Circadian clocks in crustaceans: identified neuronal and cellular systems

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1. ABSTRACT

Circadian rhythms are known for locomotory and reproductive behaviours, and the functioning of sensory organs, nervous structures, metabolism and developmental processes. The mechanisms and cellular bases of control are mainly inferred from circadian phenomenologies, ablation experiments and pharmacological approaches. Cellular systems for regulation summarised here comprise the retina, the eyestalk neuroendocrine X-organ-sinus gland system, several neuropeptides such as red pigment concentrating, hyperglycaemic and pigment-dispersing hormones, and factors such as serotonin and melatonin. No master clock has been identified, but a model of distributed clockwork involves oscillators such as the retinular cells, neurosecretory systems in the optic lobes, putative brain pacemakers, and the caudal photoreceptor. Extraretinal brain photoreceptors mediate entrainment. Comparative analyses of clock neurons and proteins known from insects may allow the identification of candidate clock neurons in crustaceans as putative homologues in the two taxa. Evidence for the existence of "insect-like" intracellular clock proteins and (light sensitive) transcription factors is scarce, but *clock*-, *period*-, and *cryptochrome*-gene products have been localised in the CNS and other organs rendering further investigations into crustacean clockwork very promising.

2. INTRODUCTION: CRUSTACEAN CIRCADIAN BIOLOGY

2.1. Rhythms and circadian phenomena

Habitats of almost all organisms are exposed to daily changes of light and dark phases, the photoperiod, caused by the earth`s rotational movements. The organisms have to adapt to these natural physical constraints by performing and regulating their activities in a circadian rhythm (“circa dies”: approximately a day). Therefore, circadian clocks that regulate internal synchrony and are entrained to external diurnal parameters have evolved in all domains of life (1, 2). However, circadian rhythms are only one out of several types of rhythms controlled by distinct well investigated clocks in arthropods, including several

crustacean species, such as circalunar (3-6), circatidal (6-8), seasonal or circannual rhythms (6, 9). Rhythms shorter than a day are referred to as ultradian. The circadian rhythm has been assumed to be the basic unit of all these rhythms (10), but evidence has been provided that some circadian rhythms at least during certain developmental stages in crustaceans or in distinct sensory neurons result from the coupling of higher frequency ultradian oscillators (11, 12) (see 3.3., 3.4., 3.5., 5.).

Crustaceans predominantly inhabit all marine and freshwater environments of the earth, but also live in terrestrial or semi-terrestrial habitats. They usually occur in large numbers and are, thus, of considerable ecological and often economical importance. In this review, the known cellular systems and mediators underlying circadian rhythms in crustaceans will be covered. Rhythmicity as an oecophysiological adaptation involves almost every facet of crustacean biology and comprises developmental, physiological, sensory, and behavioural aspects of several species. Of special importance are photoperiodically controlled rhythmical phenomena in locomotory activity, moulting, and reproduction including the release of eggs or dispersal of larvae, not at last interesting for fisheries in terms of predictions of catches. Rhythmical activities can save energy for the individual animals during their locomotory or reproductive activities, but may under certain circumstances such as during moulting still expose them to extreme stress and vulnerability (13); moulting behaviour is, furthermore, intricately dependent on the relative length of the light and dark phases which defines the progression of seasons (see 14). As many crustaceans live in intertidal habitats, circatidal rhythms play pivotal roles and govern rhythms of locomotory activity, food intake, moulting and reproduction, in addition to circadian rhythms. One extensively investigated species is the shore crab *Carcinus maenas* which displays reliable patterns of activity as it seeks shelter during low tide and displays major locomotory activity during high tide when water covers its habitat. This activity pattern is retained under constant laboratory conditions as a free-running circatidal rhythm (6-8, 15). Circadian rhythms of spontaneous locomotion in the crayfish *Procambarus clarkii* show bimodal patterns with increased activity both after onset and offset of light, with highest activity after offset of light, a behaviour that persists under constant darkness (16, 17). Thus, rhythmic behaviours exploit temporal niches within a fluctuating environment in order to most efficiently support survival and reproduction. Moreover, circadian activity patterns may serve as separation mechanisms for sympatric species, e.g. those using identical food recourses, in that they demand or constitute temporal niches, thereby reducing competition for potentially limited resources (18).

2.2. Chronobiological systems in Crustacea

Several crustacean clocks have previously been reviewed with regard to both circadian (10, 19-22) and circatidal (3, 4, 6, 8, 19) rhythms, just to mention only some more recent or extensive works. Whilst ultradian rhythms have been detected in many systems (12), their functional significance has yet to be determined. They are often excluded from further analyses (23), which may well leave aspects of great biological importance unrecognised. We have addressed these phenomena where described.

Although crustaceans are a species-rich and highly diverse group of organisms, concise data on circadian rhythmicity have mainly been obtained from decapod species. Model organisms in many neurobiological studies have been and still are crayfish, mainly the species *Procambarus clarkii, Procambarus bouvieri,* and *Cherax destructor*. However, chronobiological studies have involved multiple crustacean species and are preferably comparative. Most common are studies on locomotory patterns, which are perhaps best documented for the shore crab *Carcinus maenas* (7, 24, 25), crayfish (26), and lobsters (27-30). For *C. maenas* and species of commercial value such as *Homarus americanus*, rhythms related to moulting and reproduction have been well documented (31-35). Some data have also been obtained from prawns, e.g. *Penaeus* (or *Litopenaeus*) species (36, 37), ditch shrimps (*Palaemonetes varians* (38), glass shrimps (*Palaemonetes pugio*; 39) or night shrimps (*Processa canaliculata*; 40).

Diurnal pigment colour change has been extensively studied in diverse fiddler crabs of the genus *Uca* and other crustaceans (41, 42). As many crustacean species are nocturnal or active during dim or twilight hours, a very interesting aspect of crustacean chronobiology and of considerable oecological importance concerns the diel vertical migration (DVM) performance of many small chiefly planktonic crustacean species in marine and freshwater environments. This is well investigated for marine decapod larvae such as crab zoeae (43), calanid copepods (44), krill species (45-47), and freshwater micro-crustaceans such as cladocerans, e.g. *Daphnia* species (48). Another aspect is the diel horizontal migration (DHM) more recently studied in nocturnal cave dwelling mysid crustaceans, which show a surprisingly strict regulatory adaptation of certain neurotransmitter expressions to changes in light-dark conditions (49). Circadian rhythmic activities are well documented for locomotory, sensory, and central nervous systems in many crustacean species. The different systems (see Table 1) evidently involve almost all biological functions in crustaceans.

The focus of this review is explicitly on the identified cellular and molecular mechanisms and mediators underlying these circadian rhythms. We will not address the overt phenomena accessible to behavioural observation. Many of these were covered previously by excellent reviews concerning aspects of circatidal, circalunar or circadian rhythms of locomotion (3, 4, 6, 7, 15), reproduction (30, 50), moulting (9, 50), and hatching or larval release (6, 51). In certain cases, reference will be given only to landmark papers or reviews to provide further detail. Thus, our aim is not to merely summarise well-known adaptations to environmental rhythms, but to outline the known cellular and intercellular mechanisms responsible for these adaptations. We will summarise circadian chronobiological systems of crustaceans and partly use comparative approaches to highlight the commonalities underlying diverse systems.

2.3. Pacemakers in crustacean circadian systems

True endogenous rhythmicity, in general, persists free-running even in absence of entraining environmental cues. It is controlled by internal pacemakers, or clocks, which autonomously control cellular activity levels and thereby regulate physiological and behavioural events in an oscillatory pattern. As in many other animal taxa (52), such oscillators are mainly located in the nervous system of crustaceans (20, 21).

The core concept of the biological clock builds upon autonomously oscillating neurons or sets of neurons showing circadian rhythms of activity. If certain rhythmicity is lost after accidental loss or experimental inactivation of the respective cells, thereby showing to be both necessary and sufficient to initiate and maintain that particular rhythmicity, these neurons are termed master clocks, or pacemakers. Pacemakers have been identified in both invertebrates and vertebrates, in which they show endogenous oscillatory activity and can thus give timing information (53). Criteria for a pacemaker are (1) autonomy of circadian oscillatory activity in constant darkness or light conditions, (2) entrainment by external cues to adjust the phase to environmental parameters such as light and temperature, (3) loss of circadian/rhythmic behaviour after ablation of specific tissues or cell groups, (4) reactivation of the rhythmicity after transplantation of the specific cells, and (5) autonomy of circadian activity *in vitro* of the isolated pacemaker tissue for a reasonable time span (10, 54-56).

The neuronal basis of circadian rhythms is considered to be restricted to a specific neural network of identified clock neurons both in arthropods and vertebrates. Especially the use of genetically tractable model organisms has greatly facilitated the identification of molecules, neuronal and glial cells and their interactions that underlie circadian rhythmicity. A biological clock in insects that is located in ~150 identified neurons has been studied in great detail in the fruit fly *Drosophila* (57-59). It consists of distinct neuron populations that control morning and evening peaks of locomotory activity, respectively (60-62). In mammals, the pacemaker is located in the suprachiasmatic nucleus of the hypothalamus (63-66). Remarkably, intracellular key regulatory molecules exist as orthologous genes in both invertebrates (*Drosophila melanogaster*) and vertebrates (*Mus musculus*), but only very few of these have so far been investigated in crustaceans (see also 4.3.). The circadian pacemaker systems of species in arthropods and vertebrates have been compared extensively in the recent literature, highlighting the function of orthologous genes between the two taxa. Moreover evolutionary conservation has been suggested for the circadian regulation at the molecular level (52, 53, 67-70) as well as for functional and neuroanatomical similarities (53). This evolutionary conservation of genes and their functions implies that insect clock gene orthologues likely also occur in crustaceans and invites screening for the locations of potential pacemakers by use of molecular criteria. Based upon these phylogenetic considerations, some clock genes as well as the neuropeptide PDH, which is a known clock output in insects, have recently been analysed in crayfish and lobsters (see 4.3.).

In insects and mammals, the circadian pacemakers are located in the brain, associated with the visual system, and are bilaterally coupled (53). A crucial but still unanswered question in crustacean chronobiology is whether circadian rhythmicity is controlled by a single pacemaker or master clock located in the brain, or alternatively by several oscillators of (relative) independence. Ablation of clock neurons in mutant flies or by gene knock-outs, e.g. genetically driven via the introduction of cell death signals, toxins or RNA-interference (RNAi) using Gal4-UAS-system approaches, usually led to complete or partial loss of rhythmicity, which proved the control functions of central pacemakers (71, 72). Unfortunately, mutant and genetic (e.g. RNAi) analyses comparable with those in *Drosophila* have only rarely (in mutant crayfish; 73) or not (yet) been possible in crustaceans. Thus, the evidence for distinct pacemaker locations is mainly derived from experiments performed after ablation of structures accessible with relative ease such as eyestalks, brains and their connectives, and usually in conjunction with locomotor actogram analyses (review: 21).

Several studies have combined organ ablations with pharmacological approaches (74-80), but the definite origin of circadian rhythmicities has so far not been possible to trace down to the cellular level. Crude ablation experiments often led to ambiguous results with respect to pacemaker location (21, 22), since ablation or deafferentiation of specific tissues (e.g. eyestalk or brain centres) were able to suppress a given rhythm, but sometimes such rhythms became restored following lesions, as was known for both circadian locomotor and electroretinogram (ERG; see 3.1.2.) rhythms (81, 82). Depending on the method of severance (e.g. by squeezing of nerves), this restoration was likely caused by regenerative events via reorganising axons and terminals (83). Experiments showing that brain resections are not sufficient to suppress locomotor rhythmicity in crayfish (84) rendered the existence of a pacemaker exclusively confined to the brain unlikely. The ERG circadian rhythmicity remained intact even in isolated retinae *in vitro,* which indicated autonomous retinal pacemakers for each eye (23). This conclusion was derived from *in vitro* experiments with specific ablated tissues, an approach providing important additional sources of information for testing whether tissues or organs have indeed autonomous circadian activities (23, 54, 85).

As ablation or loss of function and *in vitro* tissue culturing experiments only allow for crude tissue localisations, the ultimate goal must be to identify the specific neuronal or network origins of the oscillatory activities. Up to the present date, no candidate(s) for distinct neuronal groups forming a single central brain oscillator, or master clock, have been identified conclusively in any crustacean model. Instead, experiments conducted so far have identified several neuronal tissues containing distinctly separate circadian clocks that form complex interactions (10). These exist within the brain (supraoesophageal ganglion) (10, 86), the retina of the eye (10, 23), the eyestalk (10), and the caudal photoreceptor (12, 87). Extracellular electrophysiological recordings from isolated brain-eyestalk preparations have demonstrated circadian multiunit activities and visual evoked potential

responses of neurons within proto-, deuto- and tritocerebral areas, that showed even some possible connections with the sinus gland. These circadian activities also persisted in isolated brains after eyestalk ablations. Up to the present date, these are the only data giving physiological hints for the existence of endogenous brain pacemakers (88). There are still open questions about how many oscillators exist and how they are coupled in each system or model animal, i.e. how many separate clocks do actually exist in crustaceans. Experimental approaches studying circatidal rhythms by artificially separating tidal cues such as pressure, temperature, and salinity have clearly indicated the presence of multiple, separate clocks in *C. maenas* which lead to precise spawning times and larval dispersals (4, 89). However, the interactions between the oscillators and the integration of their input/output relationships are still not fully understood.

What are the distinct neuronal elements of clocks in crustaceans and how do they relate to those present in other arthropods such as insects? The known mechanisms of crustacean circadian systems and comparisons with insect circadian systems are covered in more depth in sections 3 and 4, respectively. Identified neuronal systems (putatively) responsible for circadian control and their mechanisms will be discussed, and their interactions, as inferred and validated experimentally, are summarised in section 3.5. We argue that the localisation of clock neurons and the identification of clock proteins, peptides and other factors regulating circadian rhythmicity require an integrative approach linking neuroanatomy, molecular physiology and chronobiology.

