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The circadian biology of the marbled crayfish

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1. ABSTRACT

The parthenogenetic marbled crayfish (*Procambarus* spec.) has recently been introduced as a new preparation for neuroethological studies. Since isogeneity apparently limits inter-individual variation, this otherwise typical decapod species may be especially valuable for circadian studies. Locomotor activity of isolated marbled crayfish and agonistic activity of small social groups maintain circadian rhythmicity in constant darkness. As potential signals of circadian time information, levels of 5HT, N-acetylserotonin and melatonin were determined in brains of marbled crayfish at different daytimes. However, location and structural organization of crustacean circadian pacemakers are still elusive. Immunocytochemical and backfill studies in the marbled crayfish revealed neural structures that may correspond to portions of circadian pacemaker systems in the insect optic lobe. Position and additional chemical contents in two pigment-dispersing hormone-expressing neuron groups resembled insect pigment-dispersing factor-expressing cells in the lamina and the accessory medulla, a neuropil discussed as center for integration of timing information. Here, we discuss new findings about the possible organization of the circadian system of the marbled crayfish in the light of current knowledge about circadian clocks in crustacea.

2. INTRODUCTION

Circadian pacemakers of arthropods have been extensively studied in crustacea and their apparent sister group, the insects (1, 2). Circadian rhythms have been reported for various physiological functions and behaviors in a number of crustacean species. Endogenous circadian pacemakers modulate the sensitivity of sensory organs like the eyes (3) and caudal photoreceptors (4), determine the movements of pigments (5) and regulate blood sugar (6, 7), glutathione levels (8), the capacity to take up oxygen (6, 9) and the activity of effector systems such as heart rate and ventilation (10-12). Most behavioral studies investigated spontaneous locomotor activity and found a robust circadian rhythm with maxima at the beginning and ongoing activity throughout dark phases (13-17). In addition, plugging of burrows has also been demonstrated to be controlled by circadian pacemakers (18).

In *Drosophila* and orthopteromorph insects like cockroaches and crickets, a master clock controlling circadian behavior and eclosion rhythms is located in each of the two optic lobes, which are protocerebral brain parts processing visual information from the compound eyes (19). These optic lobe pacemakers connect to various effector areas in the central brain. Disruption of these

neuronal projections in most cases lead to complete and long-lasting breakdown of behavioral circadian rhythmicity. In crustaceans, a similar master pacemaker that controls all major aspects of circadian behavior seems to be absent. Although lesion studies in crustaceans point to the central brain as well to the eyestalks of crustaceans as clock locations (20, 21), it is clear that crustacean eye stalk clocks are not sufficient to sustain circadian rhythmic locomotor behavior as they are in many insects (reviewed by (22)). It appears that the circadian system of crustacea is less centralized than that of insects, but rather comprises a network of brain oscillators to create a circadian output with additional oscillators situated in the optic lobes being important parts of this network.

In this paper we summarize current knowledge about the crustacean circadian system and compare it to the insect circadian clockwork. Additionally, we provide new data about different aspects of the circadian system of the parthenogenetic marbled crayfish (Figure 1A). This animal, which until now lacks a valid species description, was recently recognized as a promising species for neuroethological research. So far it has been thoroughly studied with regard to its anatomy, mode of reproduction, phylogeny, ecology, and development (23-26). Marbled crayfish produce isogenic offspring by parthenogenetic reproduction, which is as so far known unique for decapod crustacea, and offers the chance to generate genetically altered lines of animals. This, together with high reproduction rates and undemanding rearing conditions, make it an attractive new model animal for research on crustacean neurobiology and physiology. We successfully used it to explore behavioral, neurochemical, and anatomical aspects of the crustacean circadian system. Our results support a hypothesis of a rather dispersed circadian network in the predecessors of the crustacea/insect taxon.

3. CIRCADIAN BEHAVIOR OF THE MARBLED CRAYFISH

Most behavioral studies in crustaceans analysed the spontaneous locomotor activity, showing a robust circadian rhythm with maxima at the beginning of the dark phase (13-17). Nocturnally active crayfish display a typical bimodal rhythm under light-darkness cycles. In addition to a pronounced activity peak that starts shortly before lights off and continues for several minutes, a shorter and less intense burst of activity occurs at the beginning of light phase. Under constant darkness, this bimodal pattern of locomotor activity usually switches to an unimodal rhythm, retaining only the pronounced burst of activity associated with lights off (15, 27, 28). Crustaceans have also been used to study the control and behavioral consequences of agonistic interactions. Most species studied show stereotyped fixed action patterns consisting of behavioral sequences with increasing intensity including approach, threat display and restrained fighting until occasional brief periods of unrestrained combat (29). Victory and defeat mediate characteristic changes of behavior and physiological conditions in both opponents (30). In co-habitated groups of crayfish, agonistic encounters result in the establishment of either superdominant hierarchies or hierarchies with defined individual ranks (31, 32).

In a recent report (33) we have studied circadian aspects of both locomotion and agonistic activity in the marbled crayfish *Procambarus spec.* (Figure 1). Under 12 hours light-12 hours darkness (LD12:12) isolated marbled crayfish displayed rhythmic locomotor activity with two activity increments coinciding with light transitions (Figure 1B). Rhythmicity persisted after switching to constant darkness (DD) (Figure 1C, D) with the characteristic light-off burst, as it has previously been described for other crustaceans. Isogenetic females of parthenogenetic marbled crayfish displayed all behavioral elements known from agonistic interactions of previously studied decapod species including the formation of hierarchies. Previously isolated marbled crayfish initially displayed high frequencies of agonistic encounters during the first hour of their co-habituation in groups of six animals that declined to lower levels with the establishment of hierarchies. Group agonistic activity was entrained to periods of exactly 24 hours under LD12:12 and peaks of agonistic activity coincided with light-to-dark and dark-to-light transitions. After switching to DD, enhanced agonistic activity was only observed at times corresponding with light to dark transitions during the preceding three days in LD 12:12 and agonistic activity remained rhythmic with an average circadian period of 24.83 ± 1.22 h in all crayfish groups tested (Figure 1E, F show the results for one group of six individuals). Analysis of individual agonistic activities revealed that only the most dominant crayfish established clear endogenous rhythmicity by initiating more than half of all agonistic encounters within the group. Since marbled crayfish display all typical features of agonsitic behavior that have previously been studied in other crustaceans (mainly lobster and crayfish), it might be expected that these species exhibit similar endogenous circadian rhythms. Based on the circadian rhythmicity of locomotion and agonistic activity, marbled crayfish can be regarded as typical decapod crustaceans despite their unusual mode of reproduction.

4. POTENTIAL PARTICIPATION OF INDOLEAMINES IN THE CIRCADIAN CONTROL OF THE MARBLED CRAYFISH

Though circadian rhythmicity in crustaceans seems to be controlled by multiple independent pacemakers, their coordination by yet unknown neural or humoral pathways remains speculative. With respect to activity rhythms of locomotion and agonistic behavior biogenic amines may contribute to mediate circadian time information, since serotonin, dopamine and octopamine have been demonstrated to promote or suppress general states of activity in various organisms. Serotonin has been associated with the control of various physiological mechanisms and behaviors (reviewed by (34, 35)) and levels of both serotonin (36, 37, 38) and serotonin receptors (39) have been demonstrated to oscillate with circadian rhythms in crustacea. Moreover, serotonin has the capability of phase-shifting the circadian output of retinal

