Candidates for photic entrainment pathways to the circadian clock via optic lobe neuropils in the Madeira cockroach

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Abstract
The compound eye of cockroaches is obligatory for entrainment of the Madeira cockroach’s circadian clock, but the cellular nature of its entrainment pathways is enigmatic. Employing multiple-label immunocytochemistry, histochemistry, and backfills, we searched for photic entrainment pathways to the accessory medulla (AME), the circadian clock of the Madeira cockroach. We wanted to know whether photoreceptor terminals could directly contact pigment-dispersing factor-immunoreactive (PDF-ir) circadian pacemaker neurons with somata in the lamina (PDFLAs) or somata next to the AME (PDFMEs). Short green-sensitive photoreceptor neurons of the compound eye terminated in lamina layers LA1 and LA2, adjacent to PDFLAs and PDFMEs that branched in LA3. Long UV-sensitive compound eye photoreceptor neurons terminated in medulla layer ME2 without direct contact to ipsilateral PDFMEs that arborized in ME4. Multiple neuropeptide-ir interneurons branched in ME4, connecting the AME to ME2. Before, extraocular photoreceptors of the lamina organ were suggested to send terminals to accessory laminae. There, they overlapped with PDFLAs that mostly colocalized PDF, FMRFamide, and 5-HT immunoreactivities, and with terminals of ipsi- and contralateral PDFMEs. We hypothesize that during the day cholinergic activation of the largest PDFME via lamina organ photoreceptors maintains PDF release orchestrating phases of sleep–wake cycles. As ipsilateral PDFMEs express excitatory and contralateral PDFMEs inhibitory PDF autoreceptors, diurnal PDF release keeps both PDF-dependent clock circuits in antiphase. Future experiments will test whether ipsilateral PDFMEs are sleep-promoting morning cells, while contralateral PDFMEs are activity-promoting evening cells, maintaining stable antiphase via the largest PDFME entrained by extraocular photoreceptors of the lamina organ.

Keywords
5-HT, acetylcholinesterase histochemistry, circadian entrainment pathways, GABA, neuropeptides, optic lobe neuropils, PDF, RRID:AB_177540, RRID:AB_2313973, RRID:AB_2314414, RRID:AB_2314803, RRID:AB_2315017, RRID:AB_2532101, RRID:AB_261181, RRID:AB_477522, RRID:AB_477652, RRID:AB_528479, RRID:AB_760350
1 | INTRODUCTION

The accessory medulla (AME) is the circadian clock of the Madeira cockroach Rhyparobia (Leucophaea) maderae (Reischig & Stengl, 2003a; Stengl & Homberg, 1994). It controls sleep–wake cycles via release of pigment-dispersing factor (PDF) from neuropeptidergic circadian pacemaker neurons in synchrony with light–dark cycles (reviews: Stengl & Arendt, 2016; Stengl, Werckenthin, & Wei, 2015). The clock is innervated by eight adjacent groups of neurons that are abundant of colocalized neuropeptides (Reischig & Stengl, 2003b). Among them are four clusters of PDF-immunoreactive (PDF–ir) neurons: two in the lamina (dorsal and ventral PDFLAs) and two next to the AME (anterior and posterior PDFMEs). While the arborizations of the four groups of PDF clock neurons were reconstructed and embedded into a standard atlas of the cockroach brain (Wei, el Jundi, Homberg, & Stengl, 2010), individual branching patterns of single PDF neurons could not be resolved. Nevertheless, functional and neuroanatomical studies suggested that PDF neurons take part in different circadian clock circuits (Giese et al., 2018; reviews: Stengl & Arendt, 2016; Stengl et al., 2015). It was hypothesized that they are involved in gating of photic entrainment pathways, in synchronization among clock cells, and in gating of clock outputs. Thus, they set the phase for circadian rest–activity (sleep–wake) rhythms.

Multiple-label studies revealed that PDFLAs that mostly colocalize 5-HT and FMRFamide immunoreactivities innervate the accessory laminae and the proximal lamina (Petri, Stengl, Würden, & Homberg, 1995). Furthermore, PDFLAs connected both lamina neuropils via the anterior fiber fan to the AME without sending processes to the midbrain (Giese et al., 2018). Their general branching pattern suggests that they obtain direct input from green-sensitive short compound eye photoreceptors and carry photic-phase information to AME clock neurons. Contralaterally projecting anterior PDFMEs connect both AMEs to ipsi- and contralateral lamina- and midbrain neuropils (Reischig & Stengl, 2002; Söhler, Stengl, & Reischig, 2011). All the posterior PDFMEs remain ipsilateral with unknown branching patterns. Additionally, undiscerned PDF neurons sent a side branch into the median-layer fiber system of the medulla (Giese et al., 2018; Giese et al., 2018; Reischig & Stengl, 2002, 2003b). Based on previous findings (Gestrich et al., 2018; Page, 1983; Page, Caldarola, & Pittendrigh, 1977) it was suggested that PDFLAs are clock inputs while PDFMEs serve as clock outputs. Furthermore, it was concluded that contralaterally projecting PDFMEs gate locomotor activity rhythms via terminals in ipsi- and contralateral premotor areas of the midbrain. Instead, ipsilaterally remaining PDFMEs gate sleep-promoting neuronal circuits, in synchrony with external light–dark cycles (Gestrich et al., 2018; reviews: Stengl & Arendt, 2016; Stengl et al., 2015).

Endogenous circadian locomotor activity rhythms are relayed via unknown clock outputs to ipsi- and contralateral premotor areas (Page, 1978; Page et al., 1977). The light entrainment pathways that synchronize these endogenous rhythms of the cockroach circadian clock with external light dark cycles are not known. Undiscerned compound eye pathways from both the ipsi- and contralateral compound eyes synchronize the clock via phase advances at dawn and phase delays at dusk (Nishiitsu-tsuji-Uwo & Pittendrigh, 1968; Roberts, 1965, 1974; review: Homberg, Reischig, & Stengl, 2003). While histamine-ir short photoreceptor cells terminate in the lamina, long histamine-ir photoreceptor cells branch in a distal, so far undiscerned layer of the medulla (Lösel & Homberg, 1999). However, no direct innervation of the AME by the compound eye was found. Therefore, interneurons relay photic entrainment information from lamina and/or medulla to the AME. Intracellular recordings identified different types of light-sensitive optic lobe interneurons with somata next to the AME (Lösel & Homberg, 2001). At least two groups of light-sensitive medial neurons (MNes) connected the AME to unknown layers in the medulla and via the anterior fiber fan to lamina and accessory laminae (Lösel & Homberg, 2001). Furthermore, polarization-sensitive ventromedial neurons (VMNes) connected both AMEs via the posterior optic commissure to median layers in the ipsi- and contralateral medulla (Lösel & Homberg, 2001). Therefore, next to PDFLAs, MNes and VMNes relay ipsi- and contralateral photic input to the cockroach clock (Reischig & Stengl, 2003b). Furthermore, the accessory laminae were hypothesized to be innervated by extracellular photoreceptors also in the Madeira cockroach, as first described in beetles (Feilisser et al., 2001). There, accessory laminae are the first-order optic neuropil of the lamina organ, which is an extracellular photoreceptor organ, next to the lobula organ that innervates the AME as first-order optic neuropil (Feilisser et al., 2001).

Here, we searched further for candidates of light entrainment pathways to the cockroach circadian clock. With multiple-label immunocytochemistry, combined with histochemistry and backfills, we specified layers of lamina and medulla, identifying respective termination sites of compound eye photoreceptor neurons. Then, neuroactive substances that were suggested to be involved in photic entrainment were located to specified layers of lamina, accessory laminae, and medulla. Backfills from the contralateral optic stalk obtained detailed information where contralateral light information is processed in optic lobe neuropils. Based on these studies, we suggested a new hypothesis on how multiple parallel-light entrainment pathways entrain circadian rest–activity rhythms (graphical abstract). Furthermore, we showed for the first time that the accessory laminae have glomerular subcompartments and do not receive histaminergic innervation from the compound eye. Instead, they expressed strong acetylcholinesterase (AChE) activity, suggesting cholinergic input from the lamina organ (Feilisser et al., 2001).

