerebralis and extends towards the anterior part of the brain. These cells cannot be exactly counted as they are closely adjacent to one another, their number can only be estimated to about five neurons on each side along the brain midline (Fig. 3. D).

Two nerves coming from the most frontally positioned part of this cluster were clearly stained in the whole mounts (how many neurons participate in forming these nerves could not be determined); at a certain distance from the cluster they cross and run ventrally, in the direction of the oesophageal foramen (Fig. 3. B,C). They probably project to the contralateral corpora cardiaca through the nervi corpori cardiaci.

Another group of stained neurons have been found to be scattered in the very posterior part of protocerebrum but this one is rather variable as to the number: two to four pairs of cells in each brain hemisphere (Fig. 3. G). Deeper in the brain, there is a cluster of about four small immunoreactive neurons positioned at the margin of the protocerebrum just where the optic lobe begins (Fig. 3. M,N,O).

Some stained neurons have also been identified in the SOG, two large neurons are located centrally and one more heavily stained cell has been found in the ventral part of the ganglion (Fig. 3. J,K,L). Occasionally two or three rather weakly stained perikarya occurred in the same region but this happened only in a small portion of examined brains and no such cells were seen in the whole mounts.

A dot-like immunoreactivity representing probably sectioned axons has been found in the optic lobes and seemed to spread towards the protocerebrum. Axons have also been found to be densely stacked in CC, again, no immunoreactivity was seen in CA (Fig. 3. P).

3.1.3. *Eclosion hormone*

Antiserum against the eclosion hormone has recognized relatively few perikarya in the moth's brain. In the ventro-medial region of the protocerebrum four large neurons show the heaviest staining (Fig. 4. B,C,D). Axons from these cells fuse into two cords which run downwards along the brain midline, possibly projecting to the corpora cardiaca. These neurons certainly represent the primary source of the immunoreactive material as the other perikarya identified showed less intense staining. Weaker though still very significant reaction occurred in the SOG, rather in its ventral part. Closer to the centre of the ganglion, two large cytoplasmically stained cells are located and more frontally a foursome of a bit smaller perikarya can be found (Fig. 4. I,J). One, occasionally two small cells are found posterio-laterally at the base of the deutocerebrum in each brain hemisphere (Fig. 4. K).

Less consistent immunoreactivity was observed centrally in the pars intercerebralis, the number of cells is difficult to count and averages around four cells in each hemisphere (Fig. 4. H). Even less certain is the status of another group of perikarya located posterio-centrally in the lateral part of the protocerebrum. When present, this cluster seems to consist of about three perikarya accompanied by a group (about five) of substantially smaller cells (Fig. 4. E,F,G). Although the intensity of the staining of these cells is not very high it is still not negligible, on the other hand in a quite a number of preparations

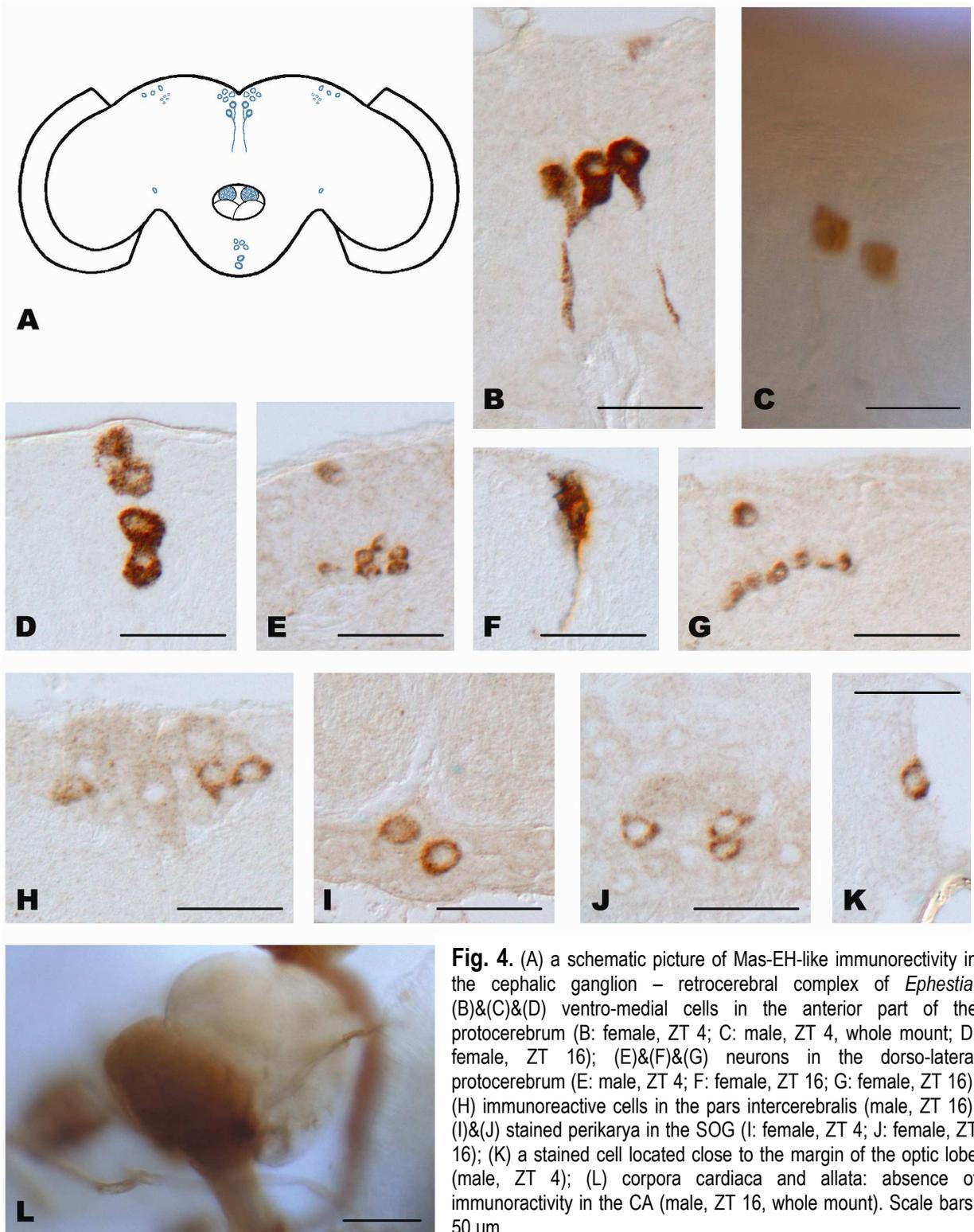


Fig. 4. (A) a schematic picture of Mas-EH-like immunoreactivity in the cephalic ganglion – retrocerebral complex of *Ephestia*; (B)&(C)&(D) ventro-medial cells in the anterior part of the protocerebrum (B: female, ZT 4; C: male, ZT 4, whole mount; D: female, ZT 16); (E)&(F)&(G) neurons in the dorso-lateral protocerebrum (E: male, ZT 4; F: female, ZT 16; G: female, ZT 16); (H) immunoreactive cells in the pars intercerebralis (male, ZT 16); (I)&(J) stained perikarya in the SOG (I: female, ZT 4; J: female, ZT 16); (K) a stained cell located close to the margin of the optic lobe (male, ZT 4); (L) corpora cardiaca and allata: absence of immunoreactivity in the CA (male, ZT 16, whole mount). Scale bars: 50 μ m.

this cluster of cells is not visible at all. This presence or absence does not seem to have any relation to neither the light/dark cycle nor the sex of the moths. It is possible that it may have some connection to the age of the animals used, but this variable was not monitored in this study and thus no conclusion can be offered on this point.