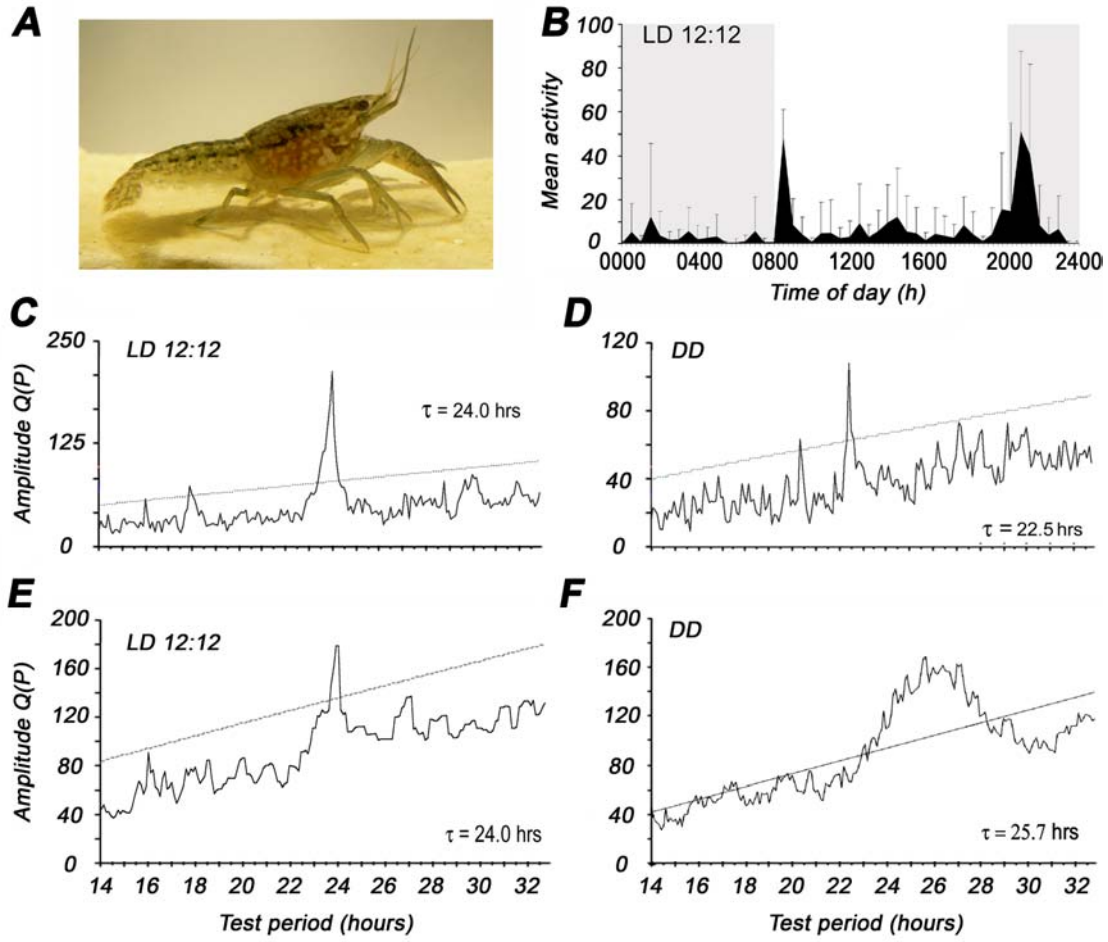

Figure 1. Circadian rhythmic activity of the marbled crayfish. (A) A semi-adult marbled crayfish *Procambarus* spec. of approximately 6 cm body length. (B) Mean locomotor activity of one individual over 12 days under LD 12:12 conditions. Dark periods are indicated by gray color. (C, D) Chi^2 periodogram analysis of a marbled crayfish's locomotor activity during three days under LD 12:12 (C) and four days in constant darkness DD (D). (E, F) Chi^2 periodogram analysis of circadian rhythmic agonistic behavior in a group of six marbled crayfish during three days under LD 12:12 (E) and four days in constant darkness (F). Diagonal lines represent Sokolove significance lines for p = 0.01 (Sokolove and Bushell 1978). tau circadian period. Figure modified from (33).

and extraretinal photosensitive neurons located in the sixth abdominal ganglion (40, 41) and electrolytic lesion of the serotoninergic system compromised locomotor rhythmicity (42). Therefore, serotonin itself or one of its metabolites *N*-acetylserotonin (NAS) and melatonin, the signal that mediates time information in vertebrates, may contribute to synchronize distributed endogenous circadian pacemakers and couple them to effector systems. Titers of serotonin, NAS and melatonin in central brains and eyestalks of the marbled crayfish were measured by ELISA at different timepoints of the light-dark cycle (2100, 0100, 0500, 0900, 1300, and 1700 h). An ELISA protocol established for measurements of these molecules in vertebrates (IBL, Hamburg) was adapted to marbled crayfish. Serotonin and N-acetylserotonin were assayed in the same samples whereas melatonin measurements were performed separately. Newly established standard curve for serotonin and NAS was sensitive to concentrations ranging from 0.2 to 258 ng/ml and that for melatonin to concentrations between 1 to 1000 pg/ml. The presence of melatonin, serotonin and NAS in the nervous system of crayfish was confirmed. Mean values of 5-HT concentrations were of 21.05 ± 0.93 (ng/ml) in brains and of 6.75 ± 0.36 in eyestalks (ng/ml) (mean ± S.E.M.); NAS titers in brains resulted in 5.65 ± 0.29 (ng/ml), and in eyestalks in 3.12 ± 0.23; melatonin in brains 30.82 ± 1,61 pg/ml and melatonin in eyestalks 21.66 ± 1.54 pg/ml. Comparing these data revealed that the average concentrations of all three substances were higher in brains as in both eyestalks by 1.5 to 3 fold. Additionally, concentrations of melatonin were one thousand times smaller in relation to 5-HT and NAS titers.

5-HT levels in the eyestalks of marbled crayfish were found to peak at the beginning of the light phase with a significant difference to its concentration at one hour after

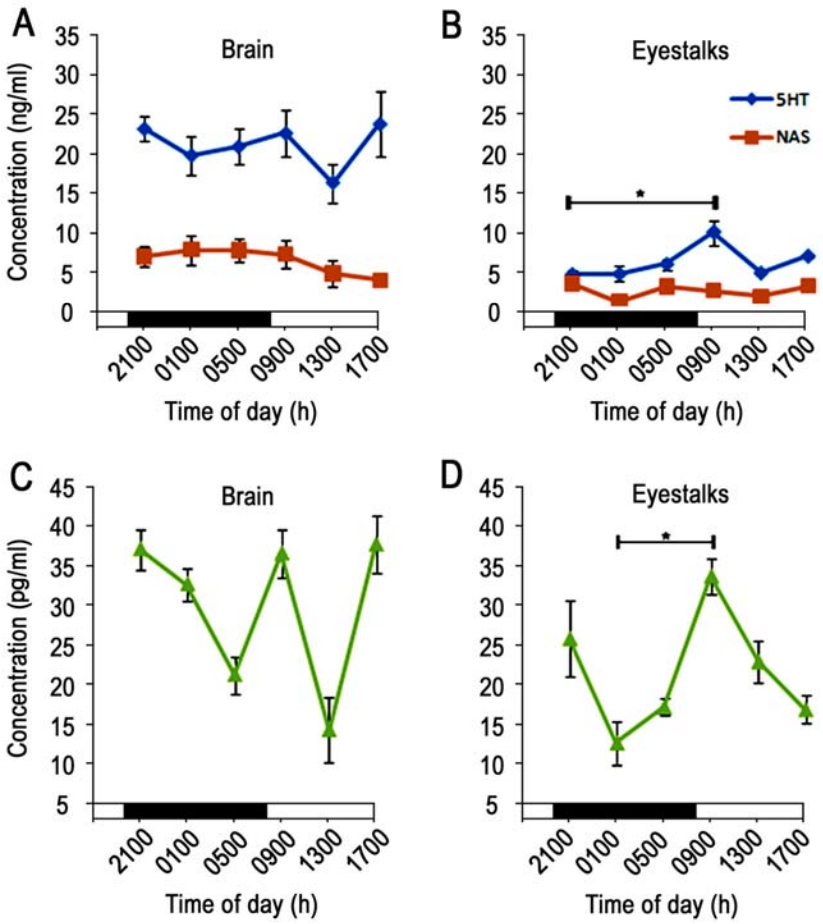

Figure 2. Diurnal changes of serotonin (A, B), N-acetylserotonin (A, B) and melatonin (C, D) titers in brains (A, C) and eyestalks (B, D) of the marbled crayfish during LD 12:12. Each point represents mean ± SEM (n = 3–4 per time point). Samples were obtained every 4 hours, starting one hour after lights on. Onset of light occurred at 0800 h. Black bar represents 12 hours of scotophase and white bar 12 hours of photophase.

the beginning of the dark phase (Figure 2B). In the central brain, no significant differences between different times of the day were detected but highest serotonin levels coincided with the onset and offset of light (Figure 2A). These results are in line with previously reported diurnal and circadian rhythms of 5-HT concentrations in the nervous system of juvenile and adult *Procambarus clarkii* (37) that have been suggested to affect motor behavior. In marbled crayfish and other species, the highest levels of serotonin accompany light-to-dark and dark-to-light changes and coincide with peaks of locomotor and agonistic activities.