3. THE CELLULAR BASIS OF CRUSTACEAN CIRCADIAN RHYTHMS

3.1. The retina of the eye

Among the important phylogenetically common features of the Pancrustacea, which embrace the arthropod groups Insecta and Crustacea (90-92); see also 4.1.), are the retinal elements of the compound eyes, i.e. the ommatidia containing a cuticle-derived cornea and a tetraconate dioptric lens apparatus (Tetraconata concept; 93), eight (in most species) rhabdomic and pigmented sensory cells together with surrounding lateral non-neural pigment cells subserving dynamic adaptations in order to adjust to environmental light conditions by intracellular shielding pigment movements (Figure 1). Involvement of the retina of the eyes as light sensor for circadian regulations can be expected, since close adaptation to the earth`s light/dark phases needs light input detection and fine adjustments during dawn and dusk to changes of the respective illumination conditions for entrainment of rhythmicities, although recent work has attributed a major role in the circadian regulation of locomotion to extraretinal brain photoreceptors in two crayfish species (see 3.4). However, retina ablations caused increases in locomotory activity in crayfish that were otherwise fully light-entrainable and immediately adjusted to phase-shifts in the LD cycle, which indicates an indispensible role of the retinal light sensors (17).

3.1.1. Eye pigment migration and its adaptive role

Proper detection of changing illumination requires distinct adaptations with regard to the response sensitivity of the photoreceptors, which is achieved mainly by the functional activities of three sets of cellular elements in the ommatidia of the crustacean retina. The first is a gain setting at the level of the photoreceptors themselves. The second is the movements of the so-called proximal pigments (PP) within the photoreceptors, and the third is the migration of the distal pigments (DP) within the slender lateral primary pigment cells, respectively (94, 95). The shielding pigments limit light exposure of the rhabdomic membranes in the photoreceptors during the light phase and optimise photon capture during the dark phase (Figure 1) as has already been shown in the earliest studies (96-98). Accordingly, the absolute sensitivity of the crustacean eye is mainly adjusted by pigment distributions (99). During the light phase (photophase) under sufficient illumination, the PP within the photoreceptors and the DP in the lateral pigment cells become evenly distributed, thereby covering and/or shielding the photoreceptive rhabdom of the retinular sensory cells and large parts of the dioptric apparatus (76). During the dark phase (scotophase), PP and DP concentrate proximally and distally in their respective cell types, thereby enabling (maximal) access of photons to the rhabdom of the photoreceptors. The rhythm of the DP persists in conditions of constant light (10, 97, 100), as does the rhythm of the PP in constant darkness, whilst pigment migration under controlled conditions is more tightly regulated for the PP in constant darkness than for the DP under constant illumination (10, 96). The movement of the PP is dependent on the photoreceptor membrane potential (101) and can be modulated by serotonin, which is apparently also contained within retinular cells themselves (102, 103). In the ditch-fencing crayfish *Faxonella clypeata,* however, the DP migration is autonomous under both constant dark and light conditions for more than a week (104). The reflecting pigment in the secondary pigment cells or tapetal cells (105) (Figure 1) does not migrate. Interestingly, the diel migrations of the screening pigments also appear to shift the spectral sensitivity of the eyes from more ultraviolet-sensitive during dark-adaptation towards more red-sensitive during light-adaptation in crayfish (106-108) and in an isopod (*Ligia exotica*) (109, 110). In crayfish, this UV-sensitivity develops only after the first 4 weeks of age of a juvenile; it reaches a maximum after about twelve weeks but decreases slightly towards the adult stage, which, surprisingly, has its maximum eye sensitivity in green light, especially so in the dark-adapted state (108).

Early investigations on pigment migration in isolated eyes have shown DP movements towards the light-adapted state even after dark adaptation (97). While light input is thought to directly control pigment distribution of the PP within the photoreceptors (10), ground breaking early studies on effects of injections of crude extracts into the prawn *Palaemonetes* (111, 112) and into crayfish (113) have shown that DP movement is regulated by the eyestalk (ganglia) and not by ommatidial cell types themselves. This can easily be tested by measuring the so-called eye-glow (or deep pseudopupil) area, which is produced when light is reflected from the tapetal cells, i.e. when DP (and PP) are in dark-adapted positions (Figure 1). The size of the glow

area reflects preferably the DP positions, and the eye glow changes with an endogenous circadian rhythm that can be phase-shifted (97, 104, 114-116). Injections of eyestalk extracts into dark-adapted animals at different circadian times elicited glow-reducing effects largely dependent upon the injection time of the day, and sinus gland-extirpated animals lose their pseudopupil circadian rhythm towards low amplitude ultradian (2-11hrs) rhythms. It was, therefore, concluded that pigment-dispersing neurosecretions diurnally released from the sinus gland are responsible for the retinal circadian pigment movements (116). In fact, two different neuropeptides with antagonistic effects were isolated from eyestalk extracts and shown to be predominantly involved, termed red-pigment concentrating hormone (RPCH) and pigment-dispersing hormone (PDH). Their eyestalk sources, release sites in the sinus gland, and actions are dealt with in more detail in the following sections (3.2.1. and 3.2.3.). Further details about circadian eye pigment movements and their still largely unclear intracellular mechanisms of cytoplasmic granule movements are provided by comprehensive reviews (95, 117).

3.1.2. Receptor potential changes of retinular cells in the electroretinogram (ERG)

The sensitivity of the eye shows circadian changes associated with the phases of light and darkness. Electroretinograms (ERG) represent light-evoked summed potentials exclusively of retinula cells (118) that can easily be recorded over long periods of time. However, when the eye is exposed to constant or defined light pulses, the ERG amplitude is modulated in a circadian rhythm. This has been shown for several crustaceans. The circadian behaviour of the ERG also persists under conditions of constant darkness (119), and ERG amplitudes are usually higher during the "expected night" than during the "expected day" (85, 120). This indicates that the intricate adaptations of the retinular cells to low light are driven by an endogenous oscillator. However, it is not clear how this adaptive regulation is achieved at the molecular level and to which extent the ERG rhythms are controlled by neuronal mediators, e.g. neuropeptides and/or neurotransmitters.

There is evidence that the rhythm of photoreceptor sensitivity is autonomously regulated by the retina itself, as has been suggested by work *in vitro* on isolated eyes (retina and lamina ganglionaris; Figure 2A) (23, 119). Whilst intact photoreceptor axons are required for ERG responses, the lamina ganglionaris or other optic ganglia supposedly do not contribute to the recorded ERG (85, 118). Entrainment with reversed light phases for a week also reverses the circadian rhythmicity *in vitro* (23). Lesion experiments in intact animals have shown that ERG oscillations persist after sectioning of the brain hemiganglia, of the protocerebrum from the deutocerebrum, and of the brain from the remaining nervous system. However, if split-brain resection is done parasagittally, merely the non-operated side remains functional (82). Interestingly, resection of distinct parts of the brain (ipsilateral globuli cells, accessory and olfactory lobes) increases the length of the nocturnal ERG phase, resulting in marked ultrastructural changes such as degranulation in certain sinus gland axon profiles and size reduction of the gland (75, 84). Brain resection or separation of protocerebral hemiganglia leads to desynchronisation of ERG (and locomotory) phases, indicating a neuronal connectivity and synchronisation between the two visual systems (Figure 2B), the phase of which is also largely temperature-insensitive. This indicates that two separate but phase-locked oscillators exist in each hemiganglion that affect the ERG (Figure 2B), and that these have likely a nervous connection to the sinus gland (75, 78, 84, 121). The presence of the sinus gland is actually indispensible for the circadian phase relationships of the ERG rhythms between both eyes, especially prominent under DD conditions (122).

3.2. Eyestalk systems and mediators of circadian rhythmicity

The decapod crustacean eyestalk contains visual ganglia or neuropils, i.e. the lamina ganglionaris, the medulla externa, the medulla interna (= lobula), and finally the medulla terminalis, which is in fact part of the lateral protocerebrum of the brain (123-125). The eyestalk proved to be an important source of neuropeptides and small modulators such as serotonin and melatonin involved in circadian regulation, that affect rhythms of locomotion, pigment distribution, and eye sensitivity (10, 20, 21, 95). Most of the neurosecretion within the eyestalk ganglia that is of relevance for circadian rhythmicity appears to be attributed to the well-established neurosecretory X-organ-sinus gland system (XOSG) (126) (e.g. somata cluster A in *P. clarkii*; 123). The X-organ somata are located at the anterior lateral cortex of the medulla terminalis proximal to the hemiellipsoid body, and the sinus gland, which is usually situated adjacent to the medial edge between the medullae externa and interna, is formed by axonal endings of XOSG and other neurons that abut upon lacunae around the large ophthalmic artery. From these neurohaemal XOSG terminals several identified neuropeptides are released into the haemolymph, such as red pigment concentrating hormone (RPCH), crustacean hyperglycaemic hormones (CHHs), moult-inhibiting hormone (MIH), vitellogenesis/gonad-inhibiting hormone (VIH/GIH), and mandibular organ-inhibiting hormone (MOIH) (127-133). However, the sinus gland receives additional inputs from other regions of the eyestalk ganglia outside the XOSG and even from the brain (134-136). An important contribution to neurohaemal sinus gland terminals arises from pigment-dispersing hormone-immunoreactive (ir) neurons. These, however, have locations, projection patterns and input regions very different from those of the XOSG neurons (137-141; see also 3.2.3.). Expression and release of neuropeptides from the XOSG, in particular RPCH and CHHs, follow a circadian rhythm as the XOSG system can be driven by retinal illumination (142).

3.2.1. Red pigment concentrating hormone (RPCH)

The octapeptide RPCH (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2) was the first invertebrate neuropeptide fully structurally elucidated from eyestalk extracts of *Pandalus borealis* (143). It shares common features with the insect adipokinetic hormone (AKH) peptide family, viz. a N-terminal pyroglutamate, an aromatic residue at the fourth and a tryptophan at the eighth position (144). This neuropeptide appears to be invariably conserved in decapod crustaceans (145) and has long been known to act antagonistically to PDH by concentrating DP within the retinal pigment cells of the ommatidia (21, 95). Albeit not affecting

the positions of the PP within the photoreceptors, RPCH enhances the ERG amplitude, i.e. the light sensitivity of the eye, especially in light-adapted animals with an effectivity five-fold over dark-adapted animals irrespective of DP positions during circadian acrophases in the early night (146, 147). The clearly dose-dependent effects of RPCH on the ERG, the DP, and even on tegumentary chromatophores were readily blocked by injections of a specific antiserum against an Y^1-RPCH analogue (147). RPCH concentrates integumentary chromatophores, again acting antagonistically to PDH (95), and is well known for its strong modulatory activity in the stomatogastric system of *Cancer borealis,* in which it leads to reversible coupling of separate previously uncoupled identified neuronal oscillators (148, 149). Furthermore, it has profound effects on circuitries controlling the crayfish swimmeret rhythm. Although in the latter rhythm RPCH lengthens both the period and the duration of bursts of action potentials, it does not alter the phase relationships (150).

In crayfish and crabs, but not in isopods, RPCH is strongly expressed in the XOSG, and several RPCH-ir interneurons can be distinguished in the optic ganglia and ventral nerve cord (VNC), some with axons running through the protocerebral tract (= optic nerve) (127, 151, 152). In decapods, up to twenty XOSG neurons project to the sinus gland, which contains the highest amounts of identified RPCH, and from which the peptide is preferably released at night (20, 127, 151). Furthermore, a distinct group of 8-12 RPCH-neurosecretory neurons next to the lateral medulla externa of crayfish have been traced to extensive branches within this neuropil and in the lamina ganglionaris. Some of their fibres even extend beyond the lamina below the retina, but none of them enters the sinus gland (153). The latter medulla-associated interneurons have endings next to the terminals of the photoreceptors in the lamina. These neurons closely resemble previously identified neurosecretory neurons that exhibit tonic activity in darkness and are inhibited under illumination. It has been argued that these neurons are involved in the feedback control of dark adaptation and/or circadian changes in visual sensitivity (154). Thus, RPCH appears to modulate DP and photoreceptors of the retina via two pathways, via the circulation from the XOSG and via specific interneurons.

RPCH contents in the eyestalks of the fiddler crab *Uca pugilator* (155) and in two crayfish species change with a circadian rhythm both at the levels of the peptide (*U. pugilator*, *P. clarkii*; 156) and the mRNA (crayfish *Cherax quadricarinatus;* 157). The peptide concentrations rise at the end of the light phase and are highest at the beginning of the dark phase followed by a marked decrease during the dark phase to a trough in the morning (*P. clarkii*), whilst in *C. quadricarinatus* the mRNA contents show an acrophase about two hours earlier. The rhythm of RPCH-concentrations is maintained in isolated eyestalks when kept for seven days under LD conditions, although the concentration differences are smaller and the night peaks broader and less well defined than in intact animals (156). These oscillations are indeed autonomous, because they persist under continuous illumination and in constant darkness. Injections of anti-RPCH-serum into the animals caused only slight reductions in the amplitude of the ERG rhythm and only a small lengthening of the circadian period (147). In the crayfish *C. quadricarinatus,* the mRNA levels clearly show circadian changes at two-fold higher levels in constant darkness but with additional bimodal ultradian components (157). Whether the mentioned RPCH-ir axon terminals at the base of the retina in fact contain and/or are under control of co-localised *period*-gene products, as has been suggested (23, 157) (see also 4.3.3.), deserves further exploration.

RPCH is, therefore, considered a key molecule in the regulation of the circadian systems of the eye. However, since it does not affect the phase of circadian rhythms, it may act on effectors of rhythmicity only but not on the pacemaker itself (10, 21, 147).