Quite a strong immunoreactivity was observed in the corpora cardiaca, corpora allata did not contain material recognized by this antiserum (Fig. 4. L).

3.1.4. Allatostatin

Allatostatin-like material has been detected mainly in the SOG and lateral protocerebrum. The total number of immunoreactive cells in the protocerebrum is no less than seven and no more than ten per each brain hemisphere. They are positioned in the central part of the protocerebrum and close to the surface of the brain. At least five cells out of this group are tightly adjacent, the remaining are placed in proximity (Fig. 5. B,C,D). Fine speckles of dispersed immunostaining form a wing-like shape in each hemisphere in the sections; it extends to the lateral protocerebrum and is based in the pars intercerebralis.

However, the main body of the allatostatin-like immunoreactivity occurred in the SOG where a cluster of about six large neurons can be seen (Fig. 5. E,F,G). This group of cells is located in the medial part of the ganglion, closer to the front. Deeper than these neurons two additional cells occurred, they are found laterally beneath the surface of SOG (Fig. 5. H,I).

Occasionally, several other cells were seen at the margin of the optic lobe in deutocerebrum and in pars intercerebralis but these were stained very weakly and visible in only few cases.

As to the neurohemal organs, heavier staining was seen in corpora cardiaca but some scattered axons occurred in corpora allata as well (Fig. 5. J,K).

➡ **Fig. 5.** (A) a schematic picture of Mas-AS-like immunoreactivity in the cephalic ganglion – retrocerebral complex of *Ephesia*; (B) a cluster of adjacent lateral cells (male, ZT 16, whole mount); (C)&(D) cytoplasmically stained neurons clustered in the lateral protocerebrum and another cell in proximity; dispersed staining extending to the pars intercerebralis (C: female, ZT 4; D: female, ZT 16); (E) immunoreactivity in the SOG: group of large immunoreactive neurons and axons in the neuropil above (male, ZT 4); (F)&(G) clusters of stained perikarya in the SOG (F: female, ZT 16; G: female, ZT 4, whole mount); (H)&(I) neurons in the lateral SOG (H: male, ZT 16; I: female, ZT 16); (J)&(K) axons in the corpus allatum (J: male, ZT 16, whole mount; K: female, ZT 4). Scale bars: G, J – 100 μm ; B-F, H, I, K – 50 μm .

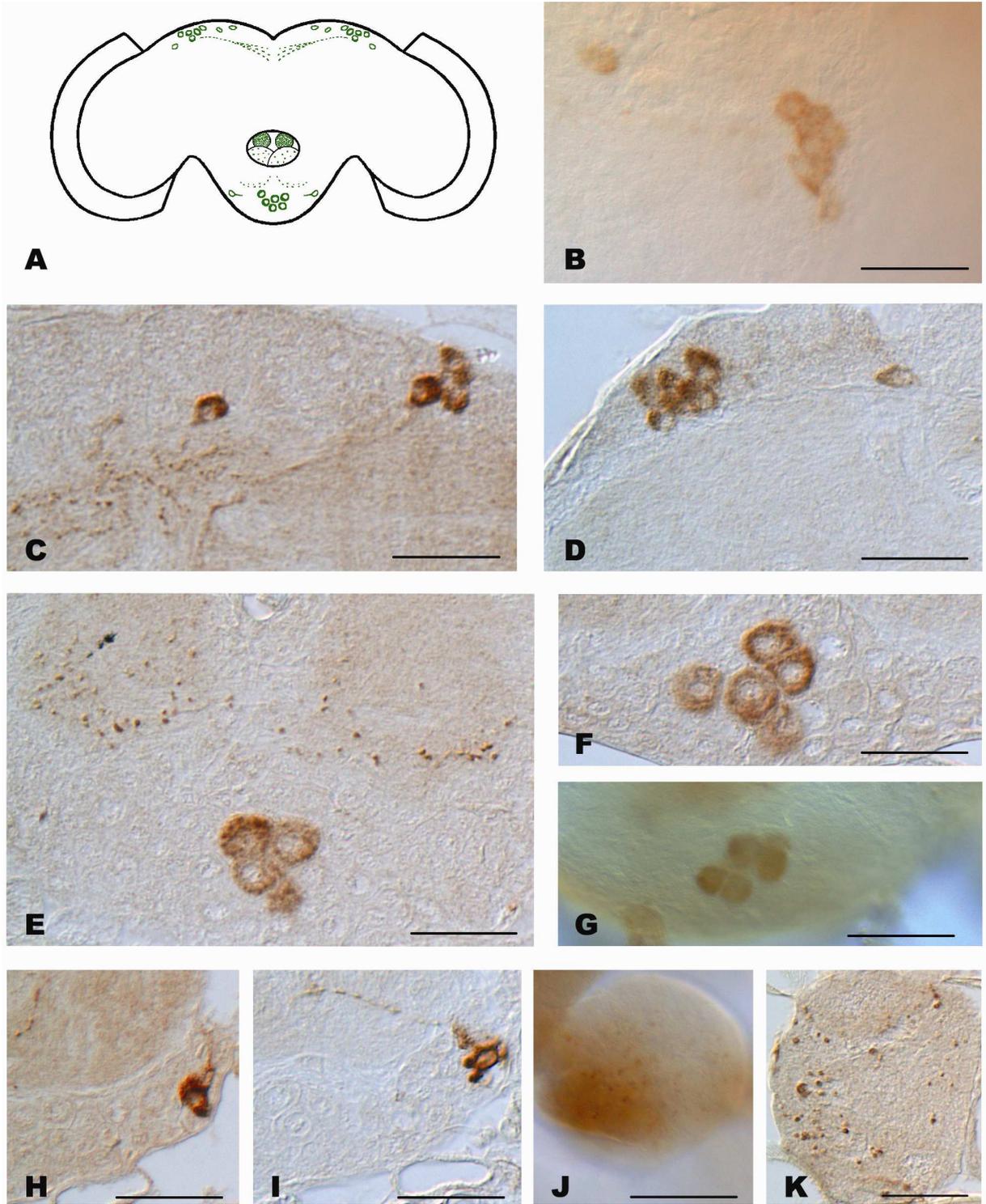


Fig. 5.

3.1.5. Allatotropin

A very high number of neurons has been recognized by the anti-allatotropin antiserum. In the lateral protocerebrum, about 15 perikarya are stained in each hemisphere. These neurons seem to be rather scattered and the whole group extends from the posterior to the centro-frontal part of the protocerebrum. Typically, two to three of the immunoreactive neurons are located most posteriorly (Fig. 6. B,C) and a cluster of about five to six small cells is placed closer to the center (Fig. 6. F). The rest of the protocerebral neurons seems to be present in pairs and dispersed in the central to frontal part of the lateral protocerebrum (Fig. 6. D). In the posterior part of the brain two smaller but heavily stained neurons can be found, these are placed closely to the brain midline and send axons in towards the oesophageal foramen (Fig. 6. G,H).