NAS, the product of 5-HT *N*-acetylation (and also melatonin demethylation), was detected in lower concentrations in the nervous system of the marbled crayfish than 5-HT (Figure 2A, B). Slightly elevated NAS levels were observed around both, the end and the beginning of dark phase but significant changes in its concentration were not detected (in eyestalks close to significance, at $p = 0.061$). Information about the presence and function of NAS in invertebrates is scarce. In vertebrates, NAS (syn. normelatonin) has received more attention because of its antioxidant properties in the nervous system (43, 44) and its function as a transcriptional inhibitor (45, 46). NAS interacts with several 5-HT receptor subtypes and may function as a potent agonist of serotonin because of its much longer half-life (45). This difference results from the fact that *N*-acetylated indoles are not substrates of monoamine oxidases (in vertebrates, MAO A). Though protein sequences and second messenger couplings of serotonin receptor subtypes seem to be conserved across species from different phyla, their pharmacological properties can vary significantly (reviewed in 47). Of the five types of serotonin receptors thought to exist in crustaceans, two have been characterized molecularly and pharmacological profiles are largely incomplete. The possibility of NAS acting as a serotonin agonist has been excluded for 5-HT_{2beta} receptors of *P. clarkii* and *P. interruptus* (48), but other types of 5-HT receptors may bind both serotonin and NAS as effective agonists. NAS, along with other 5-HT metabolites, has been shown to exhibit circadian rhythms in the retina of trout (49). In invertebrates, septiapterin reductase (50) is

known to be sensitive to NAS and melatonin, but insensitive to 5-HT. This relationship is insofar important as the suppression of sepiapterin reductase leads to declines in the tetrahydropterin cofactors of tryptophan hydroxylase and, thus, reflects a negative feedback loop limiting serotonin formation. Declines in sepiapterin reductase may have additional consequences of biological relevance, since NO synthases that generate the gaseous transmitter nitric oxide likewise depend on tetrahydropterins 51).

A recent study demonstrated that melatonin can synchronise the circadian modulation of electroretinogram amplitudes in *Procambarus clarkii* suggesting that it may function as a signal of darkness to the retina and other effectors (52). The presence and diurnal oscillation of melatonin in nervous systems of decapod crustaceans has previously been investigated with different results (7, 53-55). Our studies on the marbled crayfish revealed significant differences between melatonin concentrations in samples taken at different times of the day (Figure 2C, D). The concentrations of melatonin were comparable to those reported by Tilden and coworkers in *Cancer* (7), but were considerably lower than those reported by the groups of Agapito (53) and Balzer (54) in *Procambarus clarkii*.

When applying a cosinor analysis to diurnal variations of serotonin, NAS and melatonin in the central brain and the eyestalks of marbled crayfish, no significant rhythmicity could be detected, though there seemed to be a trend for serotonin in the eyestalks ($p = 0.067$) and melatonin in central brains ($p = 0.061$). Failure of determining a significant daily rhythm in the course of concentration changes for the three molecules was partly due to the large variability measured in individual samples. This individual variability might reflect changes of the indolamines due to physiological factors that could be related to age or social status among other factors. Though a large number of samples was assayed (more than 200 plus standard curve), data derived from only 3 to 6 animals per timepoint over one day and significance may be reached by increasing the number of samples.

In insects, specifically in *Drosophila melanogaster,* different types of Arylalkylamine-N-Acetyltransferases (AA-NAT) have been identified that convert serotonin into NAS (56, 57). AA-NAT represents the rate limiting enzyme for the formation of melatonin in vertebrates (58) but it has not been thoroughly studied for a similar role in invertebrates. However, in arrhythmic PER mutants of *Drosophila* melatonin was not detectable while normal levels of *N*-acetylserotonin were present (59), which may suggest a role for melatonin in invertebrate circadian rhythmicity.

Melatonin concentrations in eyestalks and central brains of marbled crayfish were approximately 1000-fold smaller than those of serotonin and NAS. The theoretical explanation that melatonin may be faster degraded than synthesized seems rather unlikely and would contradict all previous studies about melatonin metabolism in other organisms. The most probable interpretation would be to assume that serotonin N-acetylation does not represent the rate-limiting step of melatonin formation and, thus, crustaceans may differ in this regard from the vertebrate pineal. A similar conclusion derived from studies on *Drosophila*, in which the NAS levels were found to be three orders of magnitude higher than that of melatonin (59). This seems to result from the abesence of a functional HIOMT gene (codes for hydroxyindole-O-methyltransferase that converts NAS into melatonin) in *Drosophila*, so that melatonin can be formed from NAS solely by other O-methyltransferases (Hardeland, pers. communication). Our finding that NAS levels are higher than those of melatonin agrees with previous observations in prawns (60). The authors concluded that AA-NAT, due to its kinetics, is unlikely to be the rate limiting enzyme of melatonin formation in prawns. Successful melatonin immunostaining has been reported in the cnidarian *Renilla kollikeri* (61) but, to our knowledge, not in any crustacean or insect. The low levels of melatonin that we and others detected by ELISA could explain the difficulty reported here to locate the indoleamine in crustacean tissues by immunoreactivity. Additionally, melatonin may be difficult to fix within nervous tissue since it is a small lipophilic molecule that is sensitive to light. It has been hypothesized that homologs of vertebrate AA-NAT might exist in bacteria, fungi, green algae and amphioxus, but not in arthropods (62). However, the presence of melatonin in insects and crustaceans either suggests the presence of AA-NAT or of another enzyme that converts serotonin to NAS.

5. LOCALIZATION OF CIRCADIAN PACEMAKERS IN CRUSTACEA AND INSECTS

While insects seem to contain a central circadian pacemaker (“master clock”) in each of their optic lobes, circadian rhythmicity of crustaceans appears to be controlled by incompletely characterized sets of independent pacemakers, situated in the eyestalks, the retina, the supraesophageal ganglion, and probably other parts of the nervous system, which in the intact animal interact by largely unidentified neural and humoral pathways (10, 20, 63).

Various ablation studies, surgical interferences, and *in vitro* studies implicated the eyestalks and central brain as potential structures to contain circadian pacemakers (64, 65). However, different studies provided ambiguous information about the importance of particular nervous structures for mediating circadian rhythmicity. If eyestalks were ablated, some circadian rhythmic parameters were abolished or at least altered while others remained unaffected and different studies received varying results concerning the persistence of particular rhythms (reviewed by 22, 63, 66). In contrast, explanted retinae and eyestalks *in vitro* displayed intact circadian rhythms of light sensitivity measured by electroretinography (64). Moreover, circadian regulated glycemic responses were largely unaffected after ablation of the protocerebrum with the exception of sudden responses produced by asphyxia that were suppressed in operated animals (67). Circadian rhythmicity of locomotor activity was initially reported to be lost after eyestalk ablation (68) but was later found to persist after the same treatment (reviewed by 22).

Similarly, rhythmic locomotor activity vanished after lesions of the circumesophageal connectives but continued after complete brain removal (69). A large amount of partly contradicting results so far prevents the exact localisation of a pacemaker that coordinates an apparently dispersed system of endogenous circadian oscillators in crustaceans. Predictions from physiological and behavioral approaches should therefore be corroborated with anatomical studies and may profit from comparison with the organisation of insect circadian systems.