3.2.2. Crustacean hyperglycaemic hormone (CHH)

The CHH peptide subfamily comprises true hyperglycaemic hormones that share sequence similarities with members of a peptide subfamily comprising MIH, VIH and MOIH, especially with regard to the positions of cysteines and the disulphide-bridges. However, CHHs are distinguished when looking at the simpler gene and precursor structures of the latter subfamily peptides, which e.g. lack so-called precursor-related peptides (131, 158, 159). CHH subfamily peptides usually consist of 72-73 amino acids (aa), show a sequence similarity exceeding 55% between species, and have at least eight different established physiological functions (131, 160-162). CHHs are produced by XOSG perikarya distinct from those of RPCH neurons (163). These are usually located at the cortex of the medulla terminalis neuropile and are transported to axon terminals in the neurohaemal sinus gland (163). Here, the peptides are released directly into lacunae of the ophthalmic artery. CHHs have relatively species-specific bioactivities and occur in multiple isoforms (160, 164). These may be products of up to ten (in shrimps) slightly different genes, but only isoforms originating from alternative splicing processes of *chh*-genes are differentially distributed in sinus glands and pericardial organs (POs) (165-167). The latter organs contain and release an alternative splice form of CHH very different from that produced in the XOSG, the so-called POCHH. This peptide is derived from the same *chh*-gene but expressed only in peripheral intrinsic cells of the POs in crabs (165, 168) or in so-called thoracic root-cells in lobsters that project to release sites in the POs (169-171). None of the identified POCHHs has a hyperglycaemic effect, and nothing is known about whether they are released rhythmically or do have any function in circadian control.

Carbohydrate metabolism is under circadian regulation with even circatidal components. This is detected as rise and fall in sugar levels in the haemolymph, which depend upon CHH that itself undergoes circadian changes in concentration (163, 172-176) with the possible exception of deep-sea dwelling crustaceans, since rhythmic diurnal changes in blood sugar are lacking in Norway lobsters, *Nephrops norvegicus* (177). The centre for the control of oscillating CHH levels has been located in the medulla terminalis of crayfish by ablation experiments, and the haemolymph sugar rhythm can be manipulated by shifting the

light/dark phase or regionally applied constant darkness: the amplitude diminishes after painting over the eyestalks or totally disappears after painting over eyestalks *and* rostral cephalothorax, and a slow adaptation with a temporary loss of synchrony occurs in constant darkness. The rhythm is also disrupted by severance of the protocerebral tract connection to the brain (77). Thus, it was proven that the physiological changes in blood sugar levels follow a conspicuous endogenous circadian rhythm entrained by light and dependent upon brain inputs: prior to the onset of the dark phase, CHH is transported to the sinus gland concomitantly with an increase in XO-perikaryal CHH production. CHH is then released into the haemolymph around the onset of the dark phase (103, 163, 174). This leads to hyperglycaemia about two hours later with rising glucose levels lasting for up to 7 hours, i.e. almost synchronously with phases of high locomotory activity. A second, normally smaller and shorter secretory burst of CHH release occurs before the onset of light, again leading to hyperglycaemia with a delay of a few hours (163, 174). In the crayfish *P. clarkii*, the CHH contents of eyestalk ganglia show a bimodal circadian rhythm (peaks at 04:00hr and 16-20:00hr in LD, but at 04:00hr only in DD) and appear not to precede but to almost coincide with bimodally increasing haemolymph glucose levels (176). Interestingly, this CHH release pattern also coincides well with circadian rhythms of the XOSG neurons, which show highest bursting activity in the evening and during the early night but low level tonic activity during morning and early afternoon hours (Figure 3A) (21, 178). When recorded extracellularly from the sinus gland or the XOSG tract, or intracellularly from the perikarya, crayfish XOSG neurons show diurnal spontaneous firing predominantly between dusk and midnight and low activity during the day, whilst other sinus gland innervating neurons (likely PDH-neurons; see below) had activity peaks during the day and low activity at night (179). Diurnally active CHH- (and RPCH-) neurons receive inputs via dendrites branching off the XOSG axon tract (e.g. within the central glomeruli or neuropiles III and VII of the medulla terminalis in *P. clarkii;* 123) from input terminals of neurons releasing 5HT, biogenic amines (dopamine, octopamine) and enkephalins (see also 3.2.6.) and further inputs from areas next to the olfactory-globular tract (OGT), the likely PDH-ir (see below) optic-globular tract (OPT-GT) which is connected to the so-called diamedullary neuropil adjacent to the medulla and lobula (terminology according to (123), apart from other inputs originating in the brain (77). As judged from pharmacological experiments, serotonin (103, 180, 181), GABA (182), dopamine (183), and several other components (see 178) likely regulate CHH rhythmicity. Furthermore, axonal terminals of brain extraretinal photoreceptors contacting serotonin-ir fibres within the protocerebral bridge of the brain (184, 185) may convey circadian information via electron-microscopically proven serotonergic inputs to the XOSG (186). Synchronous bursting patterns of the XOSG neurons are further aided by the strong electrical coupling of most of these neurons (187, 188). However, varied experimental approaches in different species led to controversial ideas about the regulatory roles of 5HT, biogenic amines dopamine and octopamine and enkephalins in the functioning of the XOSG (review: 162).

In crayfish and crabs, the CHH-producing XOSG neurosecretory neurons are present shortly before and after hatching and increase in number during the post-larval phases (189, 190). A novel source for CHH-like substances has been discovered in the tapetal cells of the ommatidia (Figure 1) of post-embryonic (second stage: PO2) and juvenile crayfish *P. clarkii* (103). CHH expression in these cells has hitherto never been demonstrated in the adult stage of any crayfish species, but evidence from CHH release studies using cultured cells in reverse haemolytic plaque assays (181) and most recent Western blot studies in adult *P. clarkii* strongly suggests the presence of a CHH in retina extracts that is similar in molecular weight to those of the two known isoforms of XOSG CHH in this species (176, 191). These cells and the XOSG cells exhibit a clear-cut circadian rhythmicity, as do the supposedly 5HT-ir retinular cells (103). These latter cells in PO2 stages are a few hours earlier in acrophase than the CHH-ir tapetal and XOSG cells, which is suggestive of CHH being controlled by 5HT stimuli in the afternoon. However, this phenomenon appears timely reversed in juveniles, which may indicate that there is no functional connection between these systems (Figure 3B-E) (22, 162, 181). Daily and circadian changes in relative CHH abundance in the retina and the eyestalk ganglia are in fact negatively correlated and appear to be under different dual metabolic feedback control by circulating glucose and lactate (176). Whether other newly discovered CHH-ir cells in abdominal segments of shore crab embryos (190) exhibit circadian rhythmicity has not yet been investigated. Interestingly, however, timely surges of contents of CHH in these cells and likely their expression and release patterns appear closely related to larval eclosion and water uptake necessary for eggshell rupture and well known larval hatching rhythms (31, 190, 192). In juvenile crayfish, a pacemaker system for circadian locomotory behaviour is likely present already from the moment of eclosion (26). However, notably, for hatching larvae released from intertidal or DVM-performing crabs circatidal rhythm components are certainly more important (193-195).

3.2.3. Pigment dispersing hormone (PDH)

Pigment dispersing hormone (PDH) is a neurohormone regulating (1) the dispersion of pigment granules in the distal pigment cells of the retina, therefore previously being called light-adapting or distal retinal pigment hormone (LAH = DRPH = PDH; 196, 197), (2) the pigment dispersion in integumental chromatophores (21, 95, 198), and (3) the physiological responses and sensitivity of the compound eye directly (146, 199). PDH does not affect granule movements of the PP in retinular photoreceptive cells, but the dispersion of pigments within the primary pigment cells causes additional (protective) shielding of photoreceptors and the dioptric apparatus during the light phase and thereby increases visual acuity. Much less is known about the numerous small-field and wide-field PDH-ir interneurons that occur in all eyestalk ganglia, brains and VNCs of several crustaceans; in fact, for none of these interneurons has any function been identified up to now.

All PDHs are octadecapeptides, and by sequence comparison one can distinguish two types of PDHs, alpha- and beta-PDHs, respectively (95). The first discovered as a light-adapting hormone was an alpha-PDH of the prawn *Pandalus borealis* (200, 201) but a somewhat larger diversity is known for the so-called beta-PDHs (202-205); notably, in the shrimp *Pandalus jordani,* two isoforms of alpha-PDH and one beta-PDH (205), and up to three isoforms of a beta-PDH have been

detected in the crabs *Callinectes sapidus* (206), *Cancer borealis* (207, 208) and *Cancer productus* (140), the shrimps *Litopenaeus japonicus* (209) and *Litopenaeus vannamei* (210), the crayfish *Orconectes limosus* (211), and the lobster *H. americanus* (207). In the CNS of diverse crustacean species, multiple neurosecretory and interneurons express PDHs (137, 138, 140, 141, 152), but innervation of the sinus gland as known e.g. in the crabs *C. maenas and C. productus,* the crayfish *O. limosus* and the lobster *H. americanus* is restricted to 3-4 neurons, the origin of which is definitely not the medulla terminalis XOSG but perikarya between the medulla interna (lobula) and the medulla terminalis of the eyestalk (Figure 4A,B). These are neurons with T-shaped branches that, together with several other PDH-ir cell groups, form a prominent tract system predominantly in the medulla terminalis, which also contains axons connecting to the brain and to the contralateral eyestalks (arrows in Figure 4A,B) (138, 140, 141). Since the origins of up to three different identified PDH-isoforms in sinus glands are still not clear (140, 211), this problem presents a further complication in the analysis of all these groups of PDH-neurons and for the analysis of possible physiological effects of these hormonally released PDH-isoforms. There is also evidence that these PDH-isoforms are differentially distributed in visual eyestalk ganglia (e.g. the lamina ganglionaris; 140) that are possibly involved in circadian regulation, which but has yet to be demonstrated experimentally. The crustacean PDH-neuron systems are, thus, much more complex than similar neurons in insects that likely express only one single isoform of the orthologous insect pigment dispersing factor PDF (95). For instance, in *Drosophila*, PDF is a known clock output factor that is expressed in the well-established so-called large and small ventral lateral neurons (4 l-LN_vs and 4 s-LN_vs) next to the medullae (212-214), which are homologues of the so-called PDFMe neurons of several orthopteroid insects (215, 216). Putative homologies of the insect PDFMe/LN_v-type and crustacean lateral medulla-associated (crab C-type) neurons make PDFs and PDHs potential key circadian molecules and PDH likely a suitable marker for clock neurons in crustaceans (see 4.2., cp. 141).

Initial eyestalk ablation experiments had shown that the eyestalk endocrine system is essential for distal retinal pigment movements (114, 196, 197). PDH even causes pigment dispersion in isolated retinae of the crayfish *O. limosus* (146) and *P. clarkii* (199). It was, therefore, presumed that the sinus gland system is the only source of PDH in circadian regulatory activity (21, 116, 217). Circadian variations have been found in the chromatophorotropic potencies and contents of various eyestalk visual ganglia of the prawn *Palaemon paucidens*, crayfish *P. clarkii* and the crab *U. pugilator* (116, 155, 218-220). In the crab *Chasmagnathus granulata*, endogenous circadian rhythms of pigment migration exist, with pigments being more dispersed during the day than during the night, and seem to be largely dependent on endogenous beta-PDH release from eyestalk sources. Interestingly, the high sensitivity of melanophores to beta-PDH as displayed *in vitro* (EC_{50}=0.18µM) is higher during the day than during the night (198). Findings of certain axon profiles in the sinus gland of *P. paucidens* (221) being depleted in their granule content by prolonged illumination and enriched in dark-adapted animals were later corroborated by the observations in *C. maenas* that an identified PDH-ir granule type accumulates in distinct sinus gland terminals when crabs were kept for at least three days in constant darkness (137). Recently, however, PDH effects were tested on isolated eyestalks, not just retinae, therefore, including the sinus gland. ERG measurements after perfusion of isolated eyestalks showed significant increases in receptor potential duration but decreases in receptor potential amplitude and retinal pseudopupil area. However, since retinae were not examined after separation from the medullae of the eyestalk, PDH cannot be considered an independent clock component of the retina. Nevertheless, PDH injection in intact animals caused advances of the ERG phase. Thus, this action of the peptide meets well with the criteria for setting the phase of the crayfish ERG (199). It also compares well with the functioning of PDFs in *Drosophila* and other insects, which regulate or set the phase as well as the period of the circadian rhythm (214, 222, 223). In particular, phase locking between eyestalk ERG rhythms has been attributed to PDH being released from the sinus gland of one light-stimulated eye (122, 224). However, as an alternative pathway, large contralateral projections via the protocerebrum exist between eyestalk ganglia of both sides; these large projections are very similar to those of insect PDF-neurons (225-228) and arise from the large PDH-tracts in the medullae terminales of eyestalks formed by PDH-ir C-type cells in decapods (138, 141, 229) (arrows in Figure 4A,B) and PGR1- and PGR3-type medulla-associated PDH-ir neurons in the isopod *Oniscus asellus* (152).

How are the PDH effects in crustaceans regulated? There is only little evidence that identified PDH- (or a DRPH-) containing neuronal elements themselves show circadian oscillations in crustaceans (219), except for some recently discovered circadian variations in the numbers of a distinct medulla-associated PDH-ir neuron type in the water flea *Daphnia magna* (230). It was, therefore, suggested that PDH-neurons are regulated by neurotransmitters, which in turn could be appearing rhythmically. Serotonin as one candidate had been shown to disperse black and red pigments in fiddler crabs with a circadian rhythmicity (155, 219, 231-233) (see also 3.2.4.). Eyestalk-less animals even retain a circadian chromatophore and locomotory rhythmicity, which demanded the existence of extra-retinal photoreceptors (20, 234, 235) that regulate rhythmicity of neurosecretory chromatophorotropins in crustaceans. Most recently, such brain photoreceptors (BPRs) previously known to exist in the crayfish *C. destructor* (185) were indeed shown to be clearly responsible for circadian locomotory rhythmicity even in retina-ablated, destalked and caudal photoreceptor (CPR)-deafferentiated crayfish. Most interestingly, these authors found evidence that the BPRs are likely contacting directly the large wide-field ascending and decending PDH-interneurons in the brain that may be critical neurons in driving the locomotory rhythms (17) (cp. Figure 4A,B). Whether BPR terminals also contact the contralaterally projecting PDH-ir eyestalk neurons has not yet been established.