More laterally, between the protocerebrum and the optic lobe another cluster of a bit less intensively stained perikarya has been found. It consists of roughly eight to ten cells and is located centro-frontally (Fig. 6. E). At about the same depth at the margin of the lobula a group of small immunoreactive cells can be seen, as to the number, it probably ranges around ten per each hemisphere (Fig. 6. I).

Roughly 20 small perikarya lie posteriorly in the optic lobe beneath the medulla in each hemisphere (Fig. 6. J,K). These cells are closely adjoining and therefore difficult to count and thus their number might be well higher.

Two pairs of relatively small cells are positioned centro-frontally at the bottom of the suboesophageal ganglion (Fig. L, M). At about the same depth two thick bundles of immunoreactive fibers along the midline of SOG can be seen but these do not seem to form a direct connection with the stained neurons. In a few preparations an additional group of about four faintly stained cells were found centrally in the medial part of SOG; this appears, though, to be an exceptional case.

Frontally in the deutocerebrum, located at the base of the antennal lobes a high number (around 20) of relatively small adjacent perikarya is found in each hemisphere (Fig. 6. N,O). This cluster of cells seems to begin in the lateral deutocerebrum and extend to the margin of the antennal lobes.

➡ **Fig. 6.** (A) a schematic picture of Mas-AT-like immunoreactivity in the cephalic ganglion – retrocerebral complex of *Ephestia*; (B)&(C) a pair of most posteriorly positioned immunoreactive cells in the lateral protocerebrum (B: female, ZT 4, whole mount; C: male, ZT 16, whole mount); (D) a group of immunoreactive perikarya in the lateral protocerebrum (male, ZT 4); (E) a group of immunolabeled cells placed dorsally at the margin of the optic lobe (female, ZT 16); (F) a cluster of small adjacent perikarya in the dorsal part of protocerebrum (female, ZT 16); (G)&(H) immunoreactive neurons in the medial protocerebrum (both: male, ZT 16 – adjacent sections); (I) cells in the medial part of the lateral optic lobe (male, ZT 4); (J)&(K) neurons at the base of the optic lobes (J: male, ZT 4, whole mount; K: male, ZT 16); (L)&(M) neurons in the SOG (L: female, ZT 4; M: male, ZT 16); (N)&(O) clusters of perikarya in the antennal lobes (N: male, ZT 4, whole mount; O: male, ZT 16). Scale bars: D-I, K-M, O – 50 µm; B, C, J, N – 100 µm.

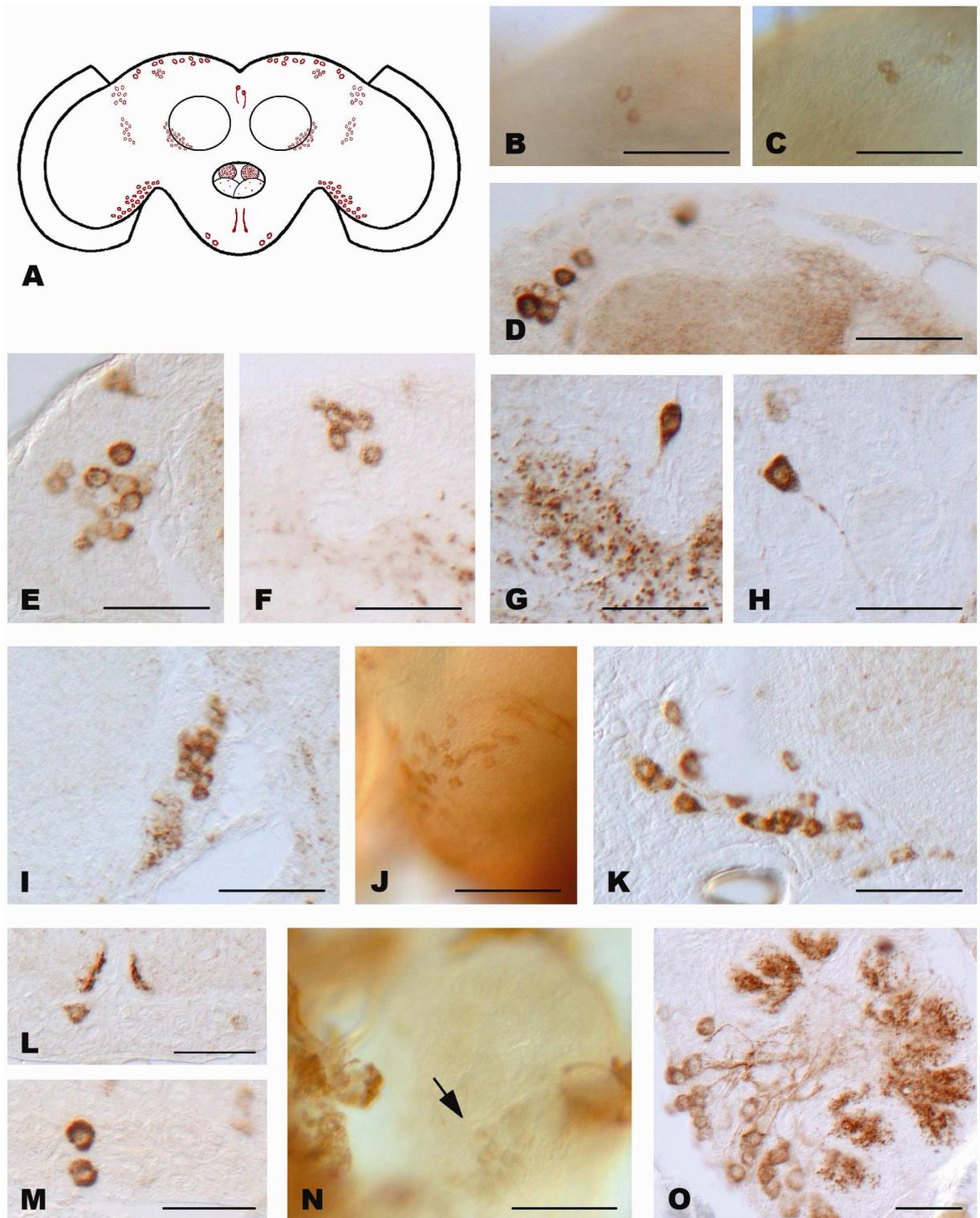


Fig. 6.

As usual, corpora cardiaca show heavier staining (no cells were observed), corpora allata contain relatively very few stained axons.

3.1.6. Prothoracicotropic hormone

Unexpectedly high number of cells has been detected using the antibody raised against the *Antherea*-PTTH. In each hemisphere, two clusters of four neurons each are situated postero-centrally in the lateral protocerebrum, they differ though in their appearance (Fig. 7. B-H). The more posteriorly located cells display even staining while the more centrally laid group has fine dark speckles in the cytoplasm. These speckles are assumed to represent secretory vesicles. Deeper in the centro-lateral protocerebrum, a cluster of about six small and also spotted cells can be seen. These cells are interconnected and a bundle of their axons project to the centre of the brain and then ventrally (Fig. 7. F,J). A group of large speckled neurons lie centrally in pars intercerebralis, they are closely adjacent and their number can only be estimated to about ten per each hemisphere (Fig. 7. I). More frontally in the same region, two pairs of heavily stained perikarya can be found, their axons run in the direction of the oesophageal foramen (Fig. 7. K).