2 | MATERIALS AND METHODS

2.1 | Animals

Cockroaches of R. maderae were reared in laboratory colonies with 12:12 hr light–dark cycles. Temperature was kept at 25°C and relative humidity at 50%. Animals were fed with dried dog food, potatoes, and carrots, and had access to water ad libitum. All experiments were performed with adult male cockroaches.
2.2 | Enzyme-mediated AChE histochemistry

The thiocholine method of Karnovsky and Roots (1964) modified by Tago, Kimura, and Maeda (1986) and described by Homberg, Hoskins, and Hildebrand (1995) was performed to detect AChE. Cockroaches were cold anesthetized and decapitated. Brains were dissected in animal-specific saline (0.128 M NaCl, 0.0027 M KCl, 0.002 M CaCl₂, 0.0012 M NaHCO₃, pH 7.25) and fixed for 2–4 hr at room temperature (RT) or overnight at 4°C in 4% formaldehyde (FA) in 0.1 M sodium phosphate buffer (PBS; Roti-Histofix, pH 7.0; Carl Roth GmbH, Karlsruhe, Germany) if not stated otherwise. After fixation, the brains were briefly rinsed in sodium phosphate buffer (NAPI; 0.019 M NaH₂PO₄·H₂O, 0.0788 M Na₂HPO₄·H₂O, pH 7.4), embedded in gelatin/albumin (4.8% gelatin, 20% ovalbumin), and postfixed overnight at 4°C in 10% FA in NAPI. Brains were sectioned with a vibrating blade microtome (VT 1000; Leica, Wetzlar, Germany) at a thickness of 40 μm. Following incubations and rinsing of sections were performed on a shaker at RT. Free-floating sections were incubated for 30 min in Karnovsky–Roots medium (100 μM sodium citrate, 60 μM copper sulfate, 36 μM acetylthiocholine iodide, 10 μM potassium ferricyanide, and 30 μM tetraisopropyl pyrophosphoramide) in maleate buffer (0.1 M maleic acid, pH 7.6) containing 0.5% Triton X-100 (TrX). After the incubation, sections were rinsed 2–4 hr at room temperature (RT) or overnight at 4°C in 4% formaldehyde (FA) in 0.1 M sodium phosphate buffer (PBS; Roti-Histofix, pH 7.0; Carl Roth GmbH, Karlsruhe, Germany) if not stated otherwise. After incubation, the brains were briefly rinsed in sodium phosphate buffer (NAPI; 0.019 M NaH₂PO₄·H₂O, 0.0788 M Na₂HPO₄·H₂O, pH 7.4), embedded in gelatin/albumin (4.8% gelatin, 20% ovalbumin), and postfixed overnight at 4°C in 10% FA in NAPI. Brains were sectioned with a vibrating blade microtome (VT 1000; Leica, Wetzlar, Germany) at a thickness of 40 μm. Following incubations and rinsing of sections was performed on a shaker at RT. Free-floating sections were incubated for 30 min in Karnovsky–Roots medium (100 μM sodium citrate, 60 μM copper sulfate, 36 μM acetylthiocholine iodide, 10 μM potassium ferricyanide, and 30 μM tetraisopropyl pyrophosphoramide) in maleate buffer (0.1 M maleic acid, pH 7.6) containing 0.5% Triton X-100 (TrX). After the incubation, sections were rinsed 6 × 10 min with 0.05 M Tris-HCl (pH 7.6). Next, 0.04% 3,3’-diaminobenzidine tetrahydrochloride (DAB) and 0.3%nickel(II)sulfate-hexahydrate in Tris-HCl were incubated for 5 min followed by 0.003% hydrogen peroxide application. Sections were incubated for up to 30 min and finally rinsed with Tris-HCl for at least 3 × 5 min. Further procedure corresponded to immunofluorescence staining.

2.3 | Immunofluorescence staining

AChE histochemistry was always combined with single/double immunofluorescence, or antibodies against synapsin (DSHB Cat# 3C11 [anti-SYNORF1], RRID:AB_528479), horseradish peroxidase (HRP; Sigma-Aldrich Cat# P7899, RRID:AB_261181), histamine (Millipore Cat# AB5885, RRID:AB_177540), pigment-dispersing factor (PDF; DSHB Cat# PDF C7, RRID:AB_760350), corazonin (CRZ; J.A. Veenstra, University of Bordeaux 1, Bordeaux, France, Cat# anti-corazonin, RRID: AB_2532101), GABA (Sigma-Aldrich Cat# A2052, RRID:AB_477652), 5-HT (Sigma-Aldrich Cat# S5545, RRID:AB_477752), orcokinin (ORC; H. Dircksen, University of Bonn, Bonn, Germany, Cat# orcokinin, RRID: AB_2315017), myoinhibitory peptide (MIP; M. Eckert, University of Jena, Jena, Germany, Cat# MIP [myoinhibitory peptide], RRID: AB_2314803), FMRFamide (E. Marder, Brandeis University, Waltham, MA, Cat# FMRF, RRID:AB_2314414), or allatotropin (AT; J.A. Veenstra, University of Bordeaux 1, Bordeaux, France, Cat# AT [allatotropin], RRID:AB_2313973) were applied without AChE histochemistry (Table 1). Usually dissection, fixation, and slicing steps were carried out as explained for AChE histochemistry. Only samples with antiserum against histamine were fixated in 4% N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDAC, Sigma-Aldrich, Munich, Germany) diluted in PBS for 3–4 hr on ice with or without one additional hour in 4% FA. Samples with antiserum against GABA were fixated in 4% FA plus 0.1% glutaraldehyde in PBS for 2–4 hr. After slicing or—if AChE histochemistry was performed—after the DAB reaction and Tris-HCl washing steps took place, sections were rinsed 3 × 10 min with saline substituted Tris-buffer (SST; 0.016 M Tris base, 0.084 M Tris-HCl, 0.3 M NaCl, pH 7.4) containing 0.1% TrX. Afterward, 5% normal goat serum (NGS) in SST 0.5% TrX was preincubated for 2 hr to block nonspecific binding sites. Sections were then incubated with one or two primary antisera raised in different species (Table 1), diluted in SST 0.5% TrX with 1–2% NGS overnight. The following day, sections were washed 3 × with SST 0.1% TrX and then incubated with secondary antibodies at working dilutions of 1:100 goat anti-mouse (GaM) Alexa 647 or 1:300 GaM Cy2/goat anti-rabbit (GaR) Cy2/Cy3 (Dianova, Hamburg, Germany) in SST 0.5% TrX with 1% NGS for 1–2 hr in a dark place. Subsequently, sections were washed 3 × 10 min with SST 0.1% TrX, mounted on chromalaun/gelatin-coated microscope slides, dehydrated, and embedded in Entellan (Merck, Darmstadt, Germany).

For double immunostaining with primary antibodies raised in the same host species, monovalent Fab fragments of secondary antibodies were employed. The first primary antibody was applied in 1–2% NGS for overnight at RT (Table 1). After washing 3 × 10 min with SST 0.1% TrX, GaR-Fab fragment labeled with Cy3 or Alexa 647 (Dianova) at a concentration of 1:100 in 1% NGS was added for 3 hr to detect the first primary antibody. Subsequently, unconjugated GaR-Fab fragment (Dianova) was applied at the same dilution in 1% NGS for one additional hour to mask remaining binding sites on the first primary antibody. Afterward, sections were rinsed 3 × 10 min with SST 0.1% TrX. The second primary antibody was then applied overnight in 1% NGS (Table 1). After repetition of washing steps, sections were incubated with Cy2-conjugated GaR-Fab fragment (Dianova) at a dilution of 1:300 for 2 hr. The rest of the procedure was done as described above. Potential colocalization of different staining was very carefully evaluated in each slice of all stacks, section per section. Nevertheless, all figures shown are based on maximum projections of 40 μm sections, to show representing amounts of layers, the complete accessory lamina, or almost complete accessory medulla (AME). In addition, colocalization/coexpression of neuropeptides was double-checked when possible by single-cell MALDI-TOF (Susanne Neupert, Julia Schendzielorz, Monika Stengl, unpublished). Thus, we are very confident that colocalizations shown are at least minimum estimates of colocalized neuroactive substances in cockroach circadian clock neurons.