3.2.4. Serotonin

Serotonin (5-hydroxytryptamin, 5-HT) is a common neurotransmitter in invertebrates and vertebrates; it is synthesised from the amino acid L-tryptophan via the rate-limiting enzyme tryptophan-hydroxylase to 5-hydroxytryptophan that is then

decarboxylated by an aromatic amino acid decarboxylase. The occurrence of 5-HT in many distinct neurones with in the entire CNS has been described for several crustacean species, including lobster, prawn and crayfish (184, 236-239). A few large neurosecretory VNC-neurons are of special importance for the control of aggressive behaviour (240-242), which in an unnamed species of marbled crayfish *Procambarus spec.* was recently shown to display circadian rhythmicity, although a dependence upon 5-HT has not yet been analysed in this species (243). Three or more 5-HT receptors are present in the crustacean CNS (244, 245), but only two, 5-HTR_{1crust} and 5-HTR_{2_Pro} (or 5-HTR_{1alpha} and 5-HTR_{2beta}), have been analysed in more detail and localised in crayfish CNS structures (246, 247).

The participation of 5-HT in circadian regulation in crustaceans is well established, and both developmental and daily rhythms have been described in crayfish. 5-HT is already detectable during post-embryonic development in the crayfish retina, eyestalk and brain, and displays circadian fluctuations in 5-HT-concentrations in each tissue, linking the 5-HT system to a pacemaker system (103, 155, 248). In the lobster *H. americanus*, light-entrainable circadian changes in 5-HT concentrations have been observed in both the central brain and the eyestalk (249). Changes in the circadian rhythm of 5-HT (and likely its rate-limiting enzyme tryptophan-hydroxylase; 250) during crayfish development according to a 9-12 hr rhythm have been interpreted as (i) being superimposed upon the basic circadian rhythm (248) or (ii) representing an example for an initially ultradian-like rhythmicity of, e.g., the ERG and retinal shielding pigment movements (15min to 4hrs), that about 30 days later is being replaced by circadian rhythmicity as has been shown for first instar crayfish *P. clarkii* (22).

Enhancing effects of 5-HT on the amplitude of the ERG have been determined after 5-HT injection into whole animals and 5-HT applications onto isolated eyestalks or retinae. 5-HT affects the positioning of the PP in the retinal photoreceptors, which becomes relocated to the position in the dark-adapted state, and increases the conductance of the photoreceptor membranes (251). These modulatory effects, however, may not only be caused by the changing 5-HT concentrations, but depend on circadian changes in concentrations observed for one of the two established 5-HT-receptor types, 5-HTR_{1Crust}. The concentration of 5-HTR_{1crust} in the retina-lamina ganglionaris system is lowest at the end of the dark phase, and reaches its highest concentration at the end of the light phase (252). A similar pattern occurs in both isolated retinae and retina-lamina ganglionaris-complexes, but the isolated retina shows a slight phase advance. This difference may be caused by the higher receptor concentration in the lamina (252). This neuropil is extensively innervated by 5-HT interneurons (184, 253) that form here electron microscopically identified terminals in close apposition to axons of retinular photoreceptor cells, the sensitivity of which is known to be strongly modulated by 5-HT (251). This role of 5-HT has even recently found support by the discovery of pronounced changes in the 5-HT-expression preferably of lamina-innervating neurons that were closely related to artificially elicited near-natural light/dark-phase-transitions in cave-dwelling diel horizontally migrating mysid crustaceans (49).

Receptor levels in the crayfish retina and the 5-HT levels in the adult eyestalk occur in similar phase (248), but a reversed phase is seen for 5-HTR receptor fluctuations in the medulla terminalis (252). This finding is particularly puzzling since the established effect of 5-HT-induced CHH release in the eyestalk leading to increased haemolymph glucose levels (103, 180, 181) requires the activation of pharmacologically characterised 5-HTR_1- and 5-HTR_2-like receptors that can be blocked by the 5-HT-antagonist methysergide (254, 255). It is still not clear whether this effect is brought about by 5-HT from neuronal sources in the brain or in the eyestalk, since the eyestalk contains a large number of 5-HT-ir neurons, which can release 5-HT in a paracrine manner depending upon activity (256). 5-HT presumably acts on many 5-HTR_{1crust}-expressing cells in the eyestalk (253). It appears to be important together with the XOSG for the control of circadian rhythms of retinal sensitivity to light, and likely so already during development (103, 248). The 5-HTR_{1crust}-receptor may, thus, maintain the circadian rhythm in the adult ERG via interneuronal and paracrine modulation of eyestalk neurons by 5-HT.

3.2.5. Melatonin

Melatonin is a highly conserved molecule universally present in eukaryotes and is an important factor in setting of the mammalian circadian clock responsible for the timing of sleep-wake-cycles (257, 258). It always derives in a two-step synthesis from 5-HT being acetylated by the rate-limiting enzyme arylakylamine N-acetyl-transferase (AA-NAT) to N-acetyl-serotonin (NAS) that is then O-methylated by hydroxyindole-O-methyl-transferase (HIOMT) to melatonin.

The presence of serotonin has been investigated in several malacostracan crustaceans, including lobster and crayfish, and documented especially in the visual system (184, 236-238). However, it cannot be excluded that many 5-HT-ir neurons, in addition, express AA-NAT and HIOMT and produce NAS and finally melatonin, respectively, because they had just been immunostained only for their 5-HT precursor. Therefore, it is certainly decisive to identify melatonin in extracts of crustacean nervous systems as has been done first in brains of crayfish and prawns by use of radioimmunoassay (RIA) combined with thin-layer chromatography (259) or later in eyestalks of crabs *U. pugilator* by RIA combined with high performance liquid chromatography (260). Even in the haemolymph of *N. norvegicus* melatonin was recently identified by liquid chromatography combined with mass spectrometry (LCMS) (261). In crabs and Norway lobsters, melatonin contents clearly showed diurnal variations (260, 261). In crabs, melatonin even increased the rate of limb regeneration (260). The other important marker indicating the presence of melatonin is AA-NAT, which has been detected in *P. clarkii* by a specific radioactive substrate reaction using ^{3}H-acetyl-CoA and 5-HT (262). However, in the brain of another crayfish, *Pacifastacus leniusculus,* only AA-NAT and its product NAS were found, surprisingly no trace of melatonin or the 5-HT-metabolite 5-hydroxyindole acetic acid (263).

In the giant freshwater prawn *Macrobrachium rosenbergii*, AA-NAT contents in the optic lobe increase under continuous light conditions but do not significantly change with a circadian rhythm in LD, whereas melatonin levels show a peak during the day (15:00hr) and a nadir during the night (24:00hr) (264, 265). In *P. clarkii*, levels of AA-NAT and melatonin change with a circadian rhythm, and the highest levels occur at the end of the light phase (262, 266). In the crab *Uca pugilator*, similarly the acrophase of eyestalk melatonin contents lies in the middle of the light phase (13:00hr), whereas an acrophase of AA-NAT contents occurs during the dark phase in the late night (04:00hr). In constant darkness, there are even two melatonin acrophases, one in the middle of the subjective day (13:00hr) and another in the late night (04:00hr) concurrent with transient increases in AA-NAT contents. In constant light, there is only an acrophase of AA-NAT during the subjective day, but a long acrophase of melatonin (ca. 20hr long) with apparently higher overall levels than during LD and DD (267). In the crab *Neohelice granulata*, melatonin contents of the optic lobes show two diel peaks of comparable values in the middle of both the light and the dark phases. This diurnal pattern, however, persists only in constant darkness, not under continuous illumination (268). Physiological studies on effects of melatonin in crustaceans are still scarce but apparently coming of age, although the exact cellular (neuronal) sources of melatonin are still unclear. Melatonin is now known to play a role in crustacean antioxidant defence systems and locomotor muscle control in the crab *N. granulata* (175, 269). Possible melatonin effects on crayfish antioxidant circadian system still wait to be tested. However, in the two species *P. clarkii* and *Procambarus digueti*, the antioxidant systems are known to be very robust in their diel changes (270) and entrain to light phases (271). Especially the glutathione-system in the eyestalk and the brain of *P. clarkii* exhibits clear-cut circadian rhythmicity (272, 273).

With regard to circadian effects in crayfish, melatonin was shown to modulate the ERG amplitude in a dose-dependent manner and depending on the circadian time. Melatonin forces an increase in the ERG amplitude and, thus, has an effect antagonistic to that of PDH. Repeated injections of melatonin synchronize the phase of the ERG (274), but also single doses affect the ERG phase towards an advance or delay depending on the time of injection. Increases in amplitude of the ERG are larger during the subjective day than during the night upon 2hr-interval-injections during a 24hr cycle. This effect is likely mediated via MT_2-like melatonin receptors, as supported by pharmacological application of selective agonist (8-M-PDOT) and antagonist (DH97) (275). Furthermore, melatonin injections can cause reversals of circadian locomotory activity and muscular glucose/lactate cycles depending on the season. Electrophysiology of motoneurons suggested that these effects of melatonin are brought about by neuromodulation, i.e. enhanced synaptic transmission at the neuromuscular junctions of the opener muscle or the extensor muscle of the first walking legs (175).

3.2.6. Further factors with possible effects on circadian rhythmicity

Many of the established factors influencing circadian rhythms described before are associated with the eyestalk and contribute to its regulatory functions. In addition, others, especially neuropeptidic factors in circadian control, may well have to be considered in the near future, which is nowadays facilitated by better and faster neuropeptide identifications by LCMS and Maldi-TOF. Interesting but still not identified factors, which were long known from co-localisation studies, are FMRFamide-related peptides (FaRPs). They are co-localised with PDH in many eyestalk and CNS neurons (138, 229) but the primary structures of the FaRPs in these PDH-neurons are hitherto unknown. A further still unidentified factor is the legendary neurodepressing hormone (NDH). Evidence for the existence of this factor has been obtained from studies in crabs *C. maenas,* crayfish *P. bouvieri*, and Norway lobsters *N. norvegicus*. Surgical removal of crab X-organs causing continuous arrhythmic locomotory hyperactivity in crabs, and crayfish eyestalk ganglia extracts and *in vitro* release experiments from sinus glands eliciting inhibition of crayfish XOSG and motor neuron activities in electrophysiological assays suggested that NDH originates from the XOSG. NDH was, furthermore, found implicated in inhibitory effects on several circadian rhythms (6, 126, 276-278). However, even the previously suggested and reasonably analysed small neuropeptide nature of NDH (279) was later totally questioned (280). Nonetheless, it would be interesting to examine whether or not NDH is similar or in fact also part of a large family of proven myoinhibitory and neuroinhibitory allatostatin-A-type neuropeptides in crayfish *O. limosus* (Orcostatins; 281) that occur identically or in many isoforms in several other crustaceans (208, 282-284). Another type of NDH may belong to a family of small inhibitory opioid neuropeptides, the enkephalins, which are known to occur in several neurons and fibre networks of the eyestalk ganglia including the medulla terminalis of lobsters (285), crabs (286-288), and crayfish *O. limosus* (289), and are co-localised with MIH in the crab XOSG-neurons (288). Identified authentic Leu- and Met-enkephalins are known to inhibit the secretory activity of crab and crayfish CHH-neurons and thereby hyperglycaemia, effects that can be antagonised by the opioid-receptor antagonist naloxone (289, 290).

3.3. The caudal photoreceptor of the crayfish terminal abdominal ganglion (CPR).

A neuron known for a long time to be involved in circadian regulation is the caudal photoreceptor (CPR) of crayfish. It is not an ommatidial photoreceptor but an interneuron directly sensitive to light stimuli with transduction processes taking place within the dendrites (291, 292). The CPR is located in anterior lateral positions of the terminal (sixth) abdominal ganglion as a single pair of neurons with large contralateral intraganglionic branchings and ascending projections which merely on that contralateral side extend to terminals next to the antennal and parolfactory lobe neuropils of the brain, on their way giving off short side branches in every VNC neuromere (293); Figure 5. The CPR neurons depolarise upon photic stimulation of the ganglion, but do not adapt over time (291, 292). The neuroanatomy of these cells has been detailed to some extent (291, 293-295), but full morphological details such as final projection sites and terminal connectivities have not been fully resolved. Some

additional interneurons located in more anterior abdominal ganglia have been described as photosensitive, but their neuroanatomy and physiological functions are not understood (291).

The CPR is primarily involved in the control of locomotion, regulating light avoidance and evasion behaviours, and can even respond to tactile stimuli or water currents (292). Ganglionectomy of the caudal ganglion affected the circadian rhythm of both the ERG, by inverting its phase in the case of dark-reared animals, and the locomotory activity, which increased after ganglionectomy but remained locked to the established phase (296). Experimental illumination of the caudal ganglion in intact animals affects both the phase of the ERG rhythm and their locomotor activity, and thus revealed the important functional role of the CPR in the coordination of circadian rhythms (84, 297, 298) (Figure 5). A single illumination of the abdominal ganglion was sufficient to induce phase changes for both locomotory and ERG rhythms, which did not affect the period of the oscillations (84). However, experimental ablation of the CPR did not affect locomotor entrainment, which has helped arguing in favour of entrainment being brought about by an extraretinal light sensor (see 3.4; 16). Illumination of the CPR not only affects the ERG phase but also the ERG receptor potential amplitude and the pseudopupil area (84, 87), thereby demonstrating an intricate functional link between the caudal ganglion and the eye. Thus, the CPR has subtle regulatory functions, which may depend upon the length and intensity of light input.

Extracellular electrophysiological recordings have recently shown that the crayfish CPR itself displays circadian rhythms of spontaneous as well as regularly light-induced action potential discharges with only slightly differing periods (24.7hr vs. 24.25hr) but very different acrophases at night time (21:40hr) or dawn (03:26hr), respectively, in isolated ganglia (12). In addition, ultradian components of ca. 12h period were observed for both rhythms and thought to underlie the circadian rhythms. The spontaneous activity of the CPR in isolated ganglia retains a circadian modulation in constant darkness, with highest firing rates at the expected night. The responses to light pulses of identical intensity also vary with a diel rhythmicity especially with regard to a difference in spike rate of up to one order of magnitude. This has been interpreted as a result of circadian changes in the sensitivity of the CPR. Thus, the CPR shows circadian rhythms in activity as well as in sensitivity, the latter being comparable to the intrinsic rhythmicity characteristic for the retinal ERG (12). It is intriguing that both the light sensitivity and the circadian activity reside in the same oscillator neuron. Although these oscillations in the CPR are indeed indicative of spontaneous endogenous activities, the question remains which relevance this photosensor precisely has in circadian regulation (see 3.5.). It will be interesting to analyse the physiological interactions between the CPR and the ERG in more detail.