➡ **Fig. 7.** (A) a schematic picture of *Antherea*-PTTH-like immunoreactivity in the cephalic ganglion – retrocerebral complex of *Ephesia*; (B) dorsal view on the lateral protocerebral immunoreactive neurons (female, ZT 4, whole mount); (C) a cluster of adjacent immunoreactive neurons in the lateral protocerebrum (male, ZT 16, whole mount); (D) a foursome of stained perikarya (indicated by the arrow) in the lateral protocerebrum (male, ZT 16, whole mount); (E) immunoreactive neurons from the bilaterally positioned groups in the lateral protocerebrum (female, ZT 4); (F) three (of four) evenly stained dorso-lateral protocerebral cells and a cluster of small adjacent speckled perikarya with their axons conjoining into a thick bundle (female, ZT 4); (G)&(H) a group of adjacent speckled immunoreactive neurons in the lateral protocerebrum (G: female, ZT 4; H: female, ZT 16); (I) a cluster of stained cells in the pars intercerebralis (female, ZT 16); (J) a group of small speckled perikarya placed deeper in the lateral protocerebrum and their axonal connections (male, ZT 16); (K) a pair of large immunolabeled neurons placed frontally in the protocerebrum (female, ZT 16); (L) a pair of lateral neurons in the SOG with their axons projecting to the centre of the ganglion (male, ZT 16); (M) arborization in the medial part of the SOG: the two lateral projections form connection with the lateral neurons in the SOG (male, ZT 4); (N) large and heavily stained perikarya in the central part of the SOG (female, ZT 4); (O) three stained cells beneath the oesophageal foramen (male, ZT 16); (P) corpora cardiaca and allata: CC is heavily stained, CA contains only scattered immunolabeled axons (male, ZT 16). Scale bars: B, E-P – 50 μ m; C, D – 100 μ m.

They probably enter to the corpora cardiaca but no direct connection was seen neither in the sections nor in the whole mounts.

In the SOG at least 12 strongly immunoreactive cells can be seen. Three pairs of perikarya lie centrally in the ganglion: one in the bottom and two laterally on each side; these send axons to the centre of the SOG (Fig. 7. L,M). More frontally, five large (Fig. 7. N) and one smaller heavier stained neuron are located roughly in the medial part and three other cells are above them, beneath the oesophageal foramen (Fig. 7. O). All these cells except for the smaller one are also speckled. The dispersed staining seen in the sections might indicate a connection to the corpora cardiaca.

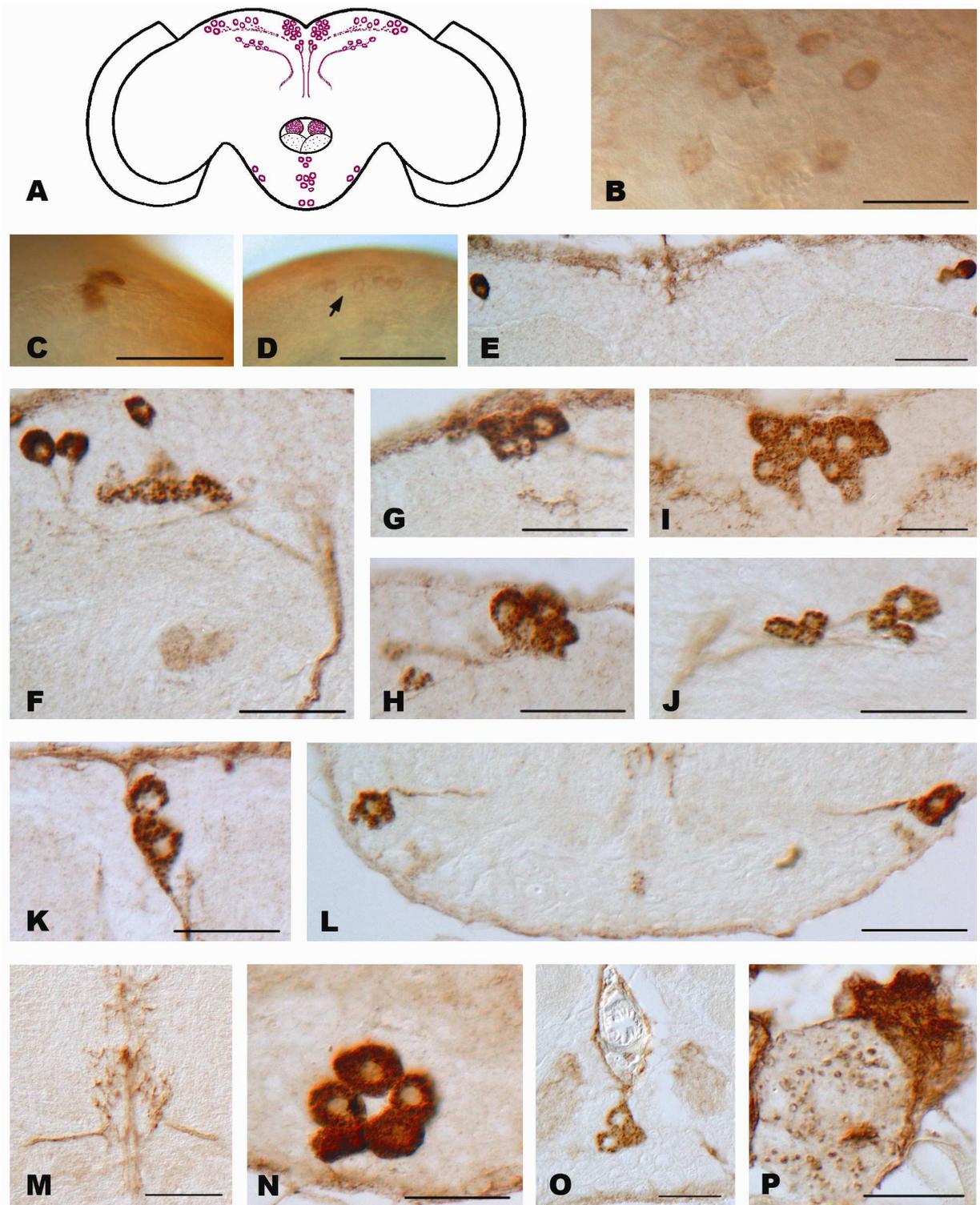


Fig. 7.

Both corpora cardiaca and allata contain material recognized by the anti-PTTH antiserum. While corpora cardiaca are heavily stained (but no immunoreactive cells were identified), corpora allata contain relatively few stained axons (Fig. 7. P).

3.1.7. Diuretic hormone

Antiserum against DH produced very significant staining throughout the brain and suboesophageal ganglion complex. Immunoreactive axons can be seen in essentially all parts of the brain, probably forming a dense network.

High number of immunoreactive cells is placed posteriorly at the base of the protocerebrum, about ten strongly immunoreactive perikarya and many just faintly stained cells can be counted in each hemisphere. Also posteriorly in the protocerebrum but right beneath the brain surface, there are four very heavily stained neurons in each brain half (Fig. 8. C-F), again surrounded by weakly immunoreactive perikarya (about four or five). At about the same depth around the oesophageal foramen two pairs of moderately stained cells can be detected (Fig. 8. H). Immunoreactivity has also been found in the optic lobes: about ten to fifteen small adjacent perikarya are clustered posteriorly at the base of the optic lobe (Fig. 8. I) and others (about ten) are scattered between medulla and lamina neuropils, occasionally, a second cluster of about five cells was found above, in the optic lobe.

