Distribution and daily oscillation of GABA in the circadian system of the cockroach *Rhyparobia maderae*

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Abstract

Gamma-aminobutyric acid (GABA) is the prevalent inhibitory neurotransmitter in nervous systems promoting sleep in both mammals and insects. In the Madeira cockroach, sleep-wake cycles are controlled by a circadian clock network in the brain's optic lobes, centered in the accessory medulla (AME) with its innervating pigment-dispersing factor (PDF) expressing clock neurons at the anterior-ventral rim of the medulla. GABA is present in cell clusters that innervate different circuits of the cockroach's AME clock, without colocalizing in PDF clock neurons. Physiological, immunohistochemical, and behavioral assays provided evidence for a role of GABA in light entrainment, possibly via the distal tract that connects the AME's glomeruli to the medulla. Furthermore, GABA was implemented in clock outputs to multiple effector systems in optic lobe and midbrain. Here, GABAergic brain circuits were analyzed further, focusing on the circadian system in search for sleep/wake controlling brain circuits. All GABA-immunoreactive neurons of the cockroach brain were also stained with an antiserum against the GABA-synthesizing enzyme glutamic acid decarboxylase. We found strong overlap of the distribution of GABA-immunoreactive networks with PDF clock networks in optic lobes and midbrain. Neurons in five of the six soma groups that innervate the clock exhibited GABA immunoreactivity. The intensity of GABA immunoreactivity in the distal tract showed daily fluctuations with maximum staining intensity in the middle of the day and weakest staining at the end of the day. Quantification via enzyme-linked immunosorbent assay and quantitative liquid chromatography coupled to electrospray ionization tandem mass spectrometry, likewise, showed higher GABA levels in the optic lobe during the inactivity phase of the cockroach during the day and lower levels during its activity phase at dusk. Our data further support the hypothesis that light- and PDF-dependently the circadian clock network of the cockroach controls GABA levels and thereby promotes sleep during the day.

Key words

accessory medulla, circadian rhythms, glutamic acid decarboxylase, immunocytochemistry, insect brain

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1 | INTRODUCTION

Circadian clocks control the timing of rhythms in physiology and behavior entrained to the 24 h cycle of day and night in virtually all organisms. In mammals and insects alike, master circadian clock networks in the brain regulate circadian sleep-wake cycles synchronized to the daily light dark cycle. They are closely associated with the visual system and are abundant of neuropeptides, with γ-aminobutyric acid (GABA) being their most prominent neurotransmitter (reviews: Helfrich-Förster, 2004, 2005; Maywood, 2018; Ono et al., 2018). In mammals, nearly all circadian clock neurons in the suprachiasmatic nucleus are GABAergic (Albers et al., 2017; Moore & Speh, 1993; Ono et al., 2018). Pharmacological studies suggest that GABA is involved in light entrainment (Gillespie et al., 1997; Novak & Albers, 2004; Ralph & Menaker, 1985, 1989), internal coupling and synchronization of circadian clock cells (Liu & Reppert, 2000; Wagner et al., 1997), as well as in gating of clock outputs (Ono et al., 2019). Furthermore, not only in mammals, but also in insects, GABA appears to be a major sleep-promoting neurotransmitter closely associated with the circadian clock network (Hamasaka et al., 2005; Helfrich-Förster, 2018; Ono et al., 2018).

In the Madeira cockroach, Rhyparobia maderae the circadian clock that controls sleep-wake cycles is located in the accessory medulla (AME) (Reischig & Stengl, 2003a; Stengl & Homberg, 1994). The cockroach AME is a small, nonretinotopically organized neuropil in the optic lobe at the anterior-ventral rim of the medulla (Reischig & Stengl, 1996, 2003b). It is abundant of GABA and partly colocalized neuropeptides, among them pigment-dispersing factor (PDF) as best characterized (Giese et al., 2018a, b; Petri et al., 1995; reviews: Stengl & Arendt, 2016; Stengl et al., 2015). The PDF expressing clock neurons take part in clock inputs, local circuits, and clock outputs, such as for the control of the cockroach’s sleep-wake rhythms (Reischig & Stengl, 2003a; Stengl & Homberg, 1994). These important PDF circadian clock neurons were suggested to closely interact with GABAergic neuronal networks in the regulation of the temporal control of information processing at multiple time scales (Schneider & Stengl, 2005, 2006, 2007; review: Stengl & Arendt, 2016).