In insects, such as crickets, cockroaches and flies, daily activity rhythms are controlled by a circadian master oscillator located in a small region of the optic lobes (reviewed by 19, 70). In *Drosophila*, a set of so-called lateral neurons (LNs) is indispensable to maintain stable circadian rhythmic behavior under non-rhythmic environmental conditions (70). These LNs are arranged in a dorsal and a ventral group within the optic lobes (LNds and LNvs, respectively) and they express a variety of substances that are components of the molecular circadian clockwork, including Period (PER), Timeless (TIM), Clock (CLK), and Cycle (CYC). The LNvs further express the neuropeptide pigment-dispersing factor (PDF), which is apparently not part of the clockwork itself, but acts as a synchronising signal between circadian oscillators in both optic lobes and an essential output signal for circadian phase information of the clock neurons to downstream effectors of the central protocerebrum (71, 72). PDF was also found in many other non-dipteran insects to be expressed by neurons that appear to be homologous to *Drosophila's* LNvs, and which are also discussed as components of the circadian clock (73-79) (Figure 3A). Morphological characteristics of these PDF-expressing medulla neurons (PDFMe) are largely conserved among non-dipteran insects. Their characteristic features include the position of the somata anteriorly between medulla and lobula, centrifugal innervations of medulla, lamina, and, in some insects, the lobula, centripetal innervations of large areas of the central protocerebrum, and commissural projections to the contralateral brain half including contralateral optic lobe. Additionally, a small neuropil compartment of the medulla near the PDFMe somata, the accessory medulla (AMe), is densely invaded by processes of the PDFMe (73, 80). An accumulating body of evidences suggests that the AMe represents an integration center for timing information in insects with the PDFMe acting as important pacemaker and/or output neurons of the neuronal assemble forming the circadian clock (e.g., 81).

6. ROLE OF PIGMENT-DISPERSING HORMONE IN CRUSTACEAN AND INSECT CIRCADIAN SYSTEMS

The PDFs expressed in the PDFMe/LNv of insects are homologous to the crustacean beta-pigment-dispersing hormones (beta-PDHs) (82). PDH was first identified and initially thought to be present only in crustaceans (83) until the beta-PDH homolog PDF was isolated in the grasshopper *Romalea microptera* (84). In insects, PDF is termed "factor" instead of "hormone" since it has not been reported to be released to the circulatory system. Moreover, PDF does not influence pigment migration in insects, though the regulation of retinal and epidermal pigment granules was the first function assigned to crustacean PDH (85). However, insect PDF causes pigment migration in bioassays using crustacean species (84). Molecular analysis revealed that both signaling molecules include variants of homologous 18-aminoacid peptides with only small differences in aminoacid sequences, that appear not to be structurally relevant. Sequence differences between crustacean beta-PDHs and insect PDFs are not greater than differences between crustacean PDHs or between insect PDFs. So far, about a dozen different beta-PDHs were isolated in a variety of crustacean species (82) and recently two β-PDH isoforms in the same species have been identified in the crab *Cancer productus* (86).

In crustacea, beta-PDH is released from the sinus gland of the optic stalk into the hemolymph to cause migration of distal retinal pigment to a more proximal position and to mediate light adapted state of the eye (85, 87). Crustacean PDH was first isolated from the shrimp *Pandalus borealis* (88), and later from other crustaceans like the crabs *Uca pugilator* and *Cancer magister* (89, 90) and the crayfish *Procambarus clarkii* (91). Crustacean PDH occurs in two classes with larger differences in amino acid sequences, namely the alpha-PDH and the beta-PDH (92). Alpha-PDH (sometimes referred to as beta-PDH II) is expressed by neurohemal cells of the sinus gland and seems to exert exclusively hormonal functions (86). In the context of this work, 'PDH' always refers to beta-PDH, being expressed by neurons and released into central nervous neuropils to act as a neuromoduator. Neurons expressing beta-PDH are distributed throughout the central nervous system (93-98) and the stomatogastric system (99) of several malacostracan crustacean species.

Crustacean beta-PDH and insect PDF are both specifically recognized by an antiserum against synthetic *Uca*-beta-PDH (83). The anatomy of PDH-ir neurons in central brains and eyestalks of decapod crustaceans was assessed with immunohistochemistry in *Orconectes limosus*, *Carcinus maenas*, and *Cancer productus* (86, 96, 97), and by *in situ* hybridization with labeled beta-PDH RNA in *Cancer productus* (86). In these species, similar groups of neurons in the optic stalks, central brain, and stomatogastric system were labelled by the antiserum (96, 97, 99, 100).

In insects, the same antiserum labels the PDFMe and, in most non-dipteran insects, two groups of neurons at the posterior lamina (dorsal and ventral PDFLa (74-77, 101, 102). Additional neurons in the central protocerebrum were labeled in some but not all insect species studied (73, 78, 79). PDF seems to function as the major output signal of a defined population of circadian clock neurons in the optic lobes (781, 103-106) that co-express all canonical clock proteins in *Drosophila* (reviewed by 70) and other insects (107).

As a general comparison, the system of beta-PDH-neurons in crustacea is larger and more complex than the PDF-expressing neuron system in insects, and