More centrally, two giant neurons are located at the bottom of the suboesophageal ganglion (Fig. 8. M). About six adjoining small cells are positioned laterally in the deutocerebrum at the margin of the optic lobes. A very significant reaction has occurred in the pars intercerebralis, about eight adjacent large neurons can be seen in each hemisphere forming two clusters along the brain midline (Fig. 8. G). Two thick nerve bundles coming from these clusters run ventrally and arborize along the oesophageal foramen as was nicely visible on the whole mounts, and possibly join the corpora cardiaca (Fig. 8. J,K). Laterally in the protocerebrum, just where the optic lobe begins several rather weakly stained perikarya can be found; their number can be estimated to about seven per each brain half (Fig. 8. L).

In the frontal part of the brain another heavily stained cells are present in the SOG, at least six of them are scattered in the medial part of the ganglion and other two are placed laterally on both sides. These cells are accompanied by a number of less intensively stained perikarya (Fig. 8. K). A staining pattern similar to that produced by the anti-allatotropin antiserum has been observed in the deutocerebrum. High numbers of immunoreactive cells are located in the lateral deutocerebrum extending to the antennal lobes (Fig. 8. N,O). About twenty of these cells can be found in each hemisphere.

Both corpora cardiaca and allata seem to contain material reactive to the anti-DH antiserum used in this study. Corpora cardiaca show very heavy staining but no immunoreactive cells have been found, corpora allata display much weaker and dispersed staining.

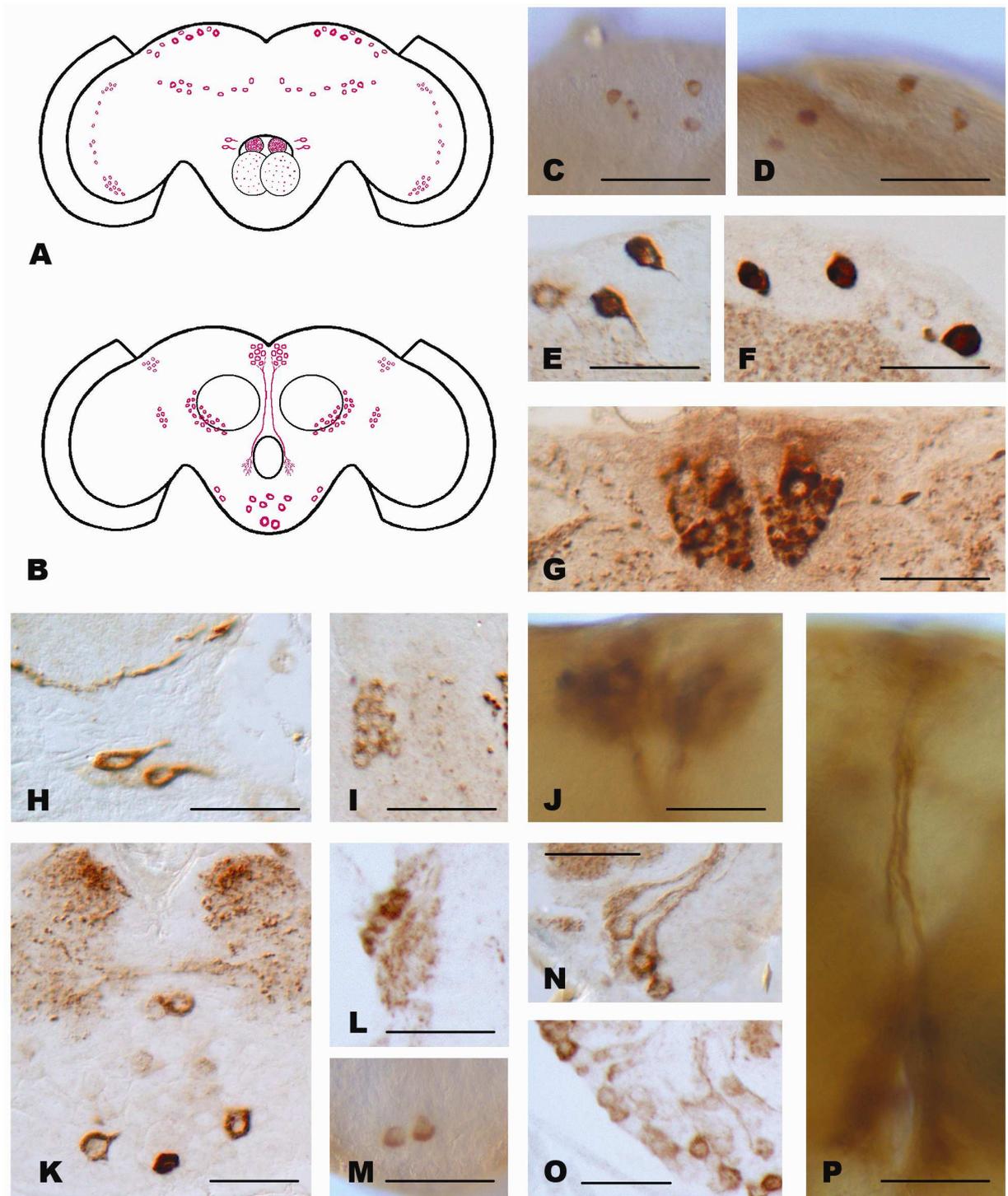


Fig. 8. (A)&(B) a schematic picture of Mas-DH-like immunoreactivity in the cephalic ganglion – retrocerebral complex of *Ephestia*: (A): posterior view; (B): anterior view; (C)&(D) neurons in the dorso-lateral protocerebrum (both: female, ZT 16); (E)&(F) perikarya in the lateral part of posterior protocerebrum (E: male, ZT 4; F: female, ZT 4); (G) immunoreactive cells in the pars intercerebralis (female, ZT 4); (H) stained perikarya around the oesophageal foramen (male, ZT 4); (I) a cluster of cells at the base of the optic lobe (male, ZT 16); (J) nerves coming from the cluster of cells in the pars intercerebralis (male, ZT 4, whole mount); (K) cells in the frontal part of the SOG (male, ZT 16); (L) perikarya located in the lateral part of the optic lobe (male, ZT 16); (M) neurons in the SOG (female, ZT 4, whole mount); (N)&(O) perikarya in the antennal lobes (N: male, ZT 4; O: male, ZT 16); (P) nerves coming from the pars intercerebralis and their arborization along the oesophageal foramen (male, ZT 4, whole mount). Scale bars: E-L, N, O – 50 µm; C, D, M, P – 100 µm.