Likewise, GABA apparently serves multiple roles in the circadian system of the Madeira cockroach. The glomeruli of the cockroach AME are densely innervated by GABA-immunoreactive (GABA-ir) processes of the distal tract that connects the AME to distal layers of the medulla where long photoreceptor neurons of the compound eye terminate (Giese et al., 2018a). Thus, it was suggested that the distal tract relays indirect light input as photic entrainment pathway into the circadian clock (Arnold et al., 2020; Petri et al., 2002; Reischig & Stengl, 1996). Furthermore, GABA-ir neurons belong to different soma groups associated with the AME that innervate glomeruli as well as interglomerular regions of the AME (Arnold et al., 2020; Giese et al., 2018a, b). GABA-ir neurites connect the AME via the anterior fiber fan to terminals of compound eye photoreceptors in the lamina and extracardiac photoreceptors in accessory laminae (Fleissner et al., 2001). In addition, the GABA-ir median-layer fiber tract links the AME and medulla with the circadian clock (Arnold et al., 2020; Giese et al., 2018a). These various GABA-ir connections to optic lobe neuropils as well as the light-like biphasic phase response curve of GABA suggested a role of GABA in light entrainment of the circadian clock (Petri et al., 2002). Furthermore, GABA was proposed to be involved in clock-dependent gating mechanisms, possibly regulating sleep-wake cycles via postsynaptic neuropils in the central brain (Giese et al., 2018a, b; Schneider & Stengl, 2005). Accordingly, circadian changes in GABA levels in the whole cockroach brain were found (McCay et al., 1996).

To further explore the roles of GABA in the circadian system of the cockroach in more detail than reported before (Arnold et al., 2020; Giese et al., 2018a; Petri et al., 2002), we mapped GABA immunostaining in the cockroach brain and compared it with glutamic acid decarboxylase (GAD) immunostaining. Possible connections between PDF-expressing clock output neurons and GABA-ir neurons in the central brain were studied by double-label immunofluorescence to search for possible sleep-promoting neuropils. Finally, to investigate whether GABA levels change in a circadian and/or daytime-dependent manner, we quantified GABA in the optic lobe as well as in the distal tract via enzyme-linked immunosorbent assay (ELISA), mass spectrometry, and immunofluorescence.

2 | MATERIALS AND METHODS

2.1 | Animals

Madeira cockroaches (R. maderae) were reared under 12:12 h light/dark (LD) cycles at 50% relative humidity and 25–26°C room temperature. To investigate daytime-dependent and circadian effects on GABA levels, two colonies were kept with inverse L:D rhythm (lights on from 08:00 to 20:00 h and from 20:00 to 08:00 h). Animals were fed with dried dog food, apples, potatoes, salad, and water ad libitum. Adult male cockroaches were used for all experiments. For basic immunolabeling, all brains were dissected out at about the same zeitgeber time (ZT) 02:00.