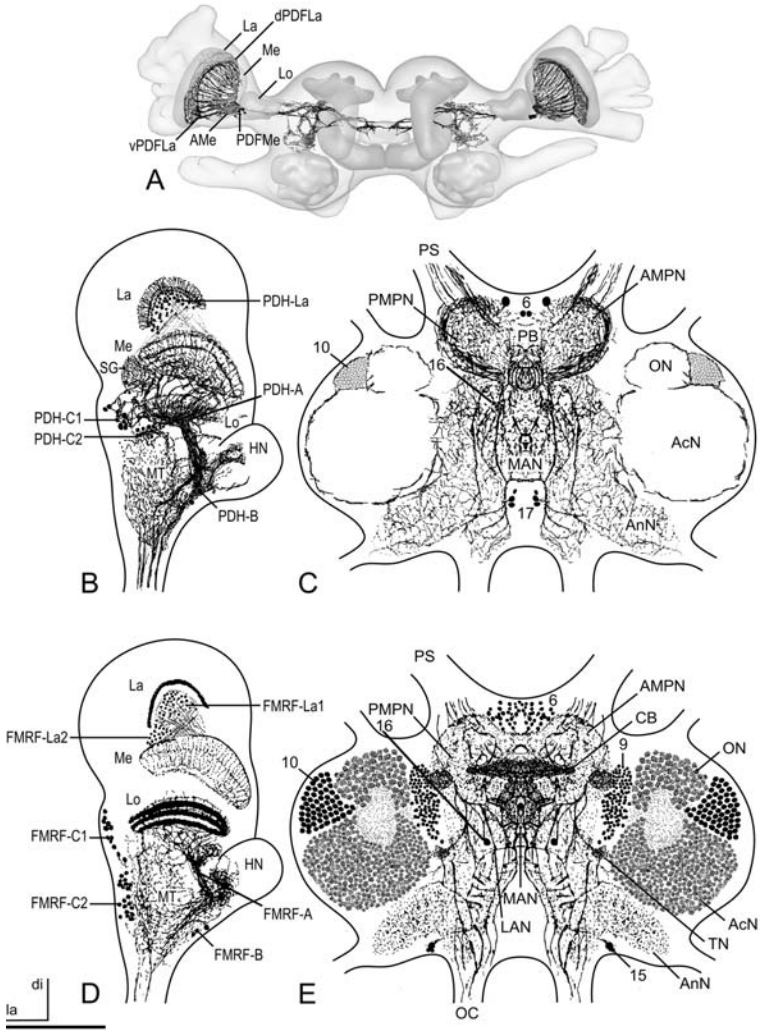

Figure 3. Schematic reconstructions of (A) pigment-dispersing hormone-immunoreactive (PDH-ir) neurons in the supraesophageal ganglion of the cockroach *Leucophaea maderae*, (B, C) PDH-ir neurons in the eyestalk and central brain of the marbled crayfish, and (C, D) FMRFamide-ir neurons in the eyestalk and central brain of the marbled crayfish. (A) In the cockroach, a group of about 16 PDH-ir neurons (PDFMe) resides at the ventro-anterior edge of the medulla (Me). They give rise to apparently all PDH-ir fiber projections that are found widespread in the central brain and most PDH-ir projections of the optic lobes including accessory medulla (AMe). PDH-ir fibers cross the brain halves via two commissures and connect both optic lobes and AMae. Further, two groups of PDH-ir neurons (dPDFLa and vPDFLa, respectively) reside at the dorsal and ventral posterior lamina (La). It is not known whether their projections leave the lamina. Lo, lobula. Modified from (112). (B) In the eyestalk of the marbled crayfish, PDH immunoreactivity was found in cells associated with the lamina (PDH-La), and in three further neuron groups: PDH-A, PDH-B, and PDH-C, the latter with subgroups PDH-C1 and PDH-C2. HN hemiellipsoid body; La, lamina; Lo, lobula; Me, medulla; MT medulla terminalis; SG sinusoidal gland. (C) In the central brain, PDH-ir somata were found in four groups named PDH-CBC6, PDH-CBC10, PDH-CBC16, and PDH-CBC17 (numbers 6, 10, 16, and 17, respectively). The labeling in PDH-CBC10 is probably not specific. AcN, accessory lobe; AMPN, anterior medial protocerebral neuropil; AnN, antenna II neuropil; MAN, median antennal I neuropil; ON olfactory lobe; PB, protocerebral bridge; PMPN posterior medial protocerebral neuropil; PS protocerebral stalk. (D) FMRFamide immunoreactivity in the eyestalk of the marbled crayfish was found in the proximity of the lamina (FMRF-La1, FMRF-La2) and in three further groups of neurons: FMRF-A, FMRF-B, an FMRF-C (the latter with subgroups FMRF-C1 and FMRF-C2). HN hemiellipsoid body; La, lamina; Lo, lobula; Me, medulla; MT medulla terminalis. (E) In the central brain, several FMRFamide-ir soma groups were found: FMRF-CBC6, FMRF-CBC9, and FMRF-CBC10, FMRF-CBC15, FMRF-CBC16 (numbers 6, 9, 10, 15, and 16, respectively). FMRF-CBC12 with one soma per side is not visible here. AcN accessory lobe; AMPN, anterior medial protocerebral neuropil; AnN, antenna II neuropil; LAN, lateral antennal I neuropil; MAN, median antennal I neuropil; OC esophageal connectives; ON olfactory lobe; PMPN posterior medial protocerebral neuropil; TN tegumentary neuropil. Coordinates: di, distal; la, lateral for B and D; Scale bar: 500 µm for all images.

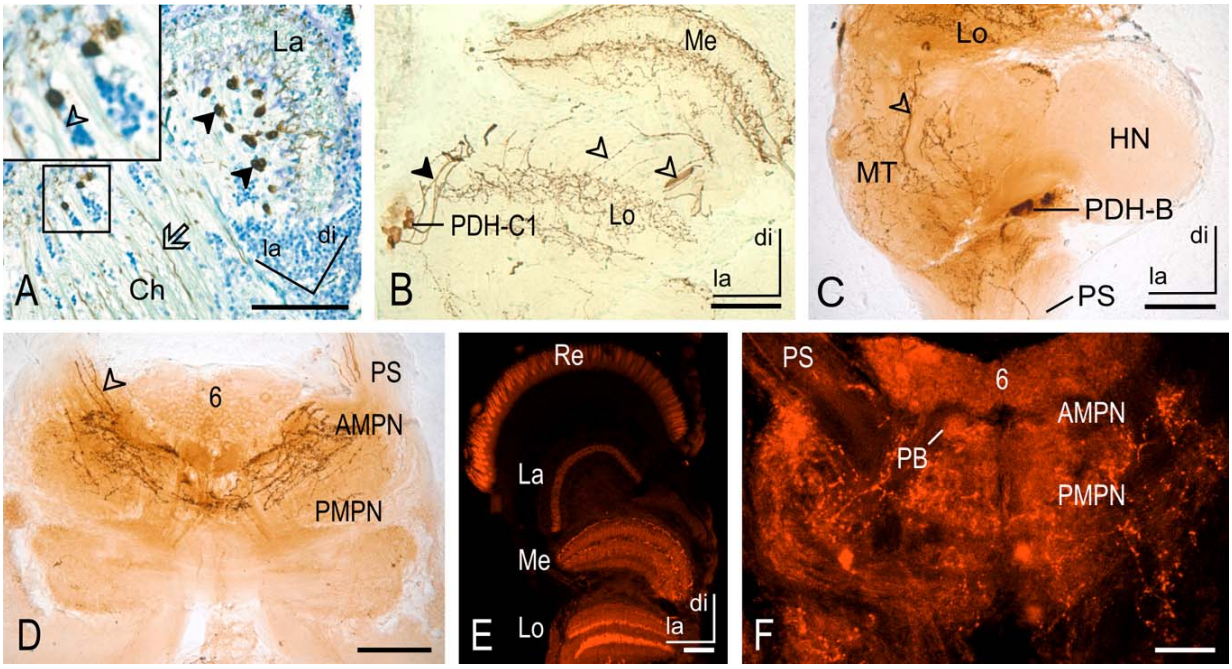

Figure 4. Pigment-dispersing hormone (PDH; A-C) and FMRFamide immunoreactivity (D-F) expressed in the eyestalk and central brain of the marbled crayfish. (A) PDH-ir lamina neurons (PDH-La, filled arrowheads) projected mainly to the lamina, but occasionally projections (open arrowhead in inset) via the first chiasm (ch) to the medulla were observed. However, most chiasmatical PDH-ir projections appeared to be centrifugal arborizations arising from the proximal PDH-ir neuron groups. (B) PDH immunoreactivity in the medulla (Me) and lobula (Lo) of the eyestalk. Both structures show a layered arborization pattern. The open arrowheads show PDH-ir fibers projecting between lobula and medulla through the second optic chiasm. The filled arrowhead shows primary neurites of the PDH-C1. (C) The PDH-B at the proximal margin of the hemiellipsoid body (HN) appears to belong to the X-organ. Single PDH-ir fibers running through the medulla terminalis (MT, arrowhead) could be traced into the protocerebral stalk (PS). Lo, lobula. (D) PDH immunoreactivity in the central brain. Fibers from the eyestalks innervate central brain regions, as the anterior medial protocerebral neuropil (AMPN) through the protocerebral stalk (PS). 6, Cell body cluster 6; PMPN posterior medial protocerebral neuropil. (E) In lamina (La), medulla (Me), and lobula (Lo) of the eyestalk a layered FMRFamid-ir arborization pattern could be observed. Re, retina. (F) In the central brain, most regions were pervaded by FMRF-ir fibers. Many FMRFamide-ir somata were located in the cell body cluster 6 (6; FMRF-CBC6). AMPN, PMPN anterior and posterior medial protocerebral neuropil, respectively; PB, protocerebral bridge. Coordinates: di, distal; la, lateral. Scale bars: 100 µm.

comprises both neuroendocrine optic stalk neurons releasing PDH to facilitate pigment migration via the sinus gland, as well as a number of optic stalk and central brain neurons utilizing PDH as paracrine neuromodulator.

7. TOWARDS CIRCADIAN PACEMAKER LOCALIZATION IN THE MARBLED CRAYFISH: AN IMMUNOHISTOCHEMICAL APPROACH

7.1. PDH-immunoreactive neurons in the brain of the marbled crayfish

The well conserved structure of the beta-PDH/PDF peptides in crustacea and insects together with functions in their circadian systems suggests a possible common origin of the circadian systems of both taxa. We were specifically interested in which of the PDH-ir neuron groups of crustacea could be homologous to the PDF neurons found in insects, and whether there exists a correlate of the insect AMe in the crustacean optic lobe. Standard immunocytochemistry was applied to microtome and vibratome sections of marbled crayfish cerebral ganglia using two different primary antibodies, a rabbit polyclonal serum directed against *Uca pugilator* beta-PDH (83) and a mouse monoclonal serum against *Drosophila* PDF (J. Blau, #C7 of Developmental Studies Hybridoma Bank, University of Iowa). Both antisera produced similar PDH-immunoreactivity in different soma clusters and processes throughout the eyestalk and median protocerebrum. A schematic reconstruction of the marbled crayfish's PDH-ir neuron system in optic lobe and median protocerebrum derived from a complete series of sections is presented in Figure 3B, C.