2.4 | Antibody characterization

The specificity of AChE histochemistry was tested and described by Schendzielorz (2013). In brief, 30 μM tetraisopropyl pyrophosphoramide in the Karnovsky–Roots medium inhibited nonspecific cholinesterases. In controls, acetylthiocholine iodide was skipped in the medium and no staining was observed. In additional control experiments, when 10⁻⁵ M AChE inhibitor 1,5-bis[4-allyldimethylammoniumphenyl]pentan-3-one
| Antibody                          | Immunogen                                           | Host species          | Working dilution | RRID, Cat          | Source/reference                                      |
|----------------------------------|-----------------------------------------------------|-----------------------|------------------|-------------------|-------------------------------------------------------|
| Anti-SYNORF1 (Synapsin)          | *Drosophila melanogaster* synapsin I isoform        | Mouse monoclonal      | 1:50             | AB_528479, Cat# 3C11 (anti-SYNORF1)                     | Developmental Studies Hybridoma Bank; Klagges et al. (1996) |
| Anti-horseradish peroxidase (HRP) | *Armoracia rusticana* peroxidase                    | Rabbit polyclonal     | 1:1000           | AB_261181, Cat# P7899                                  | Sigma-Aldrich                                                   |
| Anti-histamine                   | Histamine conjugated to keyhole limpet hemocyanin   | Rabbit polyclonal     | 1:15,000         | AB_177540, Cat# AB5885                                 | Chemicon-Millipore, Billerica, MA; Hamanaka, Kinoshita, Homberg, & Arikawa (2012) |
| Anti-Dm-pigment-dispersing factor (PDF) | *Drosophila melanogaster* PDF (NSELINSLSLPKMNNDa)   | Mouse monoclonal      | 1:1000           | AB_760350, Cat# PDF C7                                 | Developmental Studies Hybridoma Bank; Cyran et al. (2005) |
| Anti-γ-aminobutyric acid (GABA)  | GABA conjugated to bovine serum albumin (BSA) with  | Rabbit polyclonal     | 1:750            | AB_477652, Cat# A2052                                  | Sigma-Aldrich                                                   |
|                                  | glutaraldehyde                                      |                       |                  |                                                               |                                                       |
| Anti-serotonin (5-HT)            | 5-HT conjugated to BSA with glutaraldehyde         | Rabbit polyclonal     | 1:2000           | AB_477522, Cat# S5545                                  | Sigma-Aldrich                                                   |
| Anti-orcokinin (ORC)             | Orconectes limosus Asn13-ORC (NFDEIDRSGFGFN)        | Rabbit polyclonal     | 1:4,000          | AB_2315017, Cat# orcokinin                              | Heinrich Dircksen; Bungart, Dircksen, & Keller (1994)           |
| Anti-Peap-myoinhibitory peptide (MIP)-1 | *Periplaneta americana* MIP-1 (GWQDLQGGWamide)       | Rabbit polyclonal     | 1:8,000          | AB_2314803, Cat# MIP                                    | Predel, Rapus, & Eckert (2001)                                  |
| Anti-FMRFamide                   | Synthetic FMRFamide                                  | Rabbit polyclonal     | 1:2,000          | AB_23144414, Cat# FMRF                                  | Marder, Calabrese, Nusbaum, & Trimmer (1987)                     |
| Anti-corazonin (CRZ)             | *Periplaneta americana* CRZ (pQTFQYSRGWTNamide)      | Rabbit polyclonal     | 1:500            | AB_23132101, Cat# anti-corazonin                        | Veenstra & Davis (1993)                                           |
| Anti-allatotropin (AT)           | *Manduca sexta* AT (GFKNVEMMTARGFamide)              | Rabbit polyclonal     | 1:2,500          | AB_2313973, Cat#AT (allatotropin)                       | Veenstra & Hagedorn (1993)                                       |
dibromide (BW284c51) was added to the medium, specific staining was almost completely abolished. Residual nuclei labeling in cell nuclei apparently resulted from intrinsic peroxidase activity as previously reported for Manduca sexta (Homberg et al., 1995). The specificity of anti-synapsin antiserum was tested in Drosophila (Klagges et al., 1996) and it was previously used in various insect species (Gaburro et al., 2018; Groh & Rössler, 2011; Heinz, Florman, Asokaraj, el Jundi, & Reppert, 2013; Missbach, Harzsch, & Hansson, 2011; Phillips-Portillo, 2012; Zhao, Pfuhl, Surylkke, Tro, & Berg, 2013), while specificity of anti-HRP, -histamine, -PDF, -CR2, -GABA, -5-HT, -ORC, -MIP, -FMRFamide, and -AT antibodies on brain sections of R. madera were demonstrated in previous publications (Arendt, Baz, & Stengl, 2017; Giese et al., 2018; Hofer, Dircksen, Tollbäck, & Horberg, 2005; Lösel, Weigel, & Bräunig, 2006; Schendzielorz & Stengl, 2014; Schulze et al., 2012; Söhler, Neupert, Predel, & Stengl, 2008). Except for anti-HRP antiserum liquid-phase preadsorption of these antisera with different concentrations of synthetic neurotransmitters or -peptides was performed to determine whether applied antibodies bind to their corresponding antigen in R. madera.

2.5 Backfill experiments

For neurobiotin (Vector Laboratories, Burlingame, CA) backfill experiments, the animal was first anesthetized in ice water and then fixated on a wax petri dish. A constant CO2 inflow kept the animal stunned during the experiment. To expose the optic lobe, a rectangle on one side of the head capsule was excised and trachea as well as fatty tissue were carefully removed. Insect saline was used during the experiment to prevent drying. In the next step, either one optic nerve or the optic stalk was cut through with a precision shear. A glass capillary filled with a drop of neurobiotin was slipped over the severed optic nerve (0.07% neurobiotin) or the optic stalk (5%), respectively, and fixed with modeling clay. Finally, the animal was placed in a humidity box for almost 24 hr at 4°C to allow intracellular transport of the tracer. The next day, the brain was removed from the head capsule and processed for (immuno-) histochemistry. To label the neurobiotin, an optimum dilution of dye-coupled streptavidin with 1:100 for Cy2 and 1:300 for Alexa 405 (Dianova) was added in the incubation step of secondary antibodies.

2.6 Imaging

The evaluation of AChE histochemistry was carried out with the transmission mode of the confocal laser scanning microscope (CLSM; TCS SP5, Leica). All preparations labeled with fluorescence dyes were scanned with the confocal fluorescence mode. If AChE histochemistry was performed on the same section, transmission and fluorescence channels were scanned together. Preparations were scanned with a Leica HCX PL apochromate 20x/0.7 multi-immersion or 63x/1.20 water objective. Step size was 0.13–1 μm in z-direction, and the resolution was 1.024 x 1.024 or 2.048 x 2.048 pixels (Table S1). With a UV-laser Alexa 405 dye was excited at 405 nm, an argon laser-line excited Cy2 at 488 nm, and two HeNe lasers excited Cy3 at 543 nm or Alexa 647 at 647 nm, respectively. Emission was detected with photomultipliers in the range of 425–465 nm for Alexa 405, 500–540 nm for Cy2, 550–590 nm for Cy3, and 650–690 nm for Alexa 647. To avoid false-positive results, sequential scans were performed due to the overlapping emission spectra of some fluorophores. Analysis and editing of stacks was done using the ImageJ distribution Fiji (http://fiji.sc/wiki/index.php/Fiji or http://rsb.info.nih.gov/ij/). Each stack was imaged as maximum projection and adjusted concerning brightness and contrast. Figures were created with Inkscape 0.48.4 software (https://inkscape.org/de/release/inkscape-0.48.4/).

3 RESULTS

Photic entrainment pathways of the Madeira cockroach circadian clock, the accessory medulla (AME), are not well described. Compound eye photoreceptor neurons are required for photic entrainment of the cockroach clock, but they do not contact the AME directly (Lösel & Homberg, 1999). To determine which interneurons relay photic information from photoreceptor cells to the clock, multiple-label immunocytochemistry was combined with histochemistry and backfill studies. First, different layers of lamina and medulla were distinguished, as possible functional subdivisions. Then, multiple-label studies confined termination sites of short and long photoreceptor neurons to specific layers in lamina and medulla. Next, we searched for interneurons that interfaced photoreceptor neurons and circadian clock neurons. We employed antiserum against pigment-dispersing factor (PDF) as marker for cockroach circadian clock neurons and antisera against neuroactive substances predicted to be employed in photic entrainment (review: Stengl et al., 2015). Finally, backfills from the contralateral optic stalk allowed to identify arborizations of contralateral visual pathways in lamina and medulla layers.

3.1 Identification of 10 main layers in the medulla and three layers in the lamina via AChE histochemistry

With different neuroanatomical techniques we characterized distinct layers in lamina and medulla neuropils. Enzyme-mediated AChE histochemistry was employed previously to identify cholinergic neurons (Eckenstein & Sofroniew, 1983; Homberg et al., 1995; Karnovsky & Roots, 1964; Satoh, Armstrong, & Fibiger, 1983; Tago et al., 1986). It proved to be suited best for the differentiation of three lamina layers (LA1–3) and 10 main medulla layers (ME1–10) distally to proximally in horizontal optic lobe sections (Figure 1a; Table 2). AChE-labeled neuronal projections were detected in all lamina and medulla layers at varying intensities. Highest staining intensities were observed in LA1, LA3 in the accessory laminae, ME1, ME3, ME5, ME8, ME10, and the AME (Figure 1a; Table 2). Anti-synapsin (n = 10) and anti-horseradish peroxidase (HRP; n = 10) immunocytochemistry also allowed for the differentiation of 10 ME layers. However, layer boundaries were less defined compared to AChE staining (Figure 1b,c). In the lamina anti-synapsin immunocytochemistry labeled LA1 and LA2, but not LA3 (n = 10). With anti-HRP immunofluorescence
no reproducible distinction of lamina layers was possible \( n = 10 \). Consequently, in all following preparations immunocytochemistry was combined with AChE histochemistry to assign staining patterns to respective layers. In summary, AChE histochemistry distinguished three layers in the lamina, strongly stained accessory laminae, and revealed 10 layers in the medulla. The different optic neuropil layers might encode parallel retinotopic maps processing distinct visual cues together with extracellular space coordinates. Our further studies will determine in which of these optic lobe layers the spatial map is combined with temporal information provided by the circadian clock. Furthermore, future studies will determine whether AChE-stained neuropils are cholinergic.