2.2 | Immunocytochemistry

An antiserum raised in rabbit against conjugates of GABA and keyhole limpet hemocyanin (provided by Dr. T. G. Kingan, University of Arizona) was used for immunostaining on vibratome sections following the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979). Animals were anesthetized by cooling to 4°C or on ice. Brains were dissected and fixed in a solution containing one part (by volume) 25% glutaraldehyde and three parts saturated picric acid and adding acetic acid to a final concentration of 1% (Boer et al., 1979). Fixation lasted for 3 h at room temperature. The fixed brains were embedded in gelatin-albumin, postfixed in 10% formaldehyde in 0.1 mol l⁻¹ phosphate buffer overnight at 4°C, and sectioned at 30 µm with a vibrating-blade microtome (VT1000, Leica, Wetzlar, Germany). Sections were treated with a solution of 2% hydrogen peroxide, 0.3% Triton X-100, and 1% normal goat or donkey serum in Tris-buffered saline (TBS) for 30 min. After blocking with 10% normal goat or donkey serum in TBS for 2 h, sections were incubated overnight at 4°C with the primary antibody (1:2000 dilution) in the blocking solution. After washing in TBS, sections were incubated for 2 h at room temperature with secondary antisera (Alexa Fluor 488, 1:2000 dilution) in the blocking solution.
for GABA-like immunoreactivity as described by Hoskins et al. (1986) and Homberg et al. (1987). To reduce nonspecific staining, brain sections were preincubated for 1 h in SST (0.1 mol l\(^{-1}\) Tris-HCl/0.3 mol l\(^{-1}\) NaCl) with 0.5% Triton X-100 (TrX) containing 8% normal goat serum (NGS) and 2% milk powder. The incubation in GABA antiserum, diluted at 1:6000 in SST, 0.5% TrX and 5% NGS was carried out for 20 h at room temperature. Following incubation in primary antibody, the preparations were washed in SST 0.1% TrX and were incubated for 1 h at room temperature in secondary antibody, goat anti-rabbit IgG (RRID: AB_261363) diluted 1:40 in SST, 0.5% TrX and 1% NGS. After rinsing in SST, 0.1% TrX, the sections were treated for 1 h at room temperature in rabbit PAP (RRID: AB_2315056) at 1:300 in SST 0.5% TrX-100 containing 1% NGS. Sections were stained by incubation in a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.03 mg/ml) in 0.1 mol l\(^{-1}\) phosphate buffer (pH 7.4) and \(\text{H}_2\text{O}_2\) (0.02%). In some experiments, staining was intensified by adding nickel ammonium sulfate (0.3%) to the staining solution (0.03 mg/ml DAB in 0.5 mol l\(^{-1}\) Tris-HCl, pH 7.4, and 0.02% \(\text{H}_2\text{O}_2\)) resulting in a dark blue reaction product. After staining, the sections were washed, dehydrated, cleared, and mounted in Entellan (Merck, Darmstadt, Germany) under glass cover slips.

2.3 Multiple-label immunofluorescence

For comparison of GABA immunostaining with immunoreactivity for the GABA-synthesizing enzyme GAD and possible contact areas between GABA-immunostained neurons with photoreceptors and PDF-containing neurons, double- and triple-label immunostaining was performed. In these experiments, a GABA antiserum from Sigma (Sigma-Aldrich Cat# A2052, RRID: AB_477652) was used, because the antiserum from T. G. Kingan was no longer available.

For double labeling of GABA and PDF, dissected brains were fixed for 2 h at room temperature in 4% formaldehyde in 0.1 mol l\(^{-1}\) sodium phosphate buffer (Roti®Histofix; Roth, Karlsruhe, Germany). Brains embedded in gelatin-albumin were sliced in frontal plane at a thickness of 100 µm with a vibrating blade microtome (VT 1000; Leica). Sections were rinsed four times with Tris-buffered saline (TBS; 25 mmol l\(^{-1}\) Tris, 137 mmol l\(^{-1}\) NaCl, 2.7 mmol l\(^{-1}\) KCl at pH 7.4) containing 0.1% TrX, preincubated with 5% NGS in TBS containing 0.5% TrX for 2 h at room temperature and incubated in the primary antibody solution containing rabbit anti-GABA antibody (1:750; Sigma) and mouse anti-Drosophila-PDF antibody (1:500; Developmental Studies Hybridoma Bank, University of Iowa, IA) dissolved in TBS containing 2% NGS and 0.5% TrX overnight at room temperature. The next day, brain sections were rinsed four times for 10 min in TBS containing 0.1% TrX and were then transferred into the secondary antibody solution containing Cy2-conjugated goat anti-rabbit (1:300, Dianova, Hamburg, Germany, Cat #111-225-144; RRID: AB_2338021) and Alexa 647-conjugated goat-anti-mouse (1:500, Dianova, Cat # 115-605-146; RRID: AB_2338912), 1% NGS in TBS containing 0.5% TrX for 4 h. The sections were finally washed, dehydrated using a graded ethanol series, cleared, and mounted in Entellan (Merck) under glass cover slips.