Further hints about the importance of circadian CPR activity and its regulation within the caudal ganglion have been obtained by pharmacological manipulations: the addition of 5-HT or one of its agonists (8-OH-DPAT) causes a phase shift in both the spontaneous and photoreactive activities (299). This makes 5-HT a candidate for mediating the entrainment effects, although it is not yet known from where the direct 5-HT inputs to the CPR arise or whether the described 5-HTR_{1A} receptor-immunoreactive cells in the sixth abdominal ganglion (299) indeed include the CPR. However, the overall level of the serotonin receptor 5-HTR_{1A} was shown to display circadian rhythmicity (12), but the levels of 5-HT and 5-HTR_{1A} are not in phase with each other, leaving still open questions about the functional dynamics underlying oscillations of the CPR and its direct response to 5-HT modulation.

3.4. Extraretinal brain photoreceptors

The contribution of photoreceptors located outside the retina to circadian rhythm entrainment has been documented for representatives of several invertebrate taxa (300). In crustaceans, ablation experiments had indicated that photoreceptors other than the retinular cells and the CPR contribute to entrainment of ERG (234) and locomotory rhythms (16), and that entrainment in crayfish lacking retina and lamina ganglionaris persists under monochromatic blue or red light (235, 301). Previously known brain photoreceptors (BPRs) located under the front side of the crayfish cephalothorax (185) have recently been shown to be responsible for the entrainment of locomotory rhythms in a seminal study on the circadian locomotion behaviour of the two crayfish species *C. destructor* and *P. clarkii* (17) (cp. Figure 5).

In *C. destructor*, the BPRs lying on the anterior dorsal surface of the brain consist of clusters of maximally 25 photoreceptive cells. These contain readily visible brownish pigment granules, which greatly facilitate their localisation already *in vivo* in this species. The pigment granules within the BPR-cell clusters in other crayfish species such as *O. limosus*, *P. leniusculus*, and *P. clarkii* are, unfortunately, not as easily visible but recognised only by electron microscopy (302). It is not known whether the pigment granules within BPRs migrate in a similar way, as do those of the PP in the retinular cells (185, 17, 302). BPRs are histamine-ir and express cryptochrome (CRY, see also 4.3.4.), which is presumably the actual photosensitive receptor molecule. The sensory cells of the BPRs project ipsilaterally into specific brain photoreceptor neuropils (BPNs) next to the protocerebral bridge and terminate most likely on PDH-ir and/or 5-HT-ir or tachykinin-like peptide-ir fibres within the BPNs. When ablating retina and CPR thus leaving solely BPRs intact, animals not only maintain their circadian locomotory rhythm even in constant darkness (DD), but also can be entrained to 6 hr phase shifts of LD cycles. However, when more than the retina but entire eyestalks are removed, typical rhythmic responses to lights-on and lights-off are seen with some increased activity in LD, but disappear towards only arrhythmic or continuous activity in DD (17). This provides evidence that the BPRs are necessary and sufficient for light entrainment, but the endogenous rhythm generator likely resides in the eyestalks. Remarkably, the peak locomotor activities after the beginning of the light and dark phases, respectively, correlate with changes in the intensity of PDH-ir fluorescence staining. There are some species-specific differences detectable: in *C. destructor*, both BPR somata and

BPNs show an increase in PDH-ir fluorescence intensity with the beginning of the light phase; in *P. clarkii*, the beginnings of both the light and the dark phases correlate with an increase in PDH-ir fluorescence intensity, which precedes the change of illumination. Thus, these fibre stainabilities most likely represent an ongoing oscillatory neuronal activity, which is potentially involved in the initiation of behavioural rhythmicity already at the level of neuronal organisation directly following this sensory receptor. Interesting targets of BPRs in the BPNs are likely the large PDH-ir descending neurons in crayfish (17, 139) and crabs (E-type; 141), and possibly groups of wide-field eyestalk interneurons such as C-type PDH-neurons in crabs (141; Figure 4A,B) likely connecting both eyestalks via the protocerebral tract and the protocerebral bridge.

3.5. Integration of distributed circadian clock systems and rhythmicities

Persistent open questions in crustacean chronobiology concern the localisation of the circadian oscillators and their possible interaction. From our present knowledge a complex model begins to emerge, which includes three to four pairs of coupled oscillators such as the retina, the eyestalk XOSG, putative brain pacemakers, the terminal abdominal ganglion (CPR) and paired extraretinal photoreceptors in the brain (BPR), with the latter being involved in light-dependent entrainment. The highly complex circadian functions in various crustaceans thus hitherto appear to contradict a single master clock model. The autonomous oscillators in the XOSG, the caudal photoreceptor, and possibly in the brain, interact within different aspects of rhythm control. While the central players of circadian control have been identified, their mechanistic interactions and the full extent of neural and/or hormonal controls remain to be elucidated. With respect to the systems discussed above, previous research has shown a subtle network of interactions, and the interactions known from studies on crayfish (*P. clarkii*, *P. bouvieri*, and *C. destructor*) are summarised in figure 5.

The ERG pattern is arguably the most complex circadian oscillation in terms of known regulatory mechanisms, while it has hitherto not been shown to influence other oscillators. The sinus gland affects ERG rhythmicity, most likely by PDH acting on both the retinal DP and photoreceptors directly (122, 217). The CPR can set the phase of the ERG rhythm upon prolonged abdominal illumination (298) and adjust it to a 24hr rhythm (84). Thus, there is obviously no single master oscillator located outside the retina that governs the circadian rhythmicity of the ERG, but several modulatory pathways originating from different oscillators converge onto the retina and determine the different ERG parameters.

Another tightly regulated phenomenon is crayfish locomotion. The retinal light input plays a major role in affecting locomotion: in the crayfish *P. clarkii*, locomotory activity shows two main peaks closely correlated with the onset and offset of light, respectively. The lights-on-activity peak is initiated by retinal photoreceptors, because animals with ablated retinae lack this activity peak and preferably display the lights-off-activity (16). The period of this activity is supposed to be regulated by the brain, since it is altered after brain resection, while the CPR sets the phase (84). The entrainment of locomotion, however, clearly depends on extraretinal brain photoreceptors (as discussed below in more detail), but there is evidence for a presumed contribution of the sinus gland to entrainment (16).

The XOSG system is itself a proper circadian oscillator (217), yet electrophysiological recordings from single cells do not necessarily show synchronous but differing phases for individual cells, perhaps because subsets are not electrically coupled and express different peptides (179). In crayfish, XOSG outputs affect locomotion (126, 303), ERG amplitude and synchronisation (217), and the retinal pseudopupil (116). However, the neuronal origins of circadian inputs from the brain to the sinus gland as mainly affiliated with PDH or serotonin are still largely unresolved, although such connections have been described neuroanatomically (304, 305) and are most likely established by wide-field PDH-ir neurons of the eyestalk and/or the brain (see 3.2.3. and 3.4.).

The CPR affects (visual) sensory and locomotory rhythms. Ablation of the caudal ganglion showed that the CPR is not necessary for entrainment (16, 234, 296). However, with a typical transitory reaction upon strong light pulses after continuous darkness adaptation (e.g. 2000 lux for 10-30min), the CPR initiates phase shifts in locomotory (296, 303) and ERG rhythms (84, 298). In the cases of its effects on the ERG and the size of the pseudopupil (87), the CPR most likely feeds sensory information into more anteriorly located pacemaker systems via the ascending projections running through the entire VNC into the brain (293), although the postsynaptic interneuronal targets have not been identified (12).

The BPRs, on the other hand, have a clear-cut function in entrainment of locomotory rhythms (17). They relay photic information to BPNs and (indirectly) other brain neuropiles. A closer understanding of which brain (and eyestalk) centres exactly receive this input is necessary to reveal locations of putative central pacemakers and their regulatory roles. Ablation and bisection experiments indicated a role of the protocerebrum, but a distinct clock neuron as demanded by the pacemaker hypothesis has not been identified in the brain. Interaction of the brain with the other oscillators rests, therefore, on circumstantial evidence, but at least a circadian rhythm in spontaneous mass activities has been demonstrated (88). Thus, the origin of circadian rhythmicity in the brain in terms of pacemakers in identified neurons remains elusive. Nevertheless, several studies provided at least evidence for the coupling of ERG rhythms with origin in both eyes via projections through the brain, thus contradicting a single central brain oscillator that would provide synchronising output to both eyes (78, 84, 121). The underlying coupling mechanism may rely on PDH release only after light stimulation of the extraretinal BPR (20, 87) and the CPR (20, 87). However, it remains to be proven whether the large PDH-ir brain interneurons and/or group C-type PDH-ir eyestalk neurons (141) are indeed the prime or the only candidates responsible for this coupling between the brain hemispheres and the eyestalk ganglia (Figure 4A,B).

The discussed interconnections between different oscillators reveal principal organisations of distributed circadian control systems in crayfish. There may be redundant control mechanisms as suggested for circadian locomotor control in *P. clarkii,* which involves several interneurons (86), or in the case of the retina combined with inputs from the sinus gland and the brain, but this issue is so far unresolved and would require experimental co-manipulation of two oscillators to analyse effects on a possible third one. Most applicable up to now appears a network model of distributed interconnected oscillators. This network does not set the rhythm by a single central master clock only but modulates circadian parameters of each oscillator system via phase locking or entrainment. Unfortunately, without deeper knowledge about the neuronal nature and localisation of the central pacemakers and the precise connections between the oscillators, even this network model seems still somewhat premature. An intriguing complementary concept, based initially upon studies on the development of crayfish circadian rhythmicity, derives from the idea that it is the coupling of ultradian rhythms (e.g. of 4hr period) that most likely under the guidance of sinus gland neurosecretions gives rise to circadian rhythms (11, 116), a concept hypothesised similarly for *Drosophila* (306), and later supported by mathematical modelling in crayfish (307-309). The same concept applies to the ultradian action potential rhythms as elements of a circadian rhythm in the CPR (12). For deeper analyses of the highly complex situation presented here, the aim definitely ought to be the identification of circadian pacemakers in terms of identified neurons or neuron groups. This may be facilitated by comparative studies on arthropod clockwork elements as outlined in the following section.

4. COMPARATIVE ASPECTS OF CRUSTACEAN CLOCKS

4.1. Evolution of circadian pacemakers in arthropods

Functional studies on crustacean clocks analysing behavioural activities and experimental ablations of specific neuronal tissues or connections have not yet succeeded in clarifying the precise location and cellular identity of pacemaker elements. A suitable approach may, therefore, be to compare circadian clocks and their structural and molecular components known from insects to identify similar systems in crustaceans (141). Shared molecular and neuronal elements can be expected because of the close phylogenetic relationships between insects and crustaceans, which was recently revealed by several phylogenetic studies by use of numerous clearly detectable homologies in neuronal structures and development (reviews: 93, 310). Analyses of morphological, molecular, and neuronal characters supported a novel taxon including insects and crustaceans, the Pancrustacea or Tetraconata (91, 311-315).

The insect circadian system has been investigated in tremendous detail and allows for comparisons of neuronal and molecular elements of putative pacemaker neurons, which have been located in similar positions and share similarities in projection patterns in the CNS of several insects (cp. 57, 316-318). Notwithstanding the differences that exist between insect species with regard to the distinct cellular locations and distributions of neuronal clock elements and regulatory clock genes or their products in the brain and/or the optic lobe (57, 316-319), commonalities have perhaps best been documented for the locations of PDH/PDF-ir neurons close to the optic lobes and the role of PDF as a clock output factor (57, 316-318). The bearings on homologies and functional similarity of these neurons are discussed below (see 4.2.).

The homology of optic ganglia and the similar locations of clocks in insect optic lobes and crustacean eyestalk ganglia have already been used in an early study as arguments for a possible homology of clocks in both taxa (320). However, homology cannot be derived from crude tissue localisations alone but requires detailed comparisons of the cellular elements of neurons constituting the presumed pacemakers. These considerations have only recently stimulated investigations dedicated to no longer provide crude tissue associations of pacemakers only but to identify true homologues of clock neurons or clock neuron types in both taxa at the cellular and functional level. In addition, since peptide signalling and the occurrence of clock proteins has even been postulated as a conserved feature of all bilaterian clocks (321), the extensive similarities existing with regard to hitherto identified peptides and clock transcription factors between insects and crustaceans will allow to infer and test comparable functional aspects too. This may be even more successful when structural homologies of putatively involved cell types have already been established, although it cannot be excluded that e.g. orthologous clock genes may regulate oscillatory activities in pacemakers that are not homologues by common descent but were recruited independently in divergent evolutionary lineages. In crayfish, for instance, PDH as one known clock output factor is setting the phase of visual sensitivity (199). This function is clearly comparable with that of orthologous insect PDFs, which are well known to coordinate the phase of circadian rhythmicity in several insects (214, 222, 322). Since insect PDF-neurons and other brain neurons are well known to express clock genes such as *period, timeless and cryptochrome* (57-59, 62, 323, 324), and some crustacean neurons were recently found to express similar clock gene products (see 4.3), these neurons should be tested for co-localisations of peptide (or other marker) and clock gene expression, the oscillatory activity of the respective genes and finally for their contributions to clock functions. In the following sections, neuroanatomical and molecular studies are summarised that compare neuronal and molecular characteristics of circadian systems between crustaceans and insects. To identify clock neuron candidates, ultimately circadian changes of clock gene products or clock outputs (neuropeptides and/or neurotransmitters) will have to be demonstrated. These gene products and the expressing tissues or cells can then be linked physiologically to cellular changes and behaviours.