3.2 | PDF clock cells arborize in accessory laminae and border short photoreceptor neurons in LA3 but not long photoreceptor neurons that arborize in ME2

Histamine-ir short photoreceptor axons prominently innervated LA1 and LA2, while long photoreceptor axons terminated in ME2 (Figure 2a,b). All histamine-ir loose arborizations in proximal layers ME4, ME6, and ME7 originated from a midbrain neuron identified previously (Figure 2a,b; Table 2; Arendt et al., 2017; Lösel & Homberg, 1999). From ME6 and ME7 this centrifugal histamine-ir neuron sent few fibers with varicose branches to ME4. From ME4 fibers continued on to ME2 where they intermingled with dense arborizations of long photoreceptor cell terminals (Figure 2a,b; \( n = 8 \) of 16). With neurobiotin backfills from single optic nerves, we confirmed the branching pattern of compound eye photoreceptor neurons in LA1, LA2, and ME2 (Figure 2c,d), but not in the AME (Lösel & Homberg, 1999). To identify potential contact sites between photoreceptor axons and clock neurons, multiple-label studies were employed with PDF antiserum to mark circadian clock neurons. In LA3 PDF-ir fibers from PDF lamina (PDFLAs) and PDF medulla cells (PDFMEs) directly bordered on backfilled short photoreceptor terminals. Both PDFLAs and PDFMEs also innervated the accessory laminae. As shown in Figure 1a, accessory laminae were strongly AChE-positive, but were

FIGURE 1  (a–c) Acetylcholinesterase (AChE) histochemical staining was chosen to distinguish layers in the lamina (LA) and medulla (ME). (a) In horizontal sections (40 μm) color-inverted AChE histochemistry allowed for distinction of 3.1 ± 0.5 LA layers. Also, the accessory lamina (ALA) was strongly AChE-positive. In the ME, 10.0 ± 0.0 layers became apparent. ME1, ME3, ME5, ME8, and ME10 were more strongly labeled compared to ME2, ME4, ME6, ME7, and ME9. (b) Anti-synapsin (SYN) immunofluorescence revealed 2.2 ± 0.4 LA layers and 10.2 ± 0.7 ME layers \( n = 10 \). (c) With horseradish peroxidase (HRP) labeling differentiation of LA layers was not possible, while 10.0 ± 1.3 layers of the ME were distinguished \( n = 10 \). Confocal laser images. Lines indicate layer boundaries. AME, accessory medulla (dashed oval); LO, lobula; d, distal; p, posterior. Scale bars = 50 μm.
| Labeled neurosubstance | Acetylcholinesterase (AChE) | Histamine | Optic nerve backfill | Pigment-dispersing factor (PDF) | Contralateral optic stalk backfill | y-amino-butyric acid (GABA) | Serotonin (5-HT) | Orcokinin (ORC) | Myoinhibitory peptide (MIP) | FMRFamide | Corazonin (CRZ) | Allatotropin (AT) |
|------------------------|-----------------------------|-----------|---------------------|-----------------------------|----------------------------------|-----------------------------|----------------|----------------|-----------------------------|-----------|----------------|---------------|
| LA layers n = 10       | n = 14 of 16                | n = 13 of 16 | n = 16 of 20        | n = 3 of 9                  | n = 9 of 16                       | n = 14 of 16                 | n = 5 of 7     | n = 4 of 9      | n = 7 of 12                      | n = 5 of 13 | n = 5 of 9       |               |
| 1                      | S                           | S         | S                   | N                           | N                                 | I                           | S              | I                           | N                           | S         | N              | I             |
| 2                      | I                           | S         | S                   | N                           | N                                 | I                           | S              | I                           | N                           | S         | N              | I             |
| 3                      | S                           | N         | N                   | S                           | I                                 | S                           | S              | I                           | N                           | S         | N              |               |
| ME layers              |                             |           |                     |                             |                                   |                             |                             |                             |                             |           |                 |               |
| 1.1                    | S                           | N         | N                   | S                           | S                                 | S                           | S              | I                           | I                           | S         | I              | N             |
| 1.2                    | S                           | N         | N                   | N                           | N                                 | I                           | I              | I                           | I                           | N         | I              |               |
| 2                      | I                           | S         | S                   | N                           | N                                 | I                           | S              | I                           | I                           | S         | I              | N             |
| 3                      | S                           | N         | N                   | S                           | I                                 | S                           | I              | I                           | I                           | I         | N              | I             |
| 4                      | I                           | I         | N                   | S                           | S                                 | I                           | S              | S                           | I                           | S         | I              | N             |
| 5                      | S                           | N         | N                   | N                           | N                                 | I                           | I              | I                           | N                           | I         | N              |               |
| 6.1                    | I                           | I         | N                   | N                           | S                                 | I                           | I              | I                           | I                           | I         | N              | I             |
| 6.2                    | I                           | I         | N                   | N                           | N                                 | I                           | I              | I                           | I                           | I         | N              | I             |
| 7                      | I                           | I         | N                   | N                           | I                                 | I                           | I              | I                           | I                           | N         | I              |               |
| 8                      | S                           | N         | N                   | N                           | N                                 | I                           | N              | I                           | I                           | N         | N              | I             |
| 9                      | I                           | N         | N                   | N                           | I                                 | N                           | I              | N                           | I                           | N         | N              | S             |
| 10                     | S                           | N         | N                   | N                           | I                                 | N                           | I              | N                           | N                           | N         | N              |               |
| ALA n = 7              | n = 6                        | n = 4     | n = 15               | n = 4                        | n = 11 of 13                     | n = 9                       | n = 6 of 8     | n = 6 of 7                  | n = 11                       | n = 4     |               |               |
| Ventral                | S                           | N         | N                   | S                           | S                                 | S                           | S              | I                           | S                           | S         | N              |               |
| AME n = 4              | n = 4                        | n = 5     | n = 9                | n = 5                        | n = 8                             | n = 14                       | n = 5          | n = 5                        | n = 10                       | n = 9     | n = 9          | n = 5         |
| S                      | I                           | N         | S                   | I                           | S                                 | S                           | S              | S                           | S                           | S         | S              |               |
neither histamine-ir nor did they receive innervation by backfilled compound eye photoreceptor neurons (Figure 2). Thus, these apparently cholinergic glomerular lamina neuropils are not connected to the compound eye. Via the anterior fiber fan PDF-ir fibers from PDFLAs and PDFMEs projected over the anterior face of the medulla in sublayer ME1.1, connecting the AME to proximal lamina (LA3) and accessory laminae (Figure 2c,d; Table 2). Two to four PDFMEs appeared to send side branches from the anterior fiber fan into ME4 forming the asymmetrical median-layer fiber system with considerably more extension in a dorsal region of ME4 (Figure 3a,b; Reischig & Stengl, 2002; Wei et al., 2010). Double-label immunocytochemistry with anti-PDF and anti-CRZ antisera showed strong overlap of staining in ME4. The CRZ-ir neuron had smooth, fine arborizations in a dorsal subregion of ME4 while spreading varicose terminals in a distinct adjacent dorsal and ventral subregion of ME4 (Figure 3c). Furthermore, varicose CRZ-ir terminals invaded the AME. Both the presumptive smooth input as well as the varicose output regions of the CRZ-ir neuron were in close vicinity to PDF-ir branches in ME4 while spreading varicose terminals in a distinct adjacent dorsal and ventral subregion of ME4 (Figure 3c). Furthermore, varicose CRZ-ir terminals invaded the AME. Both the presumptive smooth input as well as the varicose output regions of the CRZ-ir neuron were in close vicinity to PDF-ir branches in ME4 while spreading varicose terminals in a distinct adjacent dorsal and ventral subregion of ME4 (Figure 3c). Furthermore, varicose CRZ-ir terminals invaded the AME. Both the presumptive smooth input as well as the varicose output regions of the CRZ-ir neuron were in close vicinity to PDF-ir branches in ME4 while spreading varicose terminals in a distinct adjacent dorsal and ventral subregion of ME4 (Figure 3c).

3.3 | One CRZ-ir MNe per optic lobe connects two distinct dorsal compartments of ME4 to the AME, strongly overlapping with PDF-ir branches

Two to four PDFMEs send a side branch from the anterior fiber fan into ME4 forming the asymmetrical median-layer fiber system with considerably more extension in a dorsal region of ME4 (Figure 3a,b; Reischig & Stengl, 2002; Wei et al., 2010). Double-label immunocytochemistry with anti-PDF and anti-CRZ antisera showed strong overlap of staining in ME4. The CRZ-ir neuron had smooth, fine arborizations in a dorsal subregion of ME4 while spreading varicose terminals in a distinct adjacent dorsal and ventral subregion of ME4 (Figure 3c). Furthermore, varicose CRZ-ir terminals invaded the AME. Both the presumptive smooth input as well as the varicose output regions of the CRZ-ir neuron were in close vicinity to PDF-ir branches in ME4 (Figure 3a,b). Furthermore, the PDF- and CRZ-ir network in ME4 both extended into neighboring layers only in the dorsal ME. Also in the AME the PDF-ir arborizations overlapped with CRZ-ir varicose arborizations (data not shown; Arendt et al., 2017). In summary, the CRZ cell and a subgroup of PDFMEs appeared to be closely interconnected via both inputs and outputs in the dorsal ME4 and in the AME, forming a...
feedback circuit with unknown function. It remains to be resolved whether this subgroup consists of ipsi- or contralateral-projecting PDFMEs.