The eyestalks of marbled crayfish contain four groups of PDH-ir neurons, that could be identified as PDH-La, PDH-A, PDH-B, and PDH-C according to the description of Harzsch *et al.* (108) derived from developmental studies on *Homarus americanus*. Three of these groups were also present in the species *O. limosus, C. maenas*, and *C. productus* (86, 96) while the PDH-A cluster on the medial-distal margin of the hemiellipsoid body was missing in these species. The largest group of PDH-ir neurons in the optic lobe of marbled crayfish was formed by the PDH-ir lamina neurons (PDH-La) (Figure 4A).

Their somata were scattered proximally to the lamina in the area of the first optic chiasm. All PDH-La were connected to the lamina through neurites extending distally, that ramified and terminated in the lamina. The lamina was additionally innervated by fine PDH-ir fibers that arose from the distal part of the medulla and passed the optic chiasm together with other fibers. However, some of these fibers appeared to originate from somata of the PDH-La. The PDH-ir fibers innervated the entire lamina in a rather reticulate manner with only faint accumulation of fibers in two layers. In contrast, tangential PDH-ir fibers innervated distinct layers of the medulla and lobula that were interconnected by columnar fibers (Figure 4B). In the medulla, the most proximal, the most distal, and two middle layers were PDH-ir. In the lobula, three layers were PDH-ir, but not the most distal and proximal layers. The medulla terminalis was differentially innervated by PDH-ir fibers, but without forming layers (Figure 4C). A conspicuous stalk of PDH-ir fibers extended into the HN1 part of the hemiellipsoid body. Most of the PDH-ir fibers that innervated medulla, lobula, medulla terminalis, and hemiellipsoid body appeared to arise from the groups PDH-A, -B, and -C, although individual projections of these groups were difficult to determine due to extensive overlap of fibers.

With close to 20 neurons the PDH-C was the second largest PDH-ir neuron group in the eyestalk (Figure 3B). This group was regularly subdivided in a group PDH-C1 lying more anteriorly of the optic lobe and consisting of larger somata (Figure 4B), and a group PDH-C2 consisting of smaller somata located more dorsally. The PDH-C group was situated close to the lobula, gave rise to most of the PDH-ir projections in medulla and lobula and appeared to innervate also the lamina, medulla terminalis, and hemiellipsoid body. For reasons explained later, this group is assumed to be homologous to the insect PDFMe/LNv. PDH-C1 neurons appeared to innervate the sinusoidal gland to release PDH into the hemolymph (also suggested by 86). Most if not all PDH-ir fibers of the sinusoidal gland originated from PDH-C1, though some additional projections from the PDH-B, which belong to the X-organ that is known to supply the sinusoidal gland with peptidic innervations, may also exist. A group similar to PDH-C owing to the location and projection areas, including projections to the sinusoidal gland, medulla, and lamina, was also described in an isopod species, the woodlouse *Oniscus asellus* (group PGR3 in 100).

The PDH-A and PDH-B consisted of 1–3 and 2 cells, respectively (Figure 3B, 4C). The PDH-A was located at the distal margin of the hemiellipsoid body, while the PDH-B was situated at its proximal margin and probably formed part of the X-organ. Most (if not all) of these groups contributed to a thick bundle of PDH-ir neurites that originated from the proximal lobula and proceeded between medulla terminalis and hemiellipsoid body to the optic peduncle. A large portion of the fibers innervating the medulla terminalis and the fibers innervating the hemiellipsoid body appeared to arise from this bundle.

Anatomical reconstruction of PDH immunoreactivity in the eyestalks of the crayfish coincided with previous description from other species. We were able to identify the main groups of PDH-ir somata described in *Homarus americanus* embryos (108). Other than in lobster, PDH-C subgroups were less clearly separated and formed a dispersed cluster of cells with different diameters in adult marbled crayfish.

In the central brain consisting of median protocerebrum, deutocerebrum, and tritocerebrum, PDH-ir cells formed four bilateral groups (Figure 3C). The first group situated in the cell body cluster (CBC) 6 (PDH-CBC6) comprised one large soma per side accompanied by one or two somata of smaller diameter (corresponds to similar structures in *Cherax destructor* (109)). All these cells projected neurites to the anterior medial protocerebral neuropil (AMPN), where they ramified and mixed with profuse PDH-ir fibers originating from the ipsilateral and possibly contralateral eyestalks. A second group found in CBC 9 (PDH-CBC9) included about 8 cells per side and projected neurites posteriorly that ramified in deutocerebral neuropil masses. Nearby in CBC 16 resided a group of about 6 neurons per side (PDH-CBC16) with apparently similar projection areas, but their posteriorly projecting neurites were positioned more medially to that of PDH-CBC9. The last cluster was situated in the CBC 17 (PDH-CBC17). It consisted of three somata per side, that projected neurites through the circumesophageal connectives to the subesophageal ganglion. Axons from PDH-CBC17 probably innervated also the antenna II neuropils (AnN) of the tritocerebrum. Nearly all neurons of the CBC 10 were faintly labeled, but this labeling was just above background level and difficult to discern as real immunostaining.

Most neuropils of the central brain contained PDH-ir fibers, except the olfactory and accessory lobes, which were surrounded by a thin sheath of PDH-ir fibers (Figure 3C). The densest projections were found in the dorsal-anterior and ventral-posterior parts of the AMPN (Figure 4D), the ventral part of the posterior medial protocerebral neuropil (PMPN), the median antenna 1 neuropil (MAN), and the AnN. A major part of the PDH-ir fibers in the AMPN originated from somata in the eyestalks. Together with neurites of the PDH-CBC6, they form a conspicuous arc of fiber bundles that run from the base of the protocerebral stalks distally and posteriorly along the AMPN and PMPN to the brain midline and into the contralateral hemisphere. Other PDH-ir fibers of the eyestalks innervated more posterior regions of the central brain, giving rise to some arborizations invading the central body, MAN and AnN. Whether innervations of these structures originated from the contralateral or ipsilateral eyestalk was difficult to asses and fibers crossing the brain midline were detected at different places. As in the optic lobes, the exact assignment of PDH-ir projections within the central brain to particular soma groups was difficult to assess owing to overlap of projection patterns.

The morphology of decapod central brain PDH-ir neurons was previously analysed in *O. limosus* and *C.*

maenas by Mangerich and Keller (96) but a clear assignment of PDH-ir neurons to known neuron groups was not given there. However, their drawings indicate that the PDH-CBC6 and PDH-CBC16 we found in the marbled crayfish coincide with two neuron groups of *O. limosus* and *C. maenas*. Contrarily, marbled crayfish PDH-CBC17 does not match a posterior paired group of PDH-ir neurons in *O. limosus* and *C. maenas*, where another group not found in marbled crayfish, laid more laterally, thus implicating some species differences in crustacean PDH expressing neurons.

7.2. Some PDH-ir neurons in the eyestalks colocalize FMRFamide related peptides

In some insects subgroups of PDFMe were found to colocalize FMRFamide immunolabeling (110, 111). FMRFamides (= FMRFamide related peptides) comprise a diverse peptide family fulfilling many and widely unknown endo- and paracrine functions. In insects, details about the function of FMRFamides in the circadian system are unknown, but anatomical and behavioral data suggest a role in bilateral pacemaker coupling in some species (112, 113) amongst other possible functions. In crustacea, 19 FMRFamides were identified until 2003 (114, 115) and at least eight of them were detected in the eyestalk. FMRFamides exert numerous physiological actions in crustacea (reviewed by 114). Despite their relevance as modulators of crustacean nervous functions, detailed anatomical studies of FMRFamides expressing neurons in crustacean central nervous systems are remarkably scarce. To fill this gap and to assess potential homologies between crayfish eyestalk PDH-neurons and insect PDFMe, we explored the brain of the marbled crayfish with anti-FMRFamide immunosera (polyclonal guinea pig anti-FMRFamide (116) and polyclonal rabbit anti-FMRFamide; ImmunoStar, Hudson, Wisconsin) and performed anti-PDH/anti-FMRFamide double labeling. Nearly all neuropils of the eyestalks and central brain contained FMRFamide-ir fibers, which originated from relatively few somata grouped in different areas of the brain (Figure 3D, E). Many FMRFamide-ir somata were found in similar locations as PDH-ir cell bodies and fibers expressing these peptides displayed similar patterns of innervation throughout the brain, although more cells and fibers were immunoreactive to anti-FMRFamide than to anti-PDH. In the eyestalks, all neuropils except the sinusoidal gland contained FMRFamide-ir fibers. In the central brain, FMRFamide-ir fibers were present in most neuropils including the olfactory and accessory lobes, which where nearly free of PDH-ir fiber projections.