4.2. Putative clock neurons conserved in crustaceans and insects

The central clock of most insects is located in the brain or next to optic ganglia of protocerebral origin (58, 59, 318). Even though the distributed location of circadian functions and the supposed lack of a conspicuous pacemaker in the brain may indicate that crustacean clock structures are significantly different from those in insects, the recent identification of some neurons in lobsters (*H. americanus*) that are homologous to clock neurons in *Drosophila* and other insects rather gives hints to expect strikingly similar organisations in insect and crustacean clocks. These PDH-ir neurons of the lobster are very similar to the s-LN_vs that express the homologous neuropeptide PDF, a clock output of a group of *Drosophila* pacemakers, which controls the morning bout of locomotory activity (60, 213, 214), the coordinative l-LN_vs of *Drosophila* (or optic lobe 2-neurons in *Phormia terraenovae* flies; 325) and the large PDFMe clock neurons of other insects innervating the accessory medulla and the contralateral optic lobes (213, 215, 216, 318).

Classical homology criteria upon which this analysis can build are the position of neuron somata next to the medullae (externa/interna) and the specific qualities of their neuronal connectivities between the visual systems and the brain and their neurochemical characteristics. These considerations define three independent criteria for putative clock neuron homologues in crustaceans. During ontogeny of the lobster, the PDH-ir C-type neurons located next to the medulla and lobula acquire all characters that identify them as potential homologues of these insect PDF-neurons. However, they are organised in three distinct groups (termed C, C` and C``), which makes their individual identification (or exclusion) still somewhat difficult, because either one or several subgroups could likewise be homologous to the insect clock neurons (141). A functional contribution of these neurons to circadian control in the lobster has yet to be demonstrated. As lobsters display circadian locomotory rhythmicity already from the time of hatching, the documented presence of potential PDH-ir clock neurons at this critical developmental time point meets a necessary requirement for considering them as functional in the context of rhythmic control (141). The presence of similar PDH-ir neurons located next to the medulla interna/lobula had also been described in other crustacean species (*C. maenas*, *O. limosus*; 138), *O. asellus* (152), and *C. productus* (140) partly long before possible clock functions of such neurons could actually be assumed. This highlights the wide distribution of very similar specific PDH-ir cell types among crustaceans, and putative clock neurons of this kind may therefore be well conserved among insects and crustaceans. The recent analysis of similar cell types in the branchiopod species *Daphnia magna* and *D. pulex*, which have a highly modified visual system with two separate optic lobe neuropiles within the fused cyclopean eye, adds further weight to such inferences. PDH-ir neurons detected lateral to the medullae of *D. magna* and *D. pulex* are fewer in number (3-6 cells per hemisphere) than in the cases of comparable decapod PDH-ir neurons and very similar to the PDF–containing clock neuron types in *Drosophila* and other insects (230). Nevertheless, a morphological differentiation of distinct PDH-ir neuronal subtypes with exclusively circadian control functions may not occur in all crustacean groups, especially since in most above-mentioned species several additional PDH-ir neurons exist, for which one may not be able to find an insect homologue. Even if clock genes and clock neuronal structures and mediators were very conserved in both taxa, the pacemakers and their circadian functions may still be differing considerably. However, the widespread occurrence among crustacean species of potential pacemaker neurons that similarly express PDH and are located lateral to the medulla allows the promising working hypothesis of such putatively peptidergic clock neurons being conserved irrespective of evolutionary divergence in the Pancrustacea.

When looking at intertidal or subtidal crustaceans we have to take circatidal clocks into consideration. Unfortunately, their neuroanatomy and association with identified chemical mediators is much less studied and only roughly located at the tissue level. In the crab *C. maenas* and the amphipod *Corophium volutator,* evidence for the location of circatidal pacemakers in the eyestalk and brain has been provided based on eyestalk extirpation and selective cooling experiments of the nervous system (74, 326). For the crab *Sesarma hematocheir*, embryonic hatching is tightly controlled in a circatidal manner correlated with nocturnal high tides and is self-sustaining under constant darkness (327). In search for the neuronal control of hatching, deafferentiation experiments have shown that mainly the medulla terminalis of the eyestalk is responsible, as the hatching process and its synchrony were consistently disrupted. Disrupted hatching behaviour clearly indicated timing signals from the medulla terminalis, and thus the location of a pacemaker therein (193). At present, we can only speculate that this circatidal clock relates to known circadian pacemakers, but investigations on its localisation and homology to circadian systems have very promising research prospects.

4.3. Clock genes in crustaceans

4.3.1. Current knowledge about insect clock genes

In *Drosophila*, seven clock genes have been identified as essential for intact circadian rhythmicity: period, timeless, clock, cycle, double-time, shaggy and vrille, as well as cryptochrome (58). The basis of oscillatory pacemaker activity is the oscillatory activity of gene expression (67, 328), and these clock genes form interactive feedback loops regulating their own transcriptional activity, which results in successive expression and repression (55, 329). Clock (CLK) and Cycle (CYC) proteins form heterodimers, which bind to regulatory sequences of *period*- and *timeless*-genes and initiate their expression. Period (PER) and Timeless (TIM) proteins in turn form a heterodimer in association with Shaggy (SGG) and Double-time (DBT). This complex enters the nucleus and binds to CLK-CYC, causing this heterodimer to dissociate from the regulatory sequences and thereby stop PER and TIM expression. Cryptochrome (CRY) can regulate this feedback loop by binding to TIM. Therefore, and induced by illumination, CRY undergoes a conformational change which ultimately leads to the degradation of TIM and the interruption of the dimerisation of PER and TIM. Thus, CRY can adapt the transitional oscillations of PER and TIM interactions to changes in light-dark phases. In a second feedback loop, CLK-CYC dimers regulate expression of Vrille (VRI). An

activational factor (Act) initiates constitutively the expression of CLK, allowing the maintenance of CLK cycling which ultimately integrates the first feedback loop and the continued activation of PER/TIM (319). The ventral lateral (LN_vs) and the dorsal neurons (DNs) of the *Drosophila* clock circuitry all express *period*- and *timeless*-genes (58). Of the seven clock genes identified in insects, only three genes (*clock*, *period* and *cryptochrome*) or their products have up to the present date been detected in crustaceans. They occur in distinct eyestalk neurons or the BPR, but neither in retinal cells nor in the CPR. In search for clock neurons which qualify as pacemakers, not just gene expression profiling of specific neurons is important but also the physiological demonstration of the actual molecular oscillation of transcription, translation and/or phosphorylation of the molecular key players. The present knowledge about crustacean clock genes and their products is scarce with respect to distribution, oscillatory activity, and chronobiological function. Detailed circadian studies may be required for every single clock gene to ensure that cycling of its mRNA or the derived protein(s) is actually functionally necessary.

4.3.2. Crustacean *clock*-gene

In crustaceans, a *clock* gene product clearly homologous to insect Clock (CLK) is so far the first and the only properly identified circadian clock protein. It has been fully sequenced only in the one single case of the precursor for a CLK protein of the prawn *M. rosenbergii* (330), termed *Mar*-Clk. When compared with CLK proteins of insects, *Mar*-CLK shows considerable sequence identity, in fact, highest to *Drosophila* CLK (~ 35%) and particularly high with regard to the functionally important domains, the DNA-binding basic helix-loop-helix (bHLH; more than 64%) and PER-ARNT-SIM (PAS) A and B protein binding domains (more than 44% and more than 80%, respectively). Interestingly, it contains an exceptionally long glutamine-rich region at the C-terminus well known, in insects, as a necessary domain to activate so-called E-boxes of six conserved nucleotides 5`-upstream of *period*- and *timeless*-genes. *Mar*-CLK mRNA is present in all tissues tested (brain, thoracic ganglia, eyestalk, gill, hepatopancreas, ovary, and muscle) and not limited to the brain or CNS. The CLK mRNA is not expressed in a circadian rhythm, neither in thoracic ganglia nor in eyestalk nervous tissue, as studied by semi-quantitative RT-PCR, but possible rhythmicity has not yet been tested at the protein level. Eyestalk-ablated animals show an increase in *clock*-gene expression in the CNS including the brain, although the putative CLK-expressing neurons involved have not yet been identified. This CLK-upregulating effect has been interpreted as being the result of the absence of light input, since intact animals kept in constant darkness conditions show a comparable increase within three days (330). These findings again underscore the importance of the eyestalk in circadian regulation, although an effect of (putatively neuronal) CLK in the eyestalk on the expression of CLK itself or other proteins within the brain, potentially by CLK acting together with Cycle as transcriptional repressor, has yet to be investigated.

4.3.3. Crustacean *period*-gene

The *period*-gene of *Drosophila* is the first clock gene for which the structure was fully determined (331); it forms the core of oscillating gene products. Its product PER is another bHLH/PAS superfamily domain protein, acting as a transcriptional repressor in heterodimers together with TIM, which can bind to regulatory DNA sequences thereby effectively regulating their own expression in a negative feedback loop. This leads to oscillatory activation and repression of transcription of these clock genes. PER is indispensible for proper circadian rhythmicity, and *Drosophila per*-mutants are affected in several biological rhythms including locomotory activity (332) as well as pupal eclosion, larval heart beat rhythm and courtship song intervals (333, 334). The proper identification of PER-like molecules and analyses of their location and biological function in crustaceans would therefore be of great value.

In fact, the first and hitherto only evidence for the existence of a PER-like protein in a crustacean was provided successfully by immunohistochemistry only using an antibody against *Drosophila* PER in the crayfish *P. clarkii*. Immunoreactive PER was detected in distinct structures of the retina and optic ganglia, chiefly in photoreceptors and monopolar neurons of the lamina, as well as in lamina glia (23). However, the authors did not find circadian rhythmicity of *per* expression, and it thus remains to be demonstrated if PER is involved in the regulation of retinal oscillations of any nature. In particular, the expression of PER in several neuronal cell types could, unfortunately, not be linked directly to the autonomous ERG circadian rhythm.

4.3.4. Crustacean *cryptochrome*-gene

Cryptochrome (CRY) was identified first in *Drosophila* as a contributor to circadian control, which is expressed in LN_v neurons (335). CRY is a flavin-adenin-dinucleotide/pterin-containing protein of ca. 60KDa capable of absorbing the light energy from blue light. Upon light stimulation, CRY undergoes conformational changes and autophosphorylation enabling direct interaction with the clock protein TIM, which results in the proteasome-mediated degradation of TIM (336). It, thus, interferes directly with the oscillation of the PER/TIM transcription factors but helps to adjust and actually reset the clock to environmental changes in light/dark-phases via light-driven interactions (337). CRY-mRNA as well as CRY-protein cycle in a circadian pattern, with highest CRY-mRNA levels at onset of light followed by a continuous decrease but rising again during the dark phase, whilst CRY protein was increasing prior to the CRY-mRNA increase in the dark phase (337). In insects other than *Drosophila*, paralogous *cry*-gene products exist (termed CRY-m mammalian like, or CRY2), which do not contribute to photic entrainment but act as transcriptional repressor of CLK/CYC-heterodimers, and, depending upon the species, even two CRY isoforms occur. The ancestral clock of insects was thus supposed to consist of two CRYs (CRY-d = *Drosophila*-like and CRY-m), together with even two similar TIM isoforms (TIM-m and TIM-d) and CYC as the main transcriptional factors (319).

Again in the crayfish *P. clarkii*, a CRY-like molecule has been detected for the first time by means of immunohistochemistry in neurons of the anterior median (AMC) and lateral olfactory cell clusters of the protocerebrum (clusters

6 and 10; 338) and close to the proximal edge of the hemiellipsoid body complex in the medulla terminalis of the optic lobe. In addition, its presence as a single CRY-ir protein only and of a size similar to that of *Drosophila* CRY has been confirmed by Western blotting of tissue extracts (339). Whilst already assumed in the latter study, a clear-cut association of crayfish CRY with the extraretinal BPRs within the AMC cluster 6 has only recently been described for the crayfish *C. destructor* and *P. clarkii* (17) (cp. 3.4.). In the developing crayfish *P. clarkii*, eyestalk and brain tissue concentrations of CRY changed with regular daily rhythms (340). In the adult brain, CRY levels change with a circadian rhythm (but only in the AMC), which was considered suggestive of a possible function of CRY in crayfish circadian control. In eyestalk locations of the adult, apparently no significant circadian changes of CRY do occur (339). However, in the eyestalk of second post-embryonic stages (PO2) under LD conditions, CRY expression oscillates with a bimodal pattern but somewhat in antiphase to that in the brain (Eyestalk: high at 15:00ZT, low at 23:00 ZT; brain: low at 11:00ZT and high at 23:00ZT). In constant darkness, the rhythm runs freely only in the brain. In juveniles, CRY did not show any circadian rhythm in the eyestalk but an obvious rhythm in the brain (340). The discovery of crayfish CRY and its circadian changes during development (i) supports earlier findings on extraretinal effects of blue light on circadian ERG rhythms which are likely driven by CRY as the blue-light-sensitive photopigment and a possible component of the CPR and a putative brain oscillator (235, 298) and (ii) gives hints about a possible developmental plasticity in clock properties e.g. with regard to different period length in PO2 post-embryonic stages vs. juveniles (340). However, the most likely functional role of CRY in controlling circadian rhythmicity resides in its expression in the BPRs, which are the extraretinal photoreceptive elements mainly responsible for the light entrainment of rhythmic locomotion in adult crayfish but show some preference for green rather than blue light (185, 17).

5. PERSPECTIVE

The circadian rhythms of various biological phenomena are well described for several crustacean species, but investigations into the regulatory basis of these behaviours have usually not reached the cellular level of identified neuronal elements of the respective endogenous circadian clocks. Thus, most of our knowledge is merely phenomenological. However, only the deeper analysis of the mechanistic control of clocks lends promise towards a better understanding of the cellular basis of circadian rhythmicity. Crustaceans are a favourable group for neurobiological investigations in the field of comparative chronobiology: there is often a well-documented neurobiology of locomotory behaviours available and easy access to *in vivo* and *in vitro* electrophysiological analyses of identified neurons and nowadays also to their molecular biological manipulations, e.g. by promising RNAi approaches, as has already been shown in the cases of successful neuropeptide knock-downs in XOSG neurons (341, 342).