3.4 Seven layers of the medulla receive contralateral optic lobe innervation, while ME4 only receives ipsilateral, but not contralateral PDF-ir branches

With neurobiotin backfills from the contralateral optic stalk, contralateral innervations of LA3 and the accessory laminae of ME1.1, ME3, ME4, ME6, ME7, ME9, ME10, and the AME were identified (Figure 4a,c–g; Table 2). ME6 could be further subdivided into contralaterally innervated ME6.1 and only ipsilaterally innervated ME6.2 (Figure 4f; Table 2). Strongest contralateral innervation received the accessory laminae, ME1.1, ME3 (not shown), ME4, and ME6.1. Neurobiotin-labeled fiber bundles in the lobula valley tract were counted (23.2 ± 4.6; Table 3). Single bundles usually contained several axons and innervated just one or two specific ME layers (Figure 4e; Table 3). All contralateral arborizations in lamina and medulla (Figure 4a,c–g; Table 2) originated from medulla cell groups I–IV (MCI–IV) next to the AME (Reischig & Stengl, 2002; Söhler et al., 2011). While it was not possible to trace fiber projections from single MC groups to specific medulla layers, however, projections of PDF-ir MCI cells were identified in double-label studies. While PDFLAs never colocalized neurobiotin (n = 4), four of the anterior PDFMEs that belonged to MCI colocalized PDF and neurobiotin, as previously reported (Reischig & Stengl, 2002; Söhler et al., 2011). The posterior PDFMEs never colocalized PDF and neurobiotin and, thus, were ipsilateral PDFMEs. The PDF-ir branches in ME4 (Figure 4c,d) were strictly ipsilateral as they never colocalized neurobiotin (n = 4). But it could not be discerned whether they belonged to anterior or posterior PDFMEs. Most but not all PDF-ir fibers connecting the AME via the anterior fiber fan to LA3 and to the accessory laminae colocalized neurobiotin, indicating that both types of neuropils received ipsi- as well as contralateral innervation (Figure 4c,d,g; Reischig & Stengl, 2002; Söhler et al., 2011). Furthermore, AChE histochemical staining and PDF immunoreactivity strongly overlapped in the accessory laminae (Figure 4h). In summary, next to LA3 and the accessory laminae, among seven layers of the medulla most strongly innervated by contralaterally projecting optic lobe neurons were ME1.1, ME3, ME4, and ME6.1. Because all PDF-ir branches in ME4 were ipsilateral, they cannot belong to contralaterally projecting PDFMEs of MCI but apparently originate from either anterior and/or posterior ipsilateral PDFMEs.

FIGURE 3 (a–c) The corazonin-immunoreactive (CRZ-ir) medulla (ME) tangential neuron appears to connect to pigment-dispersing factor ME clock cells (PDFMEs) in ME4. Overlay of PDF- (magenta) and CRZ-ir (cyan) arborizations with (a) or without (b) schematic layers in a horizontal dorsal section of lamina (LA) and ME. A maximum of four PDF-ir side branches extended to ME4, where the CRZ-ir neuron also branched. The PDF- and CRZ-ir fibers branched asymmetrically with much stronger arborizations in a dorsal field of ME4 and extending into neighboring layers of the dorsal ME. (c) Frontal section of the CRZ-ir neuron (black). It comprises three distinct arborization sites, mostly smooth or mostly varicose branching arbors at different proximal–distal levels of the dorsal ME4, and a third arbor with varicose arborizations in the accessory medulla (AME; dashed oval). The mostly smooth (open arrowheads) and varicose (filled arrowheads) branching artery of the CRZ-ir neuron overlap with arborizations of PDF-ir fibers in the dorsal ME4 (a–c). Lines indicate layer boundaries. pLA, proximal lamina; LO, lobula (dotted line); d, distal; do, dorsal (for c); p, posterior (for a,b). Scale bars = 50 μm [Color figure can be viewed at wileyonlinelibrary.com]
GABA immunoreactivity is strongest at arborization sites of PDF clock cells, while 5-HT immunoreactivity is strongest at termination sites of photoreceptor neurons

As both GABA and 5-HT were implicated in photic entrainment of the AME (Gestrich et al., 2018; Page, 1987; Petri et al., 1995; Petri, Homberg, Lösel, & Stengl, 2002), we applied antisera against GABA and 5-HT for assignment to optic lobe layers. Next to GABA-ir fiber tracts in ME1.1, belonging to the distal tract and a fiber bundle of the anterior fiber fan, the GABA-ir medial layer fiber tract connected the AME to the medulla. GABA-ir fibers innervated the AME, all layers of the medulla, and all neuropils of the lamina. Strongest GABA staining was detected in LA3 and the accessory laminae via fibers of the
anterior fiber fan projecting in ME1.1, in ME4 due to innervation via the GABA-ir medial layer fiber tract, and in the AME that is innervated by all the GABA-ir tracts (Figure 5a,b; Table 2). With antiserum against 5-HT we stained PDFLAs next to the accessory laminae, as well as somata next to the AME (not shown; Giese et al., 2018; Petri et al., 1995). Strongest expression of 5-HT immunoreactivity was observed in LA1-3, the accessory laminae, ME1.1, ME2, and the AME (Figure 5c,d; Table 2). In summary, both GABA- and even more 5-HT-ir neurons could interact with short photoreceptor terminals in the lamina, as well as with long photoreceptor neurons in ME2. While GABA appears to be dominant at arborization sites of PDF-ir clock neurons, 5-HT dominates at termination sites of photoreceptor cells. The strong innervation of the accessory laminae by 5-HT further supports their photoreceptive function.

### Table 3

| ME layer | Fiber bundles (n = 5) |
|----------|----------------------|
| 1.1      | 1.8 ± 0.4            |
| 3/4/6.1/7a | 10.0 ± 3.6          |
| 9/10b    | 8.0 ± 4.1            |
| Unknown  | 3.2 ± 2.9            |
| Total    | 23.0 ± 4.6           |

* A total of six fiber bundles in two samples were traced to one single layer.
* Two fiber bundles in one sample divided into ME7 and ME9/10.

### Figure 5

(a–d) GABA-immunoreactive (−ir) fibers were present in all layers of lamina (LA) and medulla (ME), most prominently in the proximal lamina (pLA = LA3), ME1.1, and ME4. While serotonin (5-HT) immunoreactivity stained all layers in the LA and all layers of the ME, except ME8-10, it predominated in ME1.1, ME2, and LA1-3. (a,b) GABA-ir fibers innervated all glomeruli of the accessory medulla (AME) and projected via the distal tract (DT; arrowhead) in ME1.1 to undiscerned layers in the ME. Parallel to the DT GABA-ir fibers of the anterior fiber fan (AFF of ME1.1) continued on to the pLA and accessory laminae (singular: ALA, plural: ALAs). Furthermore, GABA-ir fibers connected the AME to ME4 via the medial layer fiber tract (MLFT; stars). (c,d) LA1-3, the ALAs, the AFF in ME1.1, and ME2 were strongly 5-HT-ir. ME1.2 and ME3-7 were more weakly 5-HT-ir. Confocal laser images were of 40 μm horizontal optic lobe sections. Neurotransmitter immunoreactivities (magenta) were implemented with or without (b,d) schematic layers (a) or color-inverted acetylcholinesterase (AChE) histochemistry (c; white). Lines indicate layer boundaries. LO, lobula; d, distal; p, posterior. Scale bars = 50 μm [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 6  (a–j) Medulla (ME) layer ME4 was most strongly innervated via different neuropeptide-immunoreactive (−ir) neurons such as orcinin (ORC)-, myoinhibitory peptide (MIP)-, FMRFamide-, corazonin (CRZ)-, and allatotropin (AT)-ir neurons. (a,b) ORC-ir fibers invaded all LA and ME layers. Strongest staining was present in ME4. (c,d) MIP-ir neurons projected into ME1-4 and ME6-8 with the most prominent staining in ME4. MIP-ir fibers connected the accessory medulla (AME; dashed oval) via the anterior fiber fan (AFF) in ME1.1 to the proximal lamina (pLA = LA3). (e,f) FMRFamide-ir fibers were most prominent in the AFF in ME1.1 connecting the AME to LA1-3, and in ME2. To a lesser extent they innervated ME3-9. (g,h) CRZ-ir fibers originated from one medial neuron that connected the AME and ME4 via the medial layer fiber tract. From ME4 CRZ-ir branches projected down into ME3, ME2, and ME1.2, terminating in ME1.1 (boxed area, arrowheads), not innervating the LA. (i,j) LA1 and LA2 were weakly AT-ir (boxed area). Many AT-ir neurons projected fibers parallel to the columns of the ME and innervated ME1.2 and ME4-9. Strongest staining occurred in ME5 and ME9. Confocal laser images of 40 μm horizontal optic lobe sections. Implementation of schematic layers (a,g,i) or color-inverted acetylcholinesterase (AChE) histochemistry (c,e; white) combined with neuropeptide immunoreactivities (magenta). Lines indicate layer boundaries. d, distal; p, posterior. Scale bars = 50 μm (25 μm in boxed areas of h,j) [Color figure can be viewed at wileyonlinelibrary.com]
Strong contralateral optic lobe inputs (Figures 2a,b, 4e, and 6a–f) were innervated by pigment-dispersing factor-immunoreactive (PDF-ir) fibers that colocalized orcokinin (ORC; c,d), myoinhibitory peptide (MIP; e,f), or FMRFamide immunoreactivity (g,h). PDF-ir somata next to the ALA (PDF-LAs; c; arrowheads) branched in the ALA. (g,h) Two PDF- and FMRFamide-ir fiber bundles projected into separate glomeruli of the ALA (dashed ovals). No CRZ- (i,j) or allatotropin (AT)-ir (k,l) fibers were detected in the ALA. Confocal laser images of 40 μm horizontal (a,b,e–l) or frontal (c,d) optic lobe sections. Other neuropeptide immunoreactivities (cyan) were coexpressed with PDF immunoreactivity (a,c,e,g,i,k; magenta). LA, lamina; ME, medulla; d, distal; do, dorsal (for c,d); p, posterior (for a,b,e–i). Scale bars = 25 μm [Color figure can be viewed at wileyonelibrary.com]