In the eyestalks, five main groups of neurons were labeled, with two subgroups in the FMRF-C (Figure 3D). The locations of FMRF-La1, FMRF-A, FMRF-B, and FMRF-C1 overlapped with that of PDH-La, PDH-A, PDH-B, and PDH-C. However, double labeling with anti-PDH and anti-FMRFamide revealed that colocalization of both immunoreactivities only occurred in PDH-La and PDH-C1 somata (Figure 5A-F). This clearly differs from the situation in *C. maenas* and *O. limosus*, where FMRFamide/PDH-ir colocalization appeared in various groups of neurons within the eyestalk, including lamina neurons, a group corresponding to the PDH-C group of marbled crayfish and neurons of the X-organ (97). In contrast, X-organ neurons included in the PDH-B and FMRF-B cluster expressed either PDH or FMRFamide in marbled crayfish. Double immunolabeling revealed colocalization of both neuropeptides in many cells of the lamina. In fact all PDH-ir cells also contained FMRFamide immunoreactivity (PDH-FMRF-La) (Figure 5 A-C). Contrary, the compact cluster of cells immunoreactive to FMRFamide in the margin of the lamina and the medulla did not express PDH immunoreactivity. All of the PDH-FMRF-La cells send axons to the lamina as described before. Fibers of the medulla and lobula showed also simultaneous immunoreactivity to PDH and FMRFamide, in the same way as fibers of the MT and fibers in between these structures (Figure 5D-F). In the group FMRF-A, double immunolabeling was not observed, although differentially labeled cells were located next to each other. In group B, at least one cell was immunolabeled with both neuropeptide-antisera. In group C most of cells with large somata including their axons colocalized PDH and FMRFamide (Figure 5D-F). Few cells contained only one of the neuropeptides. A similar pattern occurred in the FMRF-ir clusters C1 and C2 where some cell bodies colocalized both peptides and others showed either FMRFamide or PDH immunoreactivity. These results point to the cells of group C as strong candidates to be homologous to medulla cells in insects, which colocalize FMRFamide and PDH immunoreactivity. Since in crustaceans this region also contains neurons with oscillating CRY expression (117), it will be interesting to determine whether some of these neurons use PDH and/or FMRFamide related pepides as output signal.

The optic neuropils lamina, medulla, and lobula revealed a layered staining pattern with both the anti-FMRFamide and the anti-beta-PDH antisera. Layers were interconnected by a network of columnar fibers. This suggests a modulatory function to all the columnar neuronal units (‘optic cartridges‘) for FMRFamides as well as for PDH. In the lamina a median FMRFamide-ir layer was flanked by two PDH-ir layers (Figure 5C). In medulla and lobula, colocalized staining appeared in particular layers, but only a portion of fibers labeled by one of the antisera contained both immunolabels. The results suggest simultaneous expression and perhaps simultaneous release of PDH and FMRFamide related peptides in various eyestalk neurons that may exert so far unknown co-modulatory functions.

In the central brain, FMRF-CBC6 and FMRF-CBC16 shared locations with PDH-CBC6 and PDH-CBC16, respectively, but colocalizing immunoreactivity was not detected in any of the neurons. These locations coincided with the FMRFamide-ir neurons described by Mangerich and Keller (96) in *C. maenas* and *O. limosus*. They also reported FMRFamide immunoreactivity in a group corresponding to FMRF-CBC9 including widespread but rather weak immunoreactivity throughout the olfactory and accessory lobes. This is one of the major differences between the otherwise rather similar pericarya locations and arborization patterns of FMRFamide- and PDH-ir neurons and suggests a prominent role of FMRFamide