For triple immunolabeling using antisera raised in the same species, fluorescently labeled and unlabeled monovalent Fab fragments were used as secondary antibodies to prevent the second secondary antibody from binding to the first primary antibody (Arendt et al., 2017; Giese et al., 2018a). For triple immunolabeling of GABA, histamine, and PDF, dissected brains were submerged in freshly prepared 4% N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDAC; E-7750; Sigma-Aldrich) in PBS for 3 h on ice. For triple immunolabeling of GABA, GAD, and PDF, brains were fixed for 2 h at room temperature in 4% formaldehyde in 0.1 mol l\(^{-1}\) sodium phosphate buffer. Following fixation, brains were embedded in gelatin-albumin, postfixed, and sectioned at 40 µm as described above. The first primary antiserum, anti-GABA, raised in rabbit (Sigma) was applied at 1:750 in TBS with 2% NGS and 0.5% TrX overnight at room temperature. After washing 4 × 10 min, sections were incubated in goat anti-rabbit (GaR)-Fab-Fragment labeled with Alexa 647 (Dianova, Cat # 111-607-003; RRID: AB_2338084) at a concentration of 1:300 in TBS containing 1% NGS and 0.5% TrX for 3 h. Subsequently, unconjugated GaR-Fab (Dianova, Cat # 111-007-003; RRID: AB_2337925) was added at a dilution of 1:100 in TBS containing 1% NGS and 0.5% TrX for 1 h to block remaining binding sites on the Fc fragment of the first primary antiserum. Afterward, sections were washed 4 × 10 min each. Then, the second primary antiserum raised in rabbit, anti-GAD (Sigma-Aldrich Cat# G5163, diluted 1:1000), or anti-histamine (Merck Millipore Cat# AB5885, diluted 1:25,000), together with monoclonal mouse anti-Drosophila PDF antibody (Developmental Studies Hybridoma Bank, 1:500) diluted in TBS containing 2% NGS and 0.5% TrX were applied simultaneously. Sections were incubated overnight at room temperature. After more washing steps, sections were incubated with Cy2 conjugated GaR-Fab (Dianova, Cat # 111–227–003) at a dilution of 1:300 and Cy3-conjugated goat anti mouse at a dilution of 1:300 for 2 h. After final washing steps (4 × 10 min) and an additional 5 min rinsing in distilled water, sections were mounted on chromalum/gelatin-coated microscope slides. They were dehydrated in a graded ethanol series and embedded in Entellan (Merck).

2.4 Tracer application combined with GABA immunofluorescence

To investigate whether GABA-ir neurons might be involved in bilateral coupling of the two AMEs, Neurobiotin tracer injection (n = 5) in one optic stalk was combined with GABA and PDF immunofluorescence. Backfills from the optic stalk were performed as described by Reischl and Stengl (2002). Cold-anesthetized cockroaches were fixed with 5% (w/v) Neurobiotin™ Tracer (Vector Laboratories Inc., Burlingame, CA, USA) was slippcd over the cut optic stalk. The capillary was fixed with modeling clay, and the animal was kept overnight at 4°C in a moist chamber to allow for intracellular transport of the tracer. The following day, the
brain was dissected out of the head capsule, fixed for 2 h at room temperature in 4% formaldehyde in 0.1 mol l\(^{-1}\) sodium phosphate buffer, embedded in gelatin-albumin, and sliced in frontal plane at a thickness of 40 \(\mu\)m with a vibrating blade microtome (VT 1000; Leica). The sections were treated for GABA and PDF labeling as described above. To visualize Neurobiotin, streptavidin coupled with Cy2 (Thermo Fisher Scientific, Waltham, MA, USA) was added to the secondary antibody solutions at the concentration of 1:300. Goat anti-rabbit secondary antibody conjugated with Cy3 (1:300) and Alexa 647 conjugated goat anti-mouse (1:500) were used to visualize GABA and PDF, respectively.

2.5 | Antibody characterization

The anti-GABA antiserum (