Strong contralateral optic lobe inputs (Figures 2a,b, 4e, and 6a–f; Table 2). Interestingly, both ME4 and AME were labeled by all neuroactive substances tested in this study, and most of them expressed strong staining intensity (Figures 1–6; Table 2). As described above (Figure 3) almost all arborizations of the single CRZ-ir MNe per optic lobe were restricted to the AME and ME4 where they overlapped with processes stained by all antiserum tested. Only few CRZ-ir fibers innervated ME1.1, where all other antiserum stained, except AT antiserum. Few CRZ-ir fibers reached ME1.2, where also ORC-, MIP-, and AT-ir processes branched, and ME2, where all antiserum stained, except AT antiserum. Furthermore, few CRZ-ir fibers branched in ME3, also overlapping with all other neuropeptidergic neurons examined, except AT-ir cells. Fine CRZ-ir processes (indicative of dendritic arbors) were found only in ME4 and in neighboring layers of the dorsal ME, while forming varicose terminals in the AME, in ME1, ME2, ME3, and also in ME4 (Figure 3 and 6g,h; Table 2). Unexpectedly, AT-ir branches just overlapped with histamine-ir short photoreceptor terminals in LA1 and LA2, but were detected neither in LA3, nor in the accessory laminae, nor in the anterior fiber fan in ME1.1, nor in ME2 (Figure 6i,j; Table 2). Instead, they arborized most strongly in ME5 and ME9, with intermediate staining intensity in ME1.2, ME4, and ME6–8. Thus, AT-ir local neurons must occur in the lamina and other AT-ir neurons only innervated proximal layers in the ipsilateral medulla. In summary, ME4 is abundant of neuropeptidergic innervation and most strongly connected to the circadian clock.

3.7 | PDF and AChE signals, but not histamine overlapped in the glomeruli of the accessory laminae

As shown previously, GABA- and 5-HT-ir terminals innervate the accessory laminae (Figure 5; Table 2). Employing multiple-label immunocytochemistry the dorsal (not shown) and ventral accessory lamina
were investigated further. PDF-ir PDFLAs and PDFMEs innervated both dorsal and ventral accessory lamina (Figure 7a,c,e,g,i,k; Table 2), which were both devoid of anti-histamine staining (Figure 7a,b; Table 2). Furthermore, ORC-ir or MIP-ir processes branched in the accessory laminae, partially colocalizing PDF, indicative of medium-sized PDFMEs (Figure 7c–f; Table 2). Strongest double labeling was observed in accessory laminae with PDF and FMRFamide antisera, which identified a subdivision of the neuropil into one distal and one proximal double-labeled glomerulus innervated by separate fiber bundles, with stronger FMRFamide immunoreactivity in the proximal glomerulus (Figure 7g,h; Table 2). In contrast, CRZ (Figure 7i,j; Table 2) and AT immunoreactivity (Figure 7k,l; Table 2) were not found in...
accessory laminae. To summarize, we found no evidence for histaminergic photoreceptor terminals in the accessory laminae, but for cholinergic, GABAergic, serotonergic, and neuropeptidergic innervation.

4 | DISCUSSION

In search for light entrainment pathways to the cockroach circadian clock, the accessory medulla (AME), it was examined whether there are direct or indirect connections between pigment-dispersing factor (PDF)-processing circadian clock neurons in the lamina (PDFLAs) or medulla (PDFMEs) and compound eye photoreceptor cells. Acetylcholinesterase (AChE) histochemistry, multiple-label immunocytochemistry, and backfills from optic nerves of the compound eye or the contralateral optic lobe were performed. Thereby, three layers of the lamina (LA1–3) and 10 layers of the medulla (ME1–10) were distinguished. While short green-sensitive photoreceptor neurons terminated in LA1 and LA2 bordering on PDF-immunoreactive (-ir) terminals in LA3, the long UV-sensitive compound eye photoreceptor neurons terminated in ME2 (Strausfeld, 2012) without direct contact to PDF-ir clock neurons that branched in ME4 (Figure 8). Evidence was presented that PDF-, FMRFamide-, and 5HT-ir lamina neurons (PDFLAs), parallel to neuropeptidergic/GABAergic medial neurons (MNe), could relay ipsi- and contralateral light information via the anterior fiber fan from LA3 to the AME. In addition, via a separate branch of the anterior fiber fan PDFLAs and MNe connect AChE innervating neurons to dorsal and ventral accessory laminae. Accessory laminae did not receive input from histaminergic compound eye photoreceptor cells. Instead they appeared to receive cholinergic photoreceptor input from the lamina organ, an extraocular photoreceptor organ (Fleissner et al., 2001). Each accessory lamina expressed differential staining in at least two glomerular subcompartments indicative of parallel information processing. Also different neuropeptidergic interneurons of the medulla interconnect ME2, ME4, and AME apparently relaying ipsi- and contralateral light information from long compound eye photoreceptor neurons in parallel lines to the circadian clock. In the context of adjustment to different light phases at dawn and dusk the corazolin (CRZ)-ir MNe seems to be part of phase advance lines, while allatotropin (AT)-ir MNe may be part of phase delay lines. It became apparent that ME4 is the main input/output region of the circadian clock in the optic lobes, next to LA3 and the accessory laminae. We present here a new testable hypothesis on how the different photic entrainment pathways could connect to different PDFMEs in the circadian clock to control rest-activity rhythms (Figure 8).

4.1 | The number of layers in lamina and medulla are conserved among hemi- and holometabolous insects

In hemi- as well as in holometabolous insects the main photoreceptor organs are the compound eyes. The lamina and medulla, as well as most nested neuropils of the lobula (Rosner, von Hadeln, Salden, & Homberg, 2017) maintain topographic organization with regular parallel columns that form a retinotopic map of the external environment, thus, encoding external space (Strausfeld, 1976). Tangential neurons interconnect columns, branching perpendicularly to the columns and, thereby forming layers (= strata; Campos-Ortega & Strausfeld, 1972). These layers serve parallel visual processing and encode different parameters of photic information such as light intensity, contrast, form, color, and motion embedded into spatial information (review: Borst, Haag, & Reiff, 2010). As tangential neurons interconnect corresponding layers between optic neuropils, they are the neuronal elements of parallel visual information processing. Because a main role of the circadian clock is to guarantee synchronization between the organism’s endogenous timing and the timing of the external light dark cycle, we searched for direct/indirect connections between the circadian clock and terminals of compound eye photoreceptor axons. Thus, we concentrated our analysis on tangential neurons connecting layers of lamina and medulla to the AME. We did not focus on the lobula which is involved in small- and large-field motion detection, looming responses, and stereopsis (Aptekar, Keles, Lu, Zolotova, & Frye, 2015; Nordström & O’Carroll, 2006). A detailed analysis of the lobula of the Madeira cockroach when compared with lobulae of other species was published recently (Rosner et al., 2017).