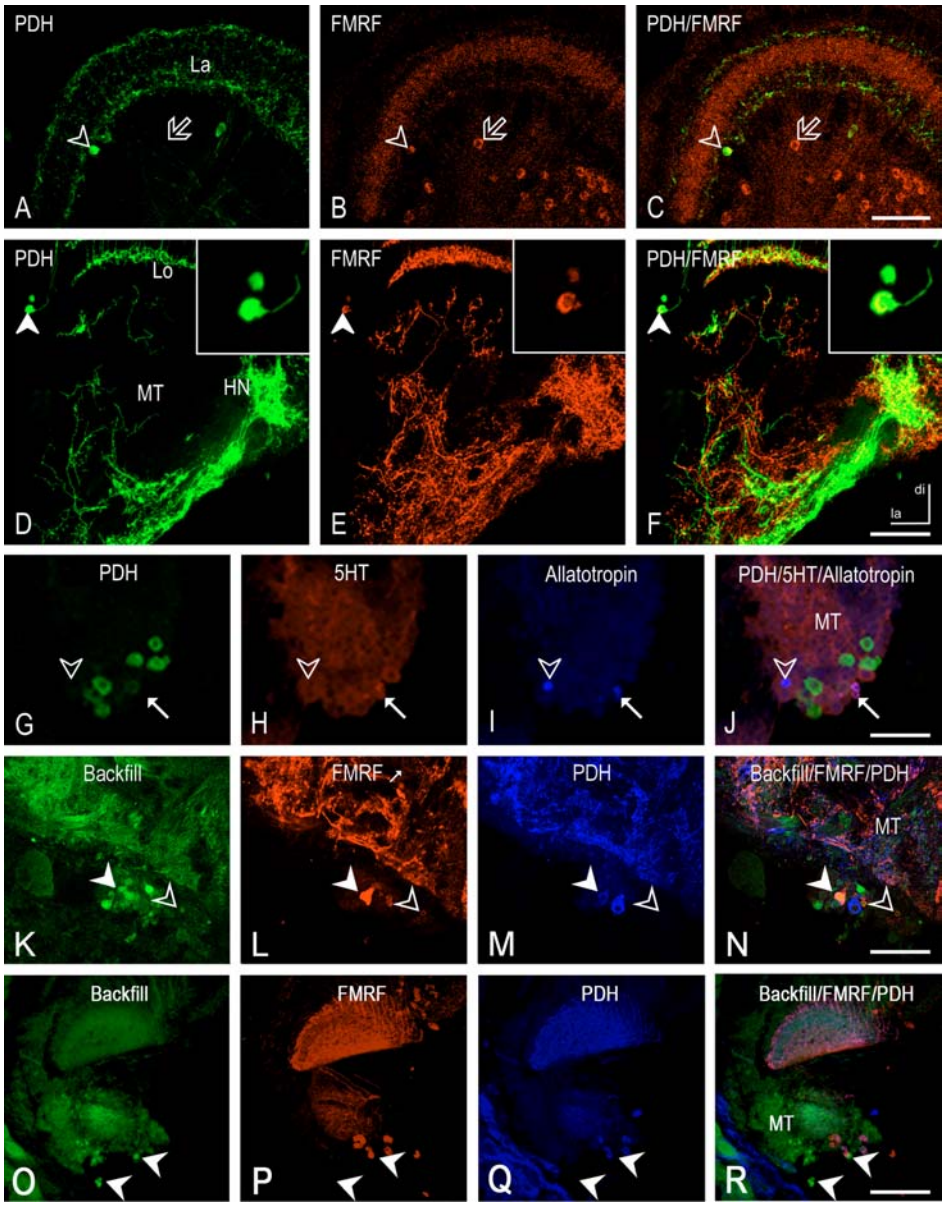

Figure 5. Immunofluorescent labelings and backfills in the eyestalk of the marbled crayfish. All images show maximum projections of several CLSM optical sections scanned at 2 µm z-distances. (A-C) All PDH-ir neurons proximal to the lamina (PDH-La, open arrowheads) showed additional FMRFamide immunoreactivity and thus, were identical with the FMRF-La1 group; however, not all FMRF-La1 neurons (including the entire FMRF-La2 group, see Figure 3D) were also PDH-ir (arrows). (D-F) Anti-PDH/FMRFamid colocalization was observed in somata of the group PDH-C (arrowheads). Details of these cells are shown in the insets. Lo lobula; MT medulla terminalis; HN hemiellipsoid body. (G–J) Triple immunolabeling against PDH, serotonin (5HT) and allatotropin with overlay. PDH-ir neurons like those of PDH-C were devoid of additional immunoreactivity against serotonin or allatotropin antisera. However, in their proximity, and scattered throughout the eyestalk, neurons showing allatotropin immunoreactivity alone (arrowheads), serotonin immunoreactivity alone (not shown here), and colocalized allatotropin and serotonin immunoreactivity (arrows) were observed. MT, medulla terminalis. (K–N) Neurobiotin backfill of the distal eyestalk via the ipsilteral protocerebral stalk combined with anti-PDH and anti-FMRFamide immunohistochemistry. Some PDH-C neurons, which were also FMRFamide-ir accumulated the neurobiotin tracer (filled arrowheads) and were therefore assumed to project to the central brain. Additionally, neurons of the FMRF-C group that were not PDH-ir showed biotin labeling (open arrowheads). In the vicinity of the PDH-C and FMRF-C additional backfilled somata were not immunolabeled. (O-R) Neurobiotin backfill of the central portion of one eyestalk via the contralateral protocerebral stalk combined with anti-PDH and anti-FMRFamide immunohistochemistry. Cell bodies in the vicinity of the PDH-C and FMRF-C group accumulated neurobiotin via projections into the contralateral eyestalk (arrowheads). These contralaterally projecting neurons were neither immunoreactive to anti-PDH nor to anti-FMRFamid antiserum. Scale bars: 100 µm.

related peptides in modulating olfactory information in the marbled crayfish. Detailed comparison between *C. maenas* and *O. limosus* (96) on the one hand and the marbled crayfish on the other revealed an exclusive presence of the clusters FMRF-CBC10 and FMRF-CBC15 in the latter (Figure 3E). Furthermore, one FMRFamide-ir group in *C. maenas* and *O. limosus* corresponded to the CBC17 cluster of PDH-ir cell bodies (Figure 3C). In general and across the three species studied in this respect, more cells and fibers were immunoreactive to anti-FMRFamid than to anti-PDH. The widespread localization of both peptides throughout the brain indicates an important role for both FMRFamides and PDH in regulating neuronal activities in the crayfish.

7.3. The eyestalks of the marbled crayfish contain no AMe-like structures

Various insects contain an accessory medulla (AMe), a small neuropil compartment of the medulla that is located near and densely invaded by processes of the PDFMe (73, 80). In contrast to other optic lobe neuropils, the AMe shows a peculiar nodular, non-retinotopic structure and is exceptionally rich in neuropeptides including members of the FMRFamide related peptides family and allatotropin (81, 110, 118). An accumulating body of evidences suggests that the AMe is an integration center for timing information in insects with the PDFMe as important pacemaker and/or output neurons of the neuronal assemble forming the circadian clock (e.g., 81). A subpopulation of PDFMe neurons has been demonstrated to couple the accessory medullae in both optic lobes of cockroaches and may serve to synchronize their pacemaker activity to generate a coherent output to midbrain and optic lobe output projections (113).

In an attempt to find a neuropil in the marbled crayfish with similarities to the insect AMe, we combined PDH- and FMRFamide immunocytochemistry with the detection of other signaling substances contained in this neuropil. In various insects, an antiserum against *Manduca sexta* allatotropin (polyclonal rabbit anti-Mas-allatotropin (119)) conspicuously marks a core structure of the AMe, which is at least partly formed by clustered allatotropin-ir neurons in the vicinity of the AMe (110, 111, and T. Reischig, unpublished). However, in the marbled crayfish allatotropin-ir cells were found in the eyestalk, but they were not localized in a particular arrangement. Some solitary allatotropin immunoreactive cells were found dispersed in the lamina and in the medulla (Figure 5I, J). In the proximity of PDH-C immunoreactive cells, some somata expressed allatotropin immunoreactivity and a subgroup of these also expressed serotonin immunoreactivity (Figure 5G-J). No characteristics of an insect AMe-like structure were found in marbled crayfish. Serotonin immunoreactivity was localized in various regions of the eyestalk, largely coinciding with the distribution of serotonin-containing neurons in *Pacifastacus leniusculus* (120) and *Cherax destructor* (121). The most prominent groups of serotonin immunoreactive cells were found in the region of the medulla terminalis, in the proximity of the hemiellipsoid body and in the region between the lamina and lobula. Serotonergic fibers were dispersed in the medulla terminalis and projected to the brain, sometimes in close proximity with PDH-ir fibers, but no colocalization with PDH was found in central brains or eyestalks of marbled crayfish.

To study whether PDH- or FMRFamide-expressing neurons may couple particular neuropils in the right and left eyestalk of marbled crayfish, neurobiotin backfills of one eyestalk were combined with immunocytochemical detection of both peptides. Although various projections contained the neurobiotin tracer applied to the proximal contralateral eyestalk, none of the labeled fibers contained PDH- or FMRFamide-associated immunoreactivity, suggesting that PDH- or FMRFamide-expressing neurons are not involved in bilateral coupling of circadian pacemaker neuropils in both eyestalks. When distal portions of the eyestalk were backfilled with neurobiotin, various cell bodies in the vicinity of the medulla terminalis were labeled by the tracer. Co-labeling with both anti-beta-PDH and anti-FMRFamide antiserum revealed that neurons of the PDH-C group that expressed both peptides and neurons of the FMRFamide-C group that did not contain PDH-ir, were among the backfilled cell bodies (Figure 5K-N). This suggests that these PDH-C and FMRFamide-C neurons project to some of the central brain regions described above, thus fostering the assumption that the PDH-C of the marbled crayfish are the homologous correlates to the PDFMe/LNv in insects.

8. CONCLUSIONS

In this report we connect recent information on the circadian regulation of behavior and physiological parameters from the marbled crayfish with earlier studies on other crustacean species. In all aspects were comparable data from other species existed the marbled crayfish turned out to be a typical decapod crustacean. Despite of its parthenogenetic mode of reproduction and the existence of pure female populations, marbled crayfish display all features of agonistic behavior, known from lobsters and various species of crayfish. Endogenous circadian rhythms were confirmed for locomotor activity of isolated marbled crayfish and newly established for agonistic interactions in small, all female populations. Marbled crayfish are relatively small and easy to rear in large numbers and can provide an alternative to larger and more demanding species for studies of circadian controlled behaviors. With respect to neuromodulatory or hormonal signals that connect circadian oscillators with effector systems, the presence of NAS as an intermediate metabolite of melatonin formation from serotonin was proven for the first time in a crustacean. As in vertebrates, NAS could act as a potent agonist at serotonin receptors to mediate behavioral states, promote particular behaviors and modulate the release of neuropeptides from neurohemal organs. Similar to *Drosophila*, melatonin can be detected in the nervous system of marbled crayfish, but at 1000 fold lower concentrations than serotonin and NAS. This contrasts with knowledge on vertebrate melatonin metabolism and suggests alternative pathways for melatonin synthesis in arthropods.

Comparison of PDH-, FMRFamide-, serotonin- and allatotropin immunocytochemical data from marbled crayfish, other crustaceans and insects suggested a possible homology of one part of the circadian pacemaker in the eyestalks of crayfish with the PDF medulla in insects. However, no structure in crustaceans could be identified that contained the characteristic features of the accessory medulla in insects, including PDFir projections connecting the PDF-FMRFamide expressing clusters in both eyestalks. Therefore, synchronisation of distributed partial circadian oscillators is more likely to occurr in the circumesophageal ganglion than in the eyestalks, probably with the participation of central brain projections of eyestalk PDH- and FMRFamide-ir neurons. Additional support of this idea arises by the requirement to integrate circadian information from eyestalks with information about light phases from brain photoreceptors in cluster 6. This cluster sends axons to protocerebral neuropils in the marbled crayfish and in other decapods and seems to be important for circadian regulated locomotor activity (101).

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