3.2. Western blot

Western blot analysis failed to produce any clear results. None of the antibodies used reacted specifically with proteins of expected molecular weights. In most cases many bands were stained and precisely the same staining pattern though perhaps somewhat fainter occurred also in the negative control where the primary antibody was not present. The reaction is therefore assumed to be specific not for the primary but rather for the secondary antibody (Jackson ImmunoResearch). This explanation is further supported by the fact that the same staining pattern was obtained for different primary antibodies. For this reason, another secondary antibody (Pierce) was employed, we had hoped that this one would better suite for the chemiluminescence detection method and produce better results. This unfortunately did not turn out to be true. The Pierce antibody produced very little background but still no

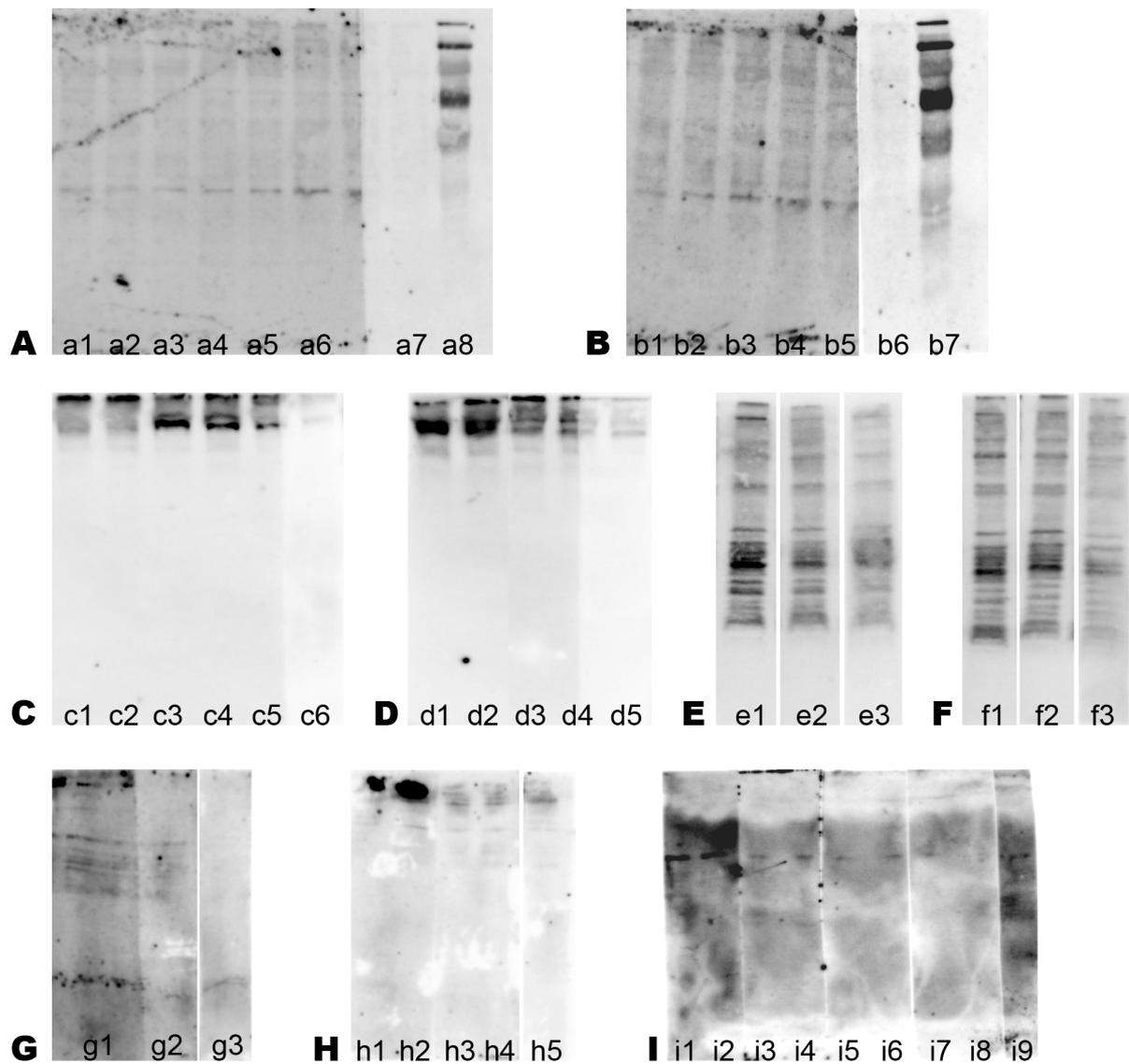


Fig. 9.

difference between the negative control and primary antibody-containing samples was observed. Several dilutions of primary and secondary antibodies and their combination were tested but none produced unambiguous results. For detailed information on dilutions of both primary and secondary antibodies, see descriptions of individual figures.

Samples for detection of allatotropin, allatostatin and corazonin were run exclusively on tris-tricine gels because the molecular weights of the proteins of interest were expected to be very low, and it was probable that these proteins would not be trapped in the classical sds-polyacrylamide gel.

◀ **Fig. 9.** (A) Detection of pigment dispersing factor. Samples from ZT 4 (run on 12% SDS-polyacrylamide gel): a1-a3 – samples from females; a4-a6 – samples from males; a7 – negative control; a8 – marker (Chemiluminiscent BlueRanger). Dilutions of antibodies: anti – *Uca*-PDH primary antibody: 1: 10,000; HRP-conjugated secondary antibody (Jackson ImmunoResearch): 1: 50,000. (B) Detection of pigment dispersing factor. Samples from ZT 16 (run on 12% SDS-polyacrylamide gel): b1-b2 – samples from females; b3-b5 – samples from males; b6 – negative control; b7 – marker (Chemiluminiscent BlueRanger). Dilutions of antibodies: see (A).

(C) Detection of prothoracicotropic hormone. Samples from ZT 4 (run on 12% SDS-polyacrylamide gel): c1-c2 – samples from females; c3-c5 – samples from males; c6 – negative control. Dilutions of antibodies: anti – *Antherea*-PTTH primary antibody: 1: 50,000; HRP-conjugated secondary antibody (Pierce): 1: 1,000. (D) Detection of prothoracicotropic hormone. Samples from ZT 16 (run on 12% SDS-polyacrylamide gel): d1-d2 – samples from females; d3-d4 – samples from males; d5 – negative control (marker not shown). Dilutions of antibodies: see (C).

(E) Detection of prothoracicotropic and diuretic hormone. Samples from ZT 4 (run on 12% SDS-polyacrylamide gel); e1 – PTTH; e2 – DH; e3 – negative control. Dilutions of antibodies: anti – *Antherea*-PTTH primary antibody: 1: 50,000; anti – *Manduca*-DH primary antibody: 1: 20,000; HRP-conjugated secondary antibody (Jackson ImmunoResearch): 1: 50,000. (F) Detection of prothoracicotropic and eclosion hormone. Samples from ZT 16 (run on 12% SDS-polyacrylamide gel); f1 – PTTH; f2 – DH; f3 – negative control. Dilutions of antibodies: see (E).

(G) Detection of corazonin and eclosion hormone. Samples from ZT 4 (run on 16,5% Tris-tricine gel); g1 – corazonin; g2 – EH; g3 – negative control. Dilutions of antibodies: anti – *Periplaneta*-CRZ primary antibody: 1: 10,000; anti – *Manduca*-EH primary antibody: 1: 20,000; HRP-conjugated secondary antibody (Jackson ImmunoResearch): 1: 50,000. (H) Detection of corazonin and eclosion hormone. Samples from ZT 16 (run on 16,5% Tris-tricine gel); h1 – corazonin, sample from females; h2 – corazonin, sample from males; h3 – EH, sample from females; h4 – EH, sample from males; h5 – negative control. Dilutions of antibodies: see (G).