Consistent with the structure of the medulla in the fruitfly Drosophila melanogaster (Fischbach & Dittrich, 1989), in butterflies (Hamanaka et al., 2012; Heinze & Reppert, 2012), in the locust Schistocerca gregaria (Homberg, Brandl, Clynen, Schoofs, & Veenstra, 2004), in the mantis Hierodula membranacea (Rosner et al., 2017), and in another study of R. maderae (Rosner et al., 2017), we distinguished 10 layers of the medulla, despite the fact that we employed different techniques compared to previously published work. However, only eight medulla layers were described in Pieris brassicae (Strausfeld & Blust, 1970) and different species of Calliphora (Strausfeld, 1970). It was suggested before that these differences do not result from different staining methods. Rather, differences in layer organization, even between related species, were assumed to result from functional adaptation to different lifestyles during evolution (Heinze & Reppert, 2012). In our study, we wanted to determine in which optic lobe neuropils/layers photoreceptor cells could contact PDF-processing circadian pacemaker neurons of the Madeira cockroach as possible circadian light entrainment pathways.

4.2 | Parallel light entrainment pathways to morning or evening oscillator circuits of the insect circadian clock

The fruitfly D. melanogaster expresses a bimodal activity pattern with a peak in the morning and a peak in the evening, anticipating lights on or off. Consistent with the two oscillator models proposed previously for vertebrates (Daan & Pittendrigh, 1976), two separate circadian clock networks were identified for the generation of the morning and evening activity peak of Drosophila (Grima, Chélot, Xia, & Rouyer,
control the activity of (E) oscillator couples to dusk, being delayed by light (Helfrich-Förster, 2004). While the morning (M) oscillator network is coupled to dawn, being advanced by light, the evening (E) oscillator couples to dusk, being delayed by light (Helfrich-Förster, 2009). In fruitflies, the PDF-ir small LNvs are M oscillator cells, which control the activity of Drosophila in the morning, while PDF-ir large LNvs together with neurons processing other neuropeptides are part of at least three E oscillator circuits controlling activity and sleep at dusk and during the night (Helfrich-Förster, 2017; Johard et al., 2009; Schlichting, Diaz, Xin, & Rosbash, 2019; Schlichting et al., 2019; Schubert, Hagedorn, Yoshii, Helfrich-Förster, & Rieger, 2018).

In contrast to the holometabolous crepuscular fruitfly, the Madeira cockroach is a hemimetabolous nocturnal insect. While cockroaches express mostly continuous activity throughout the night, nevertheless, an oscillator network coupled to dawn (M oscillator), promoting sleep and an E oscillator network coupled to dusk, promoting activity, may govern sleep–wake patterns synchronized to changing photoperiods (review: Stengl & Arendt, 2016). Since the long-living (>2 years) Madeira cockroach that radiated from the equator to moderate time zones needs to adjust to both long and short days during the turn of the year, we expect at least four parallel light input pathways relaying advancing and delaying phase information to the clock’s M and E circuits, respectively. Furthermore, there may be an additional light input pathway that signals ambient light levels distinguishing day from night. The cellular nature of M and E oscillators and parallel entrainment pathways to these oscillators is not known in the Madeira cockroach. However, based upon physiological and morphological evidence, a hypothesis was formulated (Gestrich et al., 2018) that predicted that under 12 hr light and 12 hr dark periods ipsilateral PDFMEs are sleep-rest-promoting M oscillator cells that are advanced by light at dawn. In addition, contralaterally projecting PDFMEs were predicted to be activity-promoting E oscillator cells (Figure 8) that are delayed by light at dusk. Furthermore, M cells were predicted to be active only during the light period, while antagonistic E cells were active only during the dark period per day (Gestrich et al., 2018; Page, 1978; review: Stengl & Arendt, 2016).

In the fruitfly, the AME is a hub for light inputs from different organs that guarantee photic entrainment of the circadian clock (Li et al., 2018). The Drosophila circadian clock receives monosynaptic input from the Hofbauer-Buchner (HB) eyelet, an extracellular photoreceptor organ in the brain’s optic lobes (Helfrich-Förster et al., 2002; Hofbauer & Buchner, 1989). Furthermore, the fruitfly AME receives indirect inputs from the compound eye via interneurons. Interestingly, the HB eyelet directly activates the PDF-ir M oscillators via cholinergic inputs, while via histaminergic input it directly inhibits the PDF-ir large LNvs that are arousal neurons and are part of the E oscillator circuit (Schlichting et al., 2016). Furthermore, the HB eyelet antagonizes with its differential connections the indirect photic inputs of the compound eye to the respective M and E oscillators dependent on daytime and on light regimes (Schlichting, Diaz, et al., 2019; Schlichting et al., 2016; Schlichting, Weidner, et al., 2019). Thus, in analogy to the Drosophila circadian system, we would expect that lamina and lobula organs play a similar role as the extraocular HB eyelet, providing opposite inputs to M and E oscillator circuits and antagonizing compound eye inputs clock circuit- and light level-dependently (Figure 8).

4.3 | Cholinergic lamina and lobula organs as extraretinal photoreceptor neuropils are hypothesized to relay ambient light levels indicative of day or night to the circadian clock

In beetles, lamina and lobula organs appear to be extraocular photoreceptive organs that send photoreceptor terminals to their respective first-order optic neuropils: the accessory laminae and the AME (Frisch, Fleissner, Fleissner, Brandes, & Hall, 1996). In the Madeira cockroach, the lamina and lobula organs were also suggested to house extraocular photoreceptors that transmit ambient light information to accessory laminae and to the AME, the circadian clock. Consistent with this hypothesis, they were stained with antisera against UV-opsin and CRYPTOCHROME (Fleissner et al., 2001; Hofer, 2004). In D. melanogaster, CRYPTOCHROME is a blue-light-sensitive chromophore that directly affects clock proteins in the molecular feedback loop of the circadian clockwork (Ivanchenko, Stanewsky, & Giebultowicz, 2001). Furthermore, based upon physiological and morphological evidence, a hypothesis was formulated (Gestrich et al., 2018) that predicted that under 12 hr light and 12 hr dark periods ipsilateral PDFMEs are sleep-rest-promoting M oscillator cells that are advanced by light at dawn. In addition, contralaterally projecting PDFMEs were predicted to be activity-promoting E oscillator cells (Figure 8) that are delayed by light at dusk. Furthermore, M cells were predicted to be active only during the light period, while antagonistic E cells were active only during the dark period per day (Gestrich et al., 2018; Page, 1978; review: Stengl & Arendt, 2016).

In beetles, lamina and lobula organs were labeled with antisera against UV-opsin and CRYPTOCHROME (Fleissner et al., 2001; Hofer, 2004). In D. melanogaster, CRYPTOCHROME is a blue-light-sensitive chromophore that directly affects clock proteins in the molecular feedback loop of the circadian clockwork (Ivanchenko, Stanewsky, & Giebultowicz, 2001). Furthermore, based upon physiological and morphological evidence, a hypothesis was formulated (Gestrich et al., 2018) that predicted that under 12 hr light and 12 hr dark periods ipsilateral PDFMEs are sleep-rest-promoting M oscillator cells that are advanced by light at dawn. In addition, contralaterally projecting PDFMEs were predicted to be activity-promoting E oscillator cells (Figure 8) that are delayed by light at dusk. Furthermore, M cells were predicted to be active only during the light period, while antagonistic E cells were active only during the dark period per day (Gestrich et al., 2018; Page, 1978; review: Stengl & Arendt, 2016).

In the fruitfly, the AME is a hub for light inputs from different organs that guarantee photic entrainment of the circadian clock (Li et al., 2018). The Drosophila circadian clock receives monosynaptic input from the Hofbauer-Buchner (HB) eyelet, an extracellular photoreceptor organ in the brain’s optic lobes (Helfrich-Förster et al., 2002; Hofbauer & Buchner, 1989). Furthermore, the fruitfly AME receives indirect inputs from the compound eye via interneurons. Interestingly, the HB eyelet directly activates the PDF-ir M oscillators via cholinergic inputs, while via histaminergic input it directly inhibits the PDF-ir large LNvs that are arousal neurons and are part of the E oscillator circuit (Schlichting et al., 2016). Furthermore, the HB eyelet antagonizes with its differential connections the indirect photic inputs of the compound eye to the respective M and E oscillators dependent on daytime and on light regimes (Schlichting, Diaz, et al., 2019; Schlichting et al., 2016; Schlichting, Weidner, et al., 2019). Thus, in analogy to the Drosophila circadian system, we would expect that lamina and lobula organs play a similar role as the extraocular HB eyelet, providing opposite inputs to M and E oscillator circuits and antagonizing compound eye inputs clock circuit- and light level-dependently (Figure 8).