We finally present here several open questions that can be answered with the currently available information and methods at hand. Solutions to the problems behind these questions will likely provide fundamental insights into the physiology of crustacean circadian clocks:

- What is the primary molecular basis of the pacemaker oscillations? Several molecules are expressed with a circadian rhythm, but it remains to be shown which ones of these are necessary and sufficient for a self-sustaining rhythmicity. Especially comparisons of identified crustacean clock genes with those of insects may show the conservation of functionally important domains, thus, likely indicating the conservation of protein interactions responsible for similar regulatory principles. Investigations on the expression dynamics of clock genes in crustaceans would allow first insights into the functioning of crustacean clocks at the cellular level.
- How do the distinct pacemakers connect, synchronise, and entrain? Where are the synaptic connections? As discussed above, this is a very important yet unresolved topic at the systems level, but needs to be addressed at the cellular level, although significant interspecies differences may be revealed.
- Which is the most basic oscillation of the pacemaker(s)? Ultradian rhythms have been demonstrated in different systems, such as in the cases of the ERG sensory rhythms, the spontaneous CPR activity, and the fluctuations of serotonin and other mediators. It has been hypothesised that circadian rhythms are principally based upon ultradian rhythms, and that amplitudes and phases of their coupling interactions are important for an integrative functioning of distributed pacemakers (343).
- Which function(s) do clock genes similar to those of insects have in crustaceans? With available gene sequences, soon derived from many more genomes of a growing number of crustacean species (344), RNAi approaches, for instance, together with (established) behavioural analyses appear suitable for testing the functions of the clock genes. However, such gene ablation may still be inconclusive, as proteins can be pleiotropic in different clock systems of a given organism, but the analyses of circadian phenomena will show whether redundant control molecules exist that participate in different circadian systems of a given species.
- Which neuropeptides or small signalling molecules are used by the pacemaker(s)? Identities or similarities in primary structures of neuronal signalling molecules known to exist in both insects and crustaceans invite screening for distributions in crustacean CNSs with regard to the locations and possible functions of presumptive pacemaker structures. PDH-ir neurons and their co-transmitters may serve as a suitable starting point in this respect. However, such studies must be combined with analyses of circadian behaviours of the identified cells, suitable (cultured) tissues and intact specimens themselves, e.g. in terms of their locomotory, ERG, or other rhythms. Furthermore, upon

identification of crustacean-specific neuropeptide isoforms and their precursors, injections of synthetic peptides and double-stranded RNAi preparations enable studies of manipulated circadian behaviours.

- Do circadian and circatidal clocks use identical or different molecular mechanisms? Is there mechanistic interaction or even integration between the two systems? Apart from behavioural data and entrainment experiments, the cellular localisation of distinct circatidal pacemakers has not been easily forthcoming and deserves further investigation. Clock genes that are similar in insects and crustaceans may also regulate circatidal clocks, but these have not yet been fully identified, located and/or functionally linked to well-known circatidal rhythms in crustaceans (6, 8). It is entirely possible that circadian and circatidal rhythms originate from different molecular components, and may have to be considered as convergent adaptations to different cues. Testing for the presence and functional significance of known (homologous) clock molecules in the two systems will, however, provide promising insights into their evolutionary origin, and, in particular, answer the question as to whether circadian clocks have indeed evolved from circatidal clocks (cp. 8).
- How were crustacean and insect pacemaker cells shaped during evolution, how did they gain functionality in rhythm control, and how does the distributed location of crustacean pacemakers relate to the evolution of clocks in the Pancrustacea? The *Drosophila* clock neuron clusters of a few morphologically and functionally distinct s-/l-LN_vs and LN_ds are likely a highly adapted clock system. However, PDH/PDF-ir neurons located next to the optic ganglia usually occur in most other insects (57, 316-318) and in diverse crustaceans (138, 140, 141) in much larger numbers. They could, thus, represent a clock organisation less specialised than that in *Drosophila*. These considerations ultimately address questions about the ancestral organisation of arthropod clock neurons (319, 345), and how their functional and anatomical design evolved in the different taxa. Furthermore, comparisons with other arthropod taxa such as myriapods and chelicerates will be essential to understand which features most likely were already present in the arthropod ancestor and will help to clarify whether distributed clock systems are specific to crustaceans.

5. ACKNOWLEDGEMENTS

J.S. is a stipendiary of the Swedish Carl Tryggers Foundation which is thanked for its generous financial support. The same foundation and an additional research award from the Faculty of Natural Sciences at Stockholm University provided financial support for this study to H.D., and both organisations are, therefore, gratefully acknowledged. The authors wish to thank Prof. Dr. Steffen Harzsch, University of Greifswald, Germany, for generously providing original micrographs in figure 4, and an anonymous reviewer for very thoughtful comments and helpful corrections.

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Abbreviations: Act: activating factor; AKH: adipokinetic hormone; AMC: protocerebral anterior median cluster; bHLH: basic helix-loop-helix domain; BPR: brain photoreceptor; BPN: brain photoreceptor neuropil; CHH: Crustacean hyperglycaemic hormone; CLK: Clock; CNS: central nervous system; CPR: caudal photoreceptor; CRY: Cryptochrome; CYC: Cycle; DBT: Double-time; DD: dark-dark conditions; DP: distal pigment of retinal pigment cells; DRPH = distal retinal pigment hormone: synonymous with PDH; ERG: electroretinogram; FaRP: FMRFamide-related peptide; GABA: gamma-amino butyric acid; 5-HT: 5-hydroxytryptamin or serotonin; ir: immunoreactive; LAH: light-adapting hormone, synonymous with PDH; LCMS: liquid chromatography combined with mass spectrometry; LD: light-dark conditions; l-LN_vs: large lateral ventral neurons of *Drosophila* clock; LN_ds: dorsal lateral neurons of *Drosophila* clock; MIH: moult-inhibiting hormone; MOIH: mandibular organ-inhibiting hormone; NAS: N-acetyl-serotonin; NAT: N-acetyltransferase; NDH: neurodepressing hormone; OGT: olfactory-globular tract; OPT-GT: optic-globular tract; PAS: PER-ARNT-SIM protein domain; PER: Period; PDH: pigment dispersing hormone; PO2: second post-embryonic stage of crayfish; POs: pericardial organs; PP: proximal pigment of retinal photoreceptors; RIA: radioimmunoassay; RNAi: RNA-interference; RPCH: red pigment-concentrating hormone; SGG: Shaggy; TIM: Timeless; s-LN_vs: small lateral ventral neurons of *Drosophila* clock; VNC: ventral nerve cord; XOSG: X-organ-sinus gland system.

Key Words: Crustacean, Circadian Rhythm, Ultradian Rhythm, Circadian Clock, Pacemaker, Clock Genes, Period, Cryptochrome, Pigment Migration, Red Pigment Concentrating Hormone, Crustacean Hyperglycaemic Hormone, Pigment Dispersing Hormone, Serotonin, Melatonin, Caudal Photoreceptor, Extraretinal Photoreceptor, Crayfish, *Drosophila*, *Procambarus Clarkii*, *Cherax Destructor*, *Carcinus Maenas*, X-Organ Sinus Gland System, Review

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Figure 1. Retinal pigment distribution in the ommatidium of dark-adapted (DA) and light-adapted (LA) crayfish. Both proximal pigment (pp) in the photoreceptor cells and distal pigment (dp) in the distal pigment cell change position depending on the illumination. *Abbreviations* ax: axon of the retinular cell; bm: basement membrane; ct: crystalline tract of crystalline cell; drp: distal reflecting platelets; dp: distal pigment cell; pp: proximal pigment within the photoreceptor cell; rh: rhabdom of the photoreceptor cell; tc: tapetal cell = reflecting pigment cell; adapted with permission from ref (95).

Figure 2. Circadian rhythmicity of the electroretinogram (ERG). (**A**) Circadian rhythmicity of the ERG of isolated retinae from crayfish *Procambarus clarkii* persists under constant darkness. ERG amplitude depends on stimulus intensity (bottom box) as well as circadian time. Upper trace of bars gives timing of light and dark phases prior to transfer of the specimen to constant darkness. (**B**) Coupling of circadian ERG rhythms between left and right eyes in *P. clarkii* in isolated preparations consisting of eyes, eyestalks, and protocerebrum. Parallel ERG recordings from right (upper ERG trace) and left (lower ERG trace) eye show synchronous circadian changes in ERG amplitude (arbitrary units), providing evidence for a coupling mechanism between the two hemispheres. *Abbreviations* ERG: Electroretinogram; ES: eyestalk; PC: protocerebrum; R: retina. (A,B) reproduced with permission from ref (23, 78), respectively.

Figure 3. Schematic representations of patterns of crustacean hyperglycaemic hormone (CHH) and serotonin (5-HT) circadian rhythms. (**A**) Dynamics of adult XOSG CHH neurosecretion and haemolymph hyperglycaemia during a L/D 12:12hr cycle: *1* CHH-synthesis and beginning of axonal transport, *2* axonal transport to the sinus gland, *3* CHH release into the haemolymph, *4* hyperglycaemia acrophase, 5 smaller CHH release into the haemolymph, *6* smaller hyperglycaemia; reproduced with permission from ref (163, 186). CHH (●) and serotonin (5-HT; ○) immunoreactivity in the retina of post-embryonic stage two (PO2; **B**) and juvenile (**C**) crayfish, and in the XOSGs of the PO2 stage (**D**), and the juvenile stage (**E**). Symbol density represents amount of immunoreactivity. DR, distal retina; MR, medial retina; PR, proximal retina; reproduced with permission from ref (103).

Figure 4. PDH-immunoreactive neurons in the embryo of the lobster *Homarus americanus*. (**A**) Camera lucida-reconstruction of PDH-ir neurons in the optic ganglia and the brain at 60% of embryonic development (E60%). Groups of neuronal somata denoted by letters A–E. PDH-ir neurons located laterally to the medulla externa (groups C, C`, C``) are presumptive homologues of insect clock neurons. Note the contralateral projections of type C interneurons from the large tract in the MT through brain commissures (arrow), and the large descending type E interneurons running down the entire VNC. (**B**) PDH-ir neurons in the optic ganglia and brain at 45% of embryonic development (E45%). Note the commissural PDH-ir fibre connections between eyestalks (arrow) located above the oesophageal foramen. *Abbreviations* AL: accessory lobe; AMD/CG: anterior part of the mandibular neuromere; APN anterior protocerebral neuropil; ES: oesophageal foramen; LG: lamina ganglionaris; Lo: lobula; ME: medulla externa; MT: medulla terminalis; N: nauplius eye; OL: olfactory lobe; PMD: posterior mandibular neuromere; PPN: posterior protocerebral neuropil; PT: protocerebral tract; R: retina; S: medulla satellite neuropil; SG: sinus gland; TC: tritocerebrum; scale bar: 50µm; adapted with permission from ref (141).

Figure 5. Schematic summary of known circadian oscillators and their functional correlations in crayfish. Established physiological oscillators or pacemakers (grey circles) exist in the retina, the X-organ sinus gland complex, the caudal photoreceptors (CPRs), and likely the brain, whilst the brain photoreceptor (BPR) is not an oscillator but responsible for light-driven entrainment of locomotory rhythms, and CPRs affect phase settings; arrows indicate respective effects on locomotion and ERG amplitude. See text for further details. Question mark in the abdominal ganglion refers to a possible oscillator in the ventral nerve cord. *Abbreviations* BPR: brain photoreceptor; CPR: caudal photoreceptor; R: retina; SG: sinus gland; XO: X-organ.

Table 1. Systems and/or parameters displaying circadian rhythms in crustaceans

System / parameter	Crustacean / group	Reference
Development		
Egg release	*Emerita*	(194)
Hatching	Various decapods	(346)
Larval release	Various decapods	(51)
Vitellogenin levels	*Porcellio*	(347)
Neurogenesis of olfactory projection neurons	*Homarus*	(348)
Moulting	*Homarus*	(9, 35)
Behaviour		
Burrowing	*Uca, Penaeus*	(349, 350)
Agonistic behaviour	*Procambarus spec.*	(243)
Locomotion		
Locomotory activity	Various decapods	(3, 26, 80, 303, 351-353)
Locomotory activity and orientation	Various amphipods	(354, 355)
Diel vertical migration	*e.g. Daphnia*	(48)
Diel horizontal migration	Mysid crustaceans	(49)
Phototaxis	*Crab larvae*	(356)
Sensory systems		
Electroretinogram amplitude	*Procambarus*	(23, 85)
Caudal photoreceptor amplitude	*Procambarus*	(12)
Distal retinal pigment position	*Various decapods*	(97, 220)
Proximal retinal pigment position	*Various decapods*	(96)
Neuronal parameters		
Photoreceptor rhabdomic membrane turnover	*Grapsus, Ocypode*	(357)
Photoreceptor and spectral sensitivity changes	*Cherax, Ligia, Procambarus*	(106-110)
Spike rate of X-organ neurons	*Procambarus*	(179)
Spontaneous multiunit activity of brain neurons	*Procambarus*	(88)
Integumental pigmentation		
Tegumentary chromatophores	*Procambarus, Uca*	(358, 359)
Vegetative physiological parameters		
Heart rate, haemolymph flow and cardiac performance	*Homarus, Procambarus, Cancer*	(360-363)
Blood sugar level	Various decapods	(172, 173, 364)
CHH level	*Astacus*	(174)
Free fatty acid level in abdominal muscle	*Palaemon*	(365)
Lactate level	*Uca, Procambarus*	(364, 366)
Oxygen uptake	*Orconectes, Procambarus*	(79, 366-368)
Diel ammonia excretion and glutamine nitrogen storage	*Ligidium, Armadillidium*	(369, 370)
Glutathione level	*Procambarus*	(270)
Antioxidant level	*Procambarus*	(273)
Haemolymph protein level/vitellogenin	*Porcellio*	(347)