(I) Detection of allatostatin and allatotropin. Samples from ZT 16 (run on 16,5% Tris-tricine gel); i1, i3, i5; i7 – samples from females; i2, i4, i6, i8 – samples from males. Dilutions of antibodies: anti – *Manduca*-ASH primary antibody: 1: 15,000 (i3-i4) and 1: 75,000 (i7-i8); anti – *Manduca*-ATH primary antibody: 1: 15,000 (i1-i2) and 1: 75,000 (i5-i6); HRP-conjugated secondary antibody (Pierce): 1: 1,000.

4. Discussion

Since the discovery of Kopec that brain also serves as an endocrine organ, the group of insect hormones produced by nervous tissue (neurohormones) has grown wide and diverse. Neurohormones have been proved to be important regulatory factors whose influence reaches all physiological and behavioral processes. Therefore it is no surprise that some of them have been shown to be associated also with the circadian timing system. In order to explore this connection a little further, we have picked several candidate neuropeptides and monitored their production at ZT 4 and ZT 16.

No significant differences between day and night samples have been observed for any of the neurohormones of interest. The immunoreactive material was always confined to the perikaryal cytoplasm and both the number and the localization of immunoreactive cells seemed to be identical in samples from both ZT 4 and ZT 16. The intensity of staining of the immunopositive cells also did not display any marked variations suggesting that the amount of immunoreactive material is more or less stable throughout the day. This notion however could have been confirmed only on the basis of Western blot analysis which unfortunately failed to produce any results.

4. 1. Western blot analysis

Several explanations can be offered that could account for the failure of the Western blot method. The case of PDF seems to be quite clear, the primary antibody probably cross-reacted with some other antigen since the molecular weight of the recognized protein was far beyond the range of all possibilities. The structure of β -PDHs and PDFs appears to be well conserved and there is no reason to believe that *Ephesia* should possess homologous neurohormone that would be that much larger than all PDHs so far identified.

As to the rest of the experiments, the fact that the same staining pattern as in the samples was observed also in the negative controls indicate that the primary antibodies do not recognize the protein of interest at all and the resulting staining can be assigned to the secondary antibody alone. It is possible, that the particular neurohormone is significantly different from the original antigen used for the antibody production and therefore the antibody does not recognize it. This explanation though does not seem to be very likely since all the antibodies reacted quite specifically in the other immunocytochemical techniques employed in this study. Another possibility is, that the membrane was blocked insufficiently thus leaving many sticky sites where the secondary antibody bound. Taking into account though, that milk is standard and widely used blocking reagent and the time left to it to saturate the unspecific binding sites (2 hrs) was relatively long I believe that this possibility can also be denied. Besides, the same blocking conditions and secondary antibody dilution were used also in the experiment with anti-

PDH antibody where only very faint bands were observed in the negative control. Moreover, when a different secondary antibody (Pierce) was employed the staining pattern was different, but it was still precisely the same as in the negative control, though the control was very faint indeed.

There is yet one explanation and I consider this to be the most likely. All the antibodies used in this study were raised against the protein antigen in its native state. However, in the case of Western analysis the proteins are subjected to very harsh denaturing conditions not only during the extraction from tissues but also during the subsequent electrophoresis. We are making every effort to destroy completely the spatial structure of proteins so that they would not stack in the gel and may be separated solely on the basis of their molecular weights. This makes very special demands on the antibodies. To function well on the Western blots, the antibody must recognize linear and rather short sequence of aminoacids. It is very common that antibodies working well on fixed tissues do not work at all on the blots. I believe this is exactly what happened in this study: the antibodies simply did not recognize their protein antigen because it was denaturated and its epitopes were thus destroyed. Though I favor this explanation above others it must be stated that certainly not all the possibilities to optimize the method for these particular antibodies and to fine-tune the dilutions of the sample, primary and also the secondary antibody were exploited. Yet the results we have obtained do not seem to be very promising.

4. 2. Immunocytochemistry on paraplast sections and whole mounts

Unlike the Western blot analysis, the other two methods used in this study worked very well and produced some interesting results. All the antibodies employed in this study reacted with specific groups of neurons within the *Ephestia's* cephalic ganglion.

The staining pattern obtained with the anti-corazonin antiserum was very sharp and the individual variation among samples was minimal, regardless of the time when they had been collected and also of the sex of the moths. The number and position of immunoreactive neurons is in good agreement with the previous studies on localization of this neuropeptide. The direct axonal connection to the corpora cardiaca demonstrates that at least the foursome of lateral protocerebral neurons are true neurosecretory cells. The most surprising outcome is the massive immunoreactivity occurring in the optic lobes and compound eyes. Nothing like that has been reported so far for none of the investigated insect species. Such localization may imply a role of corazonin-like substance in processing of the photic information coming from the compound eyes. Using immunocytochemical approaches it has been shown that corazonin-like substance is, at least in *Manduca* and *Antherea* co-localized with the clock-gene products (Wise *et al.*, 2002; Šauman, unpublished data). It is tempting to speculate that this may be the case also in *Ephestia* and that corazonin might possibly function in relaying (or modulating) the information on the light conditions in the outside world to the circadian clock centre. Also the position of

the corazonin-like immunoreactive neurons is in accord with this hypothesis since it is known that in some insects, including lepidopteran species, the circadian pacemaker neurons are located right in the lateral protocerebrum. Of course, there is no evidence that could support these assumptions but it would definitely be interesting to find out whether corazonin is indeed co-localized with the clock proteins and to explore its possible role within the timekeeping system.

PDH is a well established component of the circadian timing system though it is most probably not a part of the core oscillator. In *Ephestia*, this peptide has been localized in several distinct clusters. Four neurons have been heavily stained in the lateral protocerebrum further reinforcing the assumption that this may be the actual site where the clock neurons reside. These cells are found at about the same region as the corazonin-like immunoreactive neurons and there is a possibility that these two peptides may be present in the very same (clock?) neurons after all. PDH-like immunoreactivity has often been found in several clusters in the optic lobes (Závodská et al., 2003a; Sehadová et al., 2003). In *Drosophila*, one of these clusters, positioned in the accessory medulla, was demonstrated to co-localize the PDH and several clock proteins and this site is believed to be the actual pacemaker centre of the fruitfly (Helfrich-Förster, 1997). In *Ephestia*, one group of immunoreactive cells has indeed been found in the optic lobes, but these cells display much weaker staining than those in the lateral protocerebrum, suggesting that they are not the primary source of PDH-like substance in the *Ephestia*'s brain.