4.4 | The largest PDFME keeps rest-promoting M and activity-promoting E circuits in stable antiphase

As the largest contralaterally projecting cPDFME that arborized in accessory laminae, in LA3, and AME expresses excitatory ACh and inhibitory GABA receptors (Gestrich et al., 2018) it could be activated via cholinergic...
input during the day and inhibited GABA-dependently during the night. Thus, differential light inputs from extracellular lamina organ and compound eyes would orchestrate it daytime-dependently (Figure 8a). Light regime-dependent modulation of light inputs was also observed in Drosophila indicating the circadian network gates light inputs daytime-dependently and light input-dependently (Lazopulo, Lazopulo, Baker, & Syed, 2019; Schlichting, Díaz, et al., 2019; Schlichting, Weidner, et al., 2019). Thus, we predict that the largest PDFME releases PDF during the day, at all sites where it overlaps with other PDFMEs, such as in the AME. There, PDF release would affect all clock neurons that express PDF receptors. As there was a strict correlation between the branching pattern and PDF sensitivity in AME clock cells (Gestrich et al., 2018) the ipsilaterally remaining PDF-sensitive clock neurons (such as PDFMEs) were activated by PDF, while contralaterally branching PDF-sensitive AME neurons (such as PDFMEs) were inhibited by PDF (Figure 8). As PDF neurons orchestrate sleep–wake cycles in the Madeira cockroach (Reischig & Stengl, 2003a; Stengl & Homberg, 1994), we suggest that the ipsilateral PDFMEs that are active during the rest phase of the cockroach are sleep promoting. Because contralateral circadian clock neurons were suggested to control locomotor activity rhythms (Page, 1978) and because contralateral PDFMEs are inhibited during the day, when cockroaches rest, we suggest that they are activity-promoting. When light levels decline at dusk the largest PDFME is not activated any more light-dependently and its GABAergic inhibition overruns its cholinergic activation, releasing the contralateral PDFMEs from its PDF-dependent inhibition. Thus, the largest PDFME that itself does not express PDF autoreceptors could maintain sleep-promoting M and activity-promoting E circuits in stable antiphase. Future experiments will test whether indeed the largest PDFME is required for stable sleep–wake cycles and is activated during the day via cholinergic input from lamina and lobula organs.

4.6 | The CRZ-ir MNe is part of the advancing light entrainment pathway to M circuits closely interconnected with ipsilateral PDFMEs while AT neurons as part of the delaying entrainment pathway may connect to contralateral PDFMEs

In support of this hypothesis is the single CRZ processing MNe that arborizes in ME4, extends a process to ME2 where UV-sensitive long photoreceptors terminate and strongly arborizes in the AME (Arendt et al., 2017; Petri et al., 1995). As injections showed that CRZ only advances the clock at dawn this cell would be part of the M circuit that should connect to ipsilateral PDFMEs. Indeed, it overlaps with the PDF-ir median-layer fiber system in a dorsal subregion of ME4 that backfills proved to originate from ipsilateral PDFMEs only. It is not known which two to four ipsilateral PDFMEs send an ipsilateral side branch from the anterior fiber fan into ME4, forming the median-layer fiber system (Reischig & Stengl, 2002). However, because these PDF-ir fibers of the median-layer fiber system do not colocalize 5-HT, nor orcokinin (ORC), nor FMRFamide, nor leucokinin, it is likely that they originate from the posterior PDFMEs (Giese et al., 2018; Petri et al., 1995; Söhler et al., 2011). Future experiments will examine whether posterior, but not anterior PDFMEs express CRZ receptors. Also in the AME the CRZ cell could contact ipsi- as well as contralateral PDFMEs. Physiological studies will examine whether ipsi- and contralateral AME neurons respond antagonistically to CRZ, as predicted by our hypothesis.

As injections of AT revealed only phase delays, AT-ir MNe are suggested to be part of the phase delay pathway to E cells. The AT immunoreactivity was strongest in the AME, in ME5, and ME9. Thus, AT-ir neurons could contact contralaterally projecting anterior PDFMEs as part of the E circuit in the AME. Whether in ME5 and ME9 E cells arborize remains to be examined. Future experiments will test whether PDFLAs with arborizations in the proximal lamina relay input from green-sensitive photoreceptor neurons of the compound eye E circuits of the AME. Thus, in the AME both M and E circuits could be antagonistically connected via redundant neuropeptide and neurotransmitter pathways, to keep both circuits in antiphase or to shift their respective weight, depending on strength, frequency, and duration of the light inputs.

4.5 | Different parallel light entrainment pathways are suggested to antagonistically connect M and E clock circuits in the Madeira cockroach

As green light was activity promoting and UV light was inactivity promoting in cockroaches there are parallel antagonistic pathways to premotor areas connected to specific photoreceptors (Zhukovskaya, Novikova, Saari, & Frolov, 2017). Thus, we hypothesize that also antagonistic, parallel photic entrainment pathways from different wavelength photoreceptors connect to the circadian clock that mediates behavior daytime-dependently similar to Drosophila clock circuits (reviews: Helfrich-Förster, 2019; Stengl & Arendt, 2016). We predict that at night green-sensitive short compound eye photoreceptors provide excitatory input in LA3 to contralateral PDFMEs as activity-promoting E cells while inhibiting ipsilateral PDFMEs (Figure 8b). In contrast, during the day UV-sensitive long compound eye photoreceptors provide excitatory photic input to rest-promoting ipsilateral PDFMEs and inhibitory light input to activity-promoting contralateral PDFMEs in the AME via interneurons that connect ME2, ME4, and AME (Figure 8). Current experiments are testing this hypothesis.

4.7 | Multiple neuropeptidergic circuits in the medulla may relay advancing and delaying photic and nonphotic phase information via ME4 to and from the circadian clock

Injections of different neuropeptides or neurotransmitters into the vicinity of the circadian clock determined Zeitgebertime (ZT)-dependent phase shifts of locomotor activity rhythms. Therefore, these substances were part of input pathways to the clock (Arendt et al., 2017; reviews: Stengl & Arendt, 2016; Stengl et al., 2015). All phase-advancing and/or phase-delaying neuroactive agents appear to be present in different neurons innervating ME4 and AME. Thus, ME4 is a major input area for circadian clock neurons and also receives outputs from the clock. So far, the
functions of these many clock inputs and outputs are not known. However, we assume that these circadian clock neurons release their respective neuropeptides ZT-dependently to control the phase of various physiological and behavioral rhythms in the cockroach (reviews: Stengl & Arendt, 2016; Stengl et al., 2015).

The strong innervation of ME4 as transition layer between the distal and proximal ME is striking and resembles the serpentine layer in other insects (Fischbach & Dittrich, 1989; Hamanaka et al., 2012; Heinze & Reppert, 2012; Xi, Toyoda, & Shiga, 2017). ME4 is densely innervated by the GABA-ir medial-layer fiber tract and by many neuropeptidergic neurons such as the PDFMNEs. All neuropeptides and neurotransmitters examined were found in ME4, apparently processing parallel lines of clock inputs and clock outputs. Accordingly, Schubert et al. (2018) in D. melanogaster also described clock inputs and outputs in the serpentine layer. Furthermore, in ME4/serpentine layer polarized light information as important cue for spatial orientation is processed in different insect species. Previous work in locusts and flies identified the polarization vision pathway from photoreceptors in the dorsal rim area of the compound eyes to ME4/serpentine layer, to the anterior optic tubercles, to the lateral accessory lobes, and then, to the lower division of the central complex (el Jundi, Pfeiffer, & Homberg, 2011; Omoto et al., 2017). Consistently, Lösel and Homberg (2001) identified polarization-sensitive contralaterally projecting ventromedial neurons (VMNes) with arborizations in ME4 (Zeller et al., 2015; review: el Jundi, Pfeiffer, Heinze, & Homberg, 2014). The elaboration of CRZ-ir processes mostly in the dorsal area of the retinotopic map makes it likely that the CRZ neuron receives excitatory (advancing) light input from photoreceptors with dorsal receptive fields. Apparently in the same region of the medulla ipsi- and contralaterally projecting VMNes of the AME arborize that process polarization vision (Lösel & Homberg, 2001). Thus, we hypothesize that the CRZ cell receives polarization information from a so far not described dorsal area of the cockroach compound eye, reminiscent of the dorsal rim area of locusts (review: Homberg, 2015). As the polarization pattern has its highest contrast in the UV range, these cells may be connected to UV-light detecting polarization-sensitive photoreceptor neurons. Future experiments will test this hypothesis and will determine whether ME4 is the first interaction of the circadian clock with the polarization vision pathway to allow for navigation oriented in space and time, CRZ-dependently.

To summarize, we hypothesize that light inputs from cholinergic extracorollar photoreceptors in the lamina and lobula organs entrain sleep–wake rhythms of the Madeira cockroach via modulation of endogenous PDF release by the largest PDFMNE that maintains rest-or activity-promoting clock circuits in stable antiphase. Furthermore, parallel, either UV-sensitive compound eye photoreceptor neuron terminals in ME2 or green-sensitive terminals in the LA antagonistically inhibit or activate M or E oscillator circuits via different MNes or via different PDF processing neurons. Thus, ZT-dependently via distinct neuropeptide release, phase relationships of internal rhythms are controlled in synchrony with external light–dark cycles (Figure 8). Future experiments will test our hypothesis.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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