Running title: Identified circadian clock systems in crustaceans

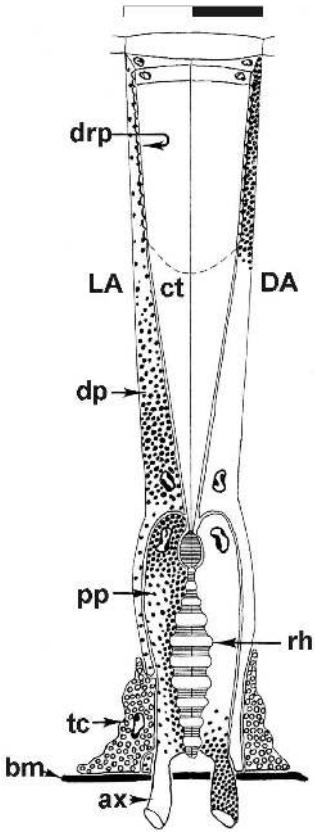


Fig.1

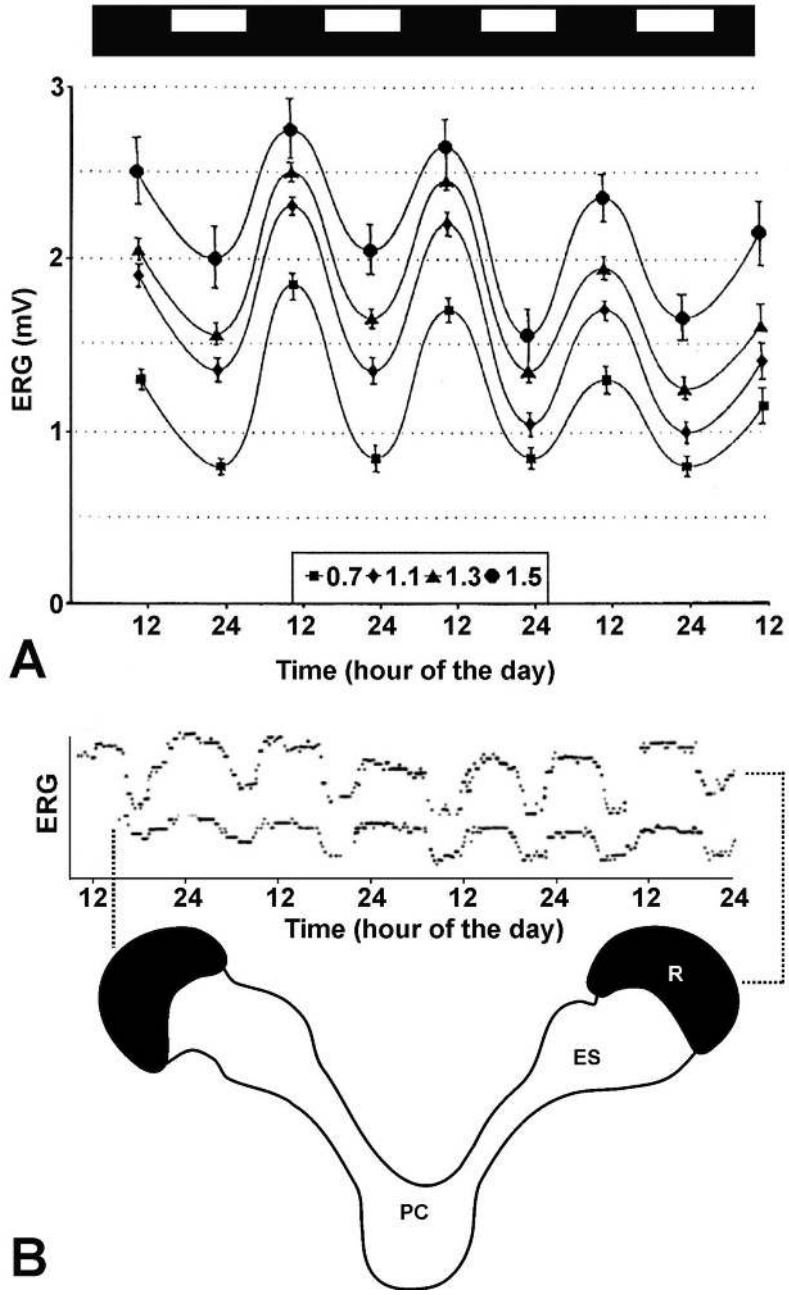

3
2
1
0
ERG (mV)
0.7 1.1 1.3 1.5
12 24 12 24 12 24 12 24 12
Time (hour of the day)
A
ERG
12 24 12 24 12 24 12 24
Time (hour of the day)
R
ES
PC
B

Fig.2

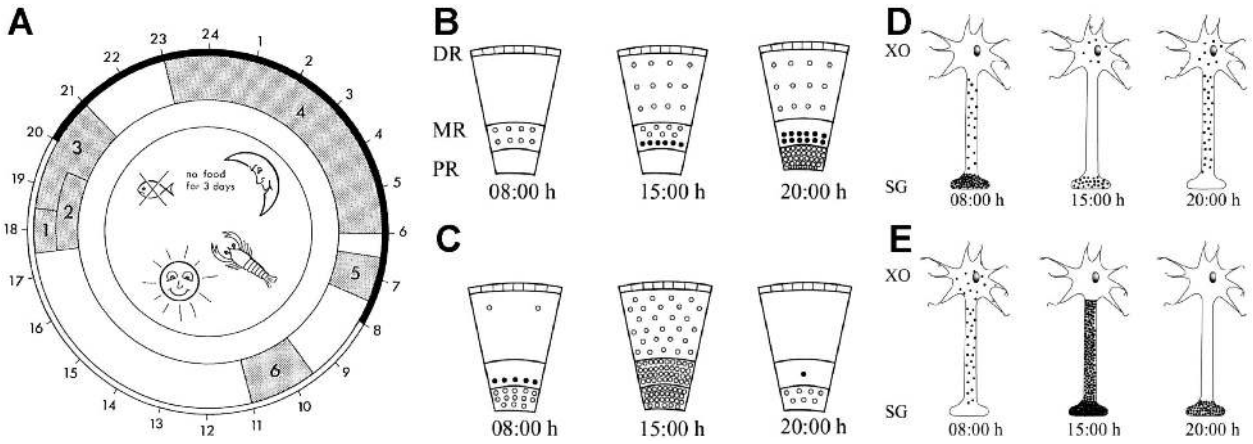
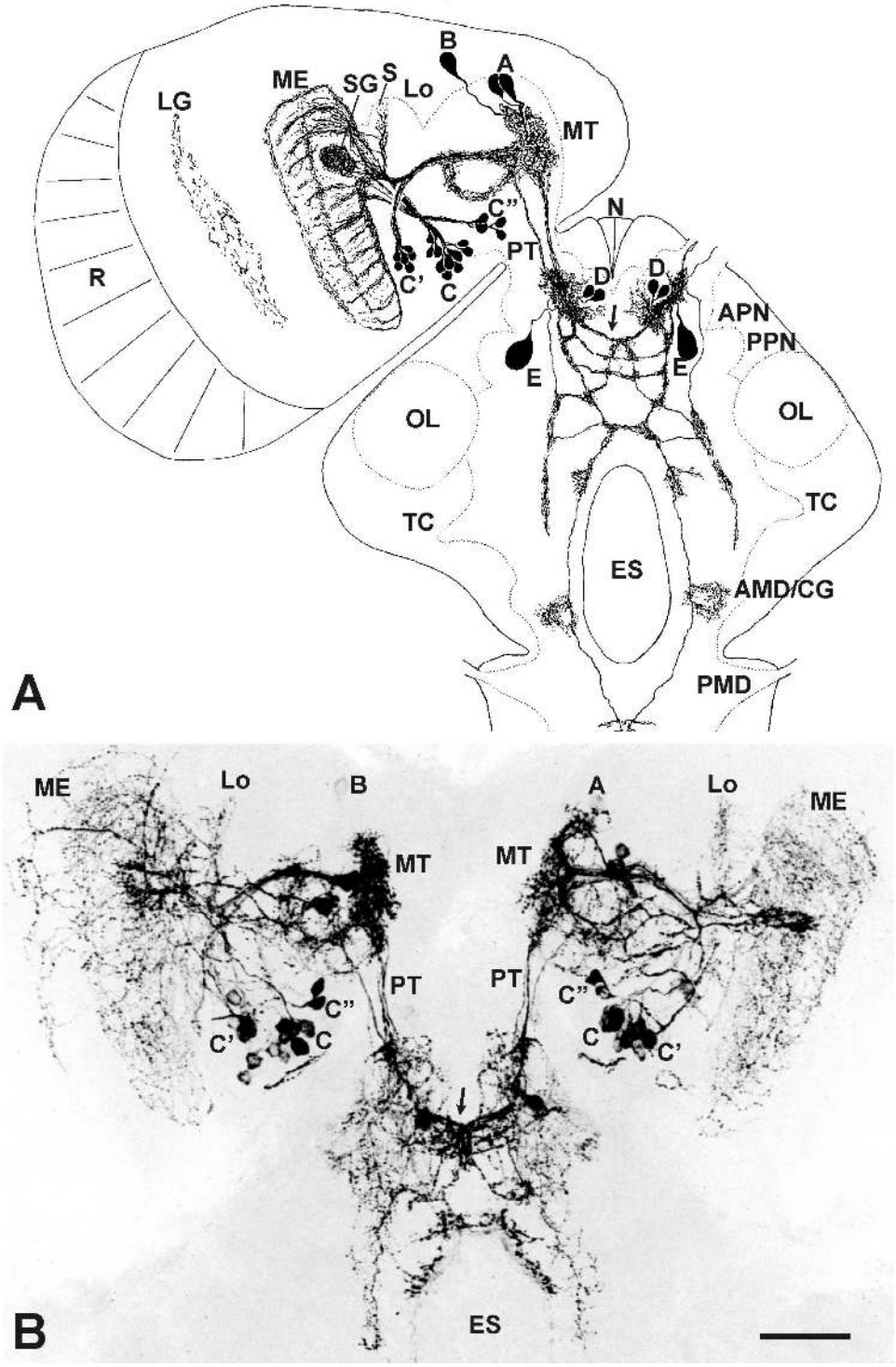


Fig.3


B
A
ME
SG
S
Lo
LG
MT
C"
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PT
R
C'
C
D
D
APN
PPN
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E
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PMD
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PT
PT
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C"
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C
C
C'
B
ES

Fig.4

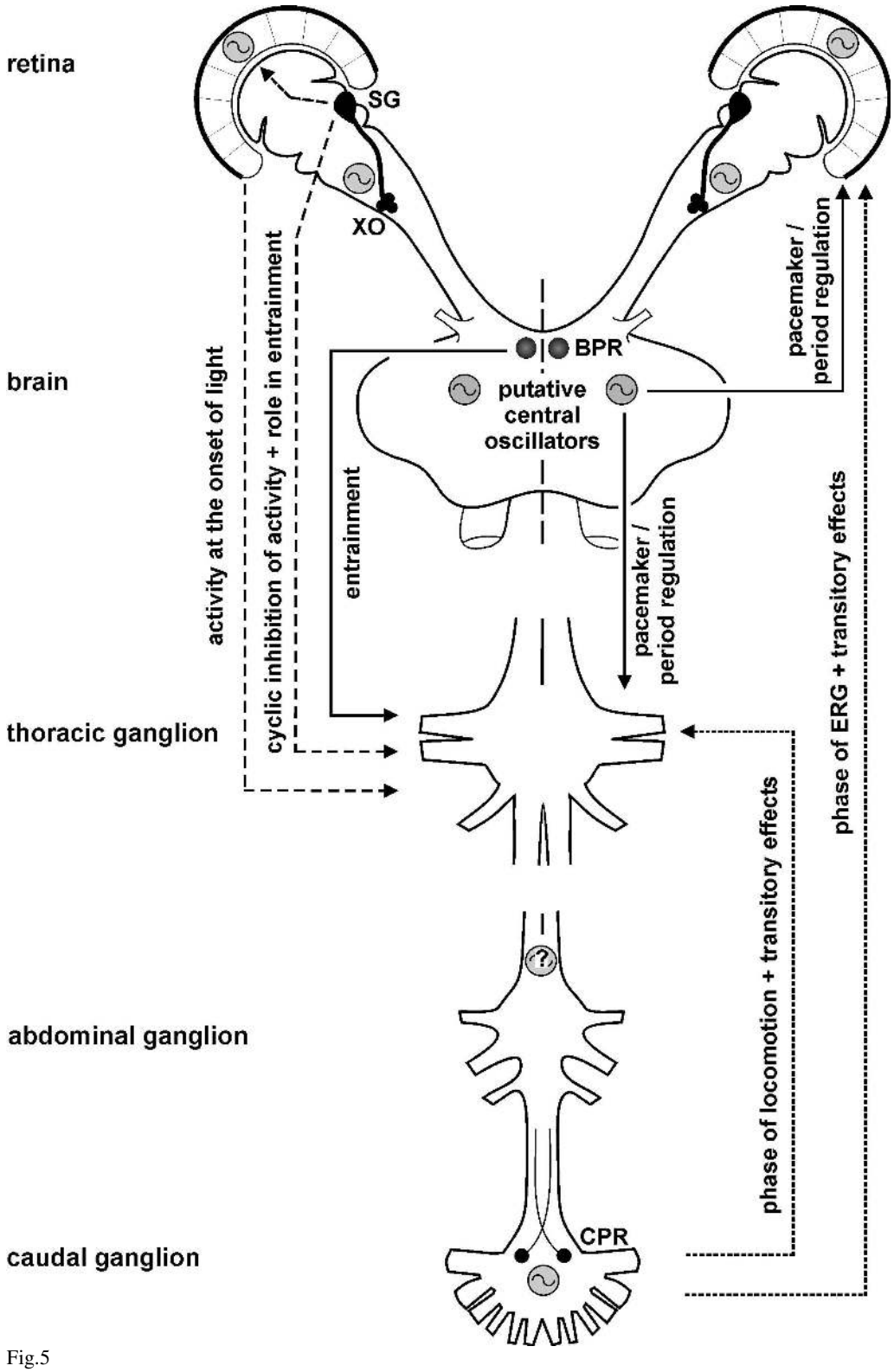
retina
brain
thoracic ganglion
abdominal ganglion
caudal ganglion
SG
XO
BPR
putative
central
oscillators
activity at the onset of light
cyclic inhibition of activity + role in entrainment
entrainment
pacemaker /
period regulation
pacemaker /
period regulation
phase of ERG + transitory effects
phase of locomotion + transitory effects
?
CPR

Fig.5