Very surprising results have been obtained with the anti *Antherea*-PTTH antiserum. The number of immunoreactive cells is much higher than would be expected on the basis of experiments on other lepidopterans (Gray et al., 1994; Shionoya et al., 2003; Šauman and Reppert, 1996b; Wei et al., 2005; Yagi et al., 1995). In view of the fact that the aminoacid sequence of the PTTHs known today does not display any dazzling similarity, the risk of cross-reactivity in the immunocytochemical studies seem to be very real. On the other hand, the staining pattern observed was very consistent and sharp; the individual differences among samples were minimal. Moreover, *Ephestia* represents a rather primitive lepidopteran so it is possible that the distribution of its own PTTH homolog may be significantly different from those seen in higher lepidopteran species such as *Manduca* or *Bombyx*. The antiserum reacted with a number of cells in the medial and lateral protocerebrum and also in the suboesophageal ganglion. According to the study of Závodská et al. (2003a), both of these locations of the PTTH-positive neurons are quite common among insects and a similarly high numbers of immunoreactive cells can be seen in some of them. A high number of PTTH-positive cells was also identified by Žitňan et al. (1993) in *Drosophila melanogaster* using antiserum against *Bombyx*-PTTH. In summary, if the immunoreactive material recognized by this antiserum is indeed functional PTTH homolog, it is very abundant. This begs the question: What is its function? Why an adult animal lacking prothoracic glands should need such high amounts of prothoracicotrophic hormone? Even if the antiserum did cross-react with other, perhaps

completely unrelated, antigen this question would still be valid. The presence of PTTH in adults' brains have been reported many times and still no function other than stimulating the synthesis of ecdysteroids by the prothoracic glands can be assigned to it at present.

We encounter a similar problem in the case of eclosion hormone. Its presence in adult individuals is well established but its function there is completely unknown. The eclosion hormone is typically produced by two pairs of ventro-medial neurons (Horodyski et al., 1989; Závodská *et al.*, 2003a,b; Zhang and Xu, 2006). In *Ephestia* these cells have also been identified as the primary source of the immunoreactive material, though some minor staining occurred also in the suboesophageal ganglion and occasionally also in the lateral protocerebrum.

Both of these hormones, PTTH as well as EH, are known to be under circadian control (at the level of their secretion into hemolymph), it is thus possible that they just mediate the information about the phase of the day between the pacemaker system and the relevant organs but the interpretation of this information as well as the recipient structures may change during development.

As to the diuretic hormone, the antiserum raised against *Manduca sexta*-DH recognized many cells throughout the brain ganglion of *Ephestia*. The available data on number and localization of DH-positive cells in other insect species are quite heterogenous and the numbers of immunoreactive cells reported in literature reach up to about 450. Veenstra and Hagerdon (1991) observed DH-positive cells only in the median protocerebrum in the brain of *Manduca sexta*. In the housefly *Musca domestica*, diuretic hormone was localized also in the lateral protocerebral cells and alongside the oesophageal foramen. Te Brugge *et al.* (1999) reported Locusta-DH positive cells to be present in the protocerebrum, at the base of the optic lobes as well as in the suboesophageal ganglion of the blood-feeding bug *Rhodnius prolixus*. In *Ephestia*, immunoreactive cells were found in all these regions and also in the antennal lobes. It is however interesting, that the staining pattern (number as well as localization of the immunoreactive cells) in the SOG of *Ephestia* is strikingly similar to that reported by Emery *et al.* (1994) to be specific for Manse-DH II in *Manduca sexta*. It is possible, that *Ephestia* also possesses two forms of diuretic hormone (which seems to be quite common among insects) and the antiserum reacts with both of them. Given the quite high overall sequence similarity of Manse-DH I and Manse-DH II, some parts being absolutely identical, this would not be that surprising. In summary, the DH-positive cells typically occur in the pars intercerebralis, otherwise their localization can be quite varied. Still some common features even in respect to the number of immunoreactive cells can be found even between so evolutionary distant species as *Ephestia* and *Musca*.

Essentially the same distribution of allatotropin-immunolabeled cells as has been observed in *Ephestia* was reported also for the true armyworm *Pseudaletia unipuncta* (Truesdell *et al.*, 2000), where the results of immunocytochemistry (using anti-MasAT antiserum) were further confirmed by the *in situ*

hybridization. In both these species, the immunoreactive cells were found in the protocerebrum, antennal lobes, optic lobes and also in SOG. The localization of AT-positive cells in the antennal lobes may imply its role in regulating the movement of the antennae and/or sensory perception. It is, of course also possible that they only have some neuromodulatory function. The localization of high numbers of immunoreactive cells in the optic lobes could mean that AT is somehow involved in the processing of photic information as was previously suggested by the experiments on the cockroach *Leucophaea maderae* (Petri *et al.*, 2002). Briefly, many roles and functions have already been proposed for allatotropin apart from its name-giving property of stimulating the production of juvenile hormone, and the distribution of this peptide within the brain seems to only confirm its pleiotropic effects.

There are three groups of allatostatins, the C-type (*Manduca sexta* type) investigated here seems to be the least popular, since most of the available literature concerns rather the A and B types of allatostatins. Very little work was done especially on characterizing the distribution of this peptide in the CNS of insects.

Most information on this subject comes, surprisingly, from the studies on *Drosophila*. Williamson *et al.* (2001) cloned C-type allatostatin from this species and learned that it is just one amino acid different from the Mas-AS. *In situ* hybridizations revealed 9 pairs of symmetrically positioned cells in the protocerebrum and three pairs in the tritocerebral part of the brain of the 3rd instar larvae. Earlier, Žitnan *et al.* (1993) used antiserum against Mas-AS to localize the immunoreactive material in both larval and adult *Drosophila*. In adults, quite a high number of immunolabeled cells were present in both lateral and medial protocerebrum, in the optic lobes and elsewhere. Abdel-latif *et al.* (2004) investigated the expression of *Spodoptera frugiperda*-AS (Spofr-AS) which is identical to that of *Manduca*. *In situ* hybridization revealed that the Spofr-AS was slightly expressed in a cluster of cells in the deutocerebrum and another group of positive cells was found in the posterior-ventral region of the optic lobes adjacent to the protocerebrum. This pattern is very different from what we have learned from the immunocytochemical experiments on *Ephesia*. As there are no other lepidopterans in which the distribution of Mas-AS was studied at the cellular level, we cannot tell which pattern is more common.

To conclude, none of the investigated neurohormones seem to be circadianly regulated at the level of their expression, since no significant differences were observed neither in the number of immunolabeled cells nor in the intensity of staining between samples from ZT 4 and ZT 16. It is very improbable that a circadianly regulated peptide would display such an expression pattern that would be the same in ZT 4 and ZT 16 but would differ between these two timepoints. This does not mean however, that these neurohormones cannot be under circadian control at other levels, possibly at the level of their secretion into hemolymph as was already demonstrated for PTTH and EH.

5. Summary

Spatial and temporal distribution of seven insect neurohormones (allatostatin, allatotropin, diuretic hormone, eclosion hormone, corazonin, pigment-dispersing factor and prothoracicotropic hormone) within the cephalic ganglion of *Ephesia kuehniella* has been explored in this study. The results can be summarized as follows:

1. All the antibodies reacted only with a restricted population of cells within the brain of *Ephesia kuehniella*.
2. The immunoreactive material was always confined to the perikaryal cytoplasm with no signs of translocation into the cell nuclei.
3. The localization pattern did not display any marked variations between night and day samples for none of the investigated neurohormones.
4. Judging by the results of immunocytochemistry on paraffin sections and whole mounts, the relative amount of the immunoreactive material does not seem to differ significantly between night and day. However, the Western blot analysis that would enable rough quantification of the particular proteins present at each timepoint failed and thus the above statement cannot be supported by clear and objective evidence.
5. It seems very likely that the expression of none of the investigated neurohormones is under direct circadian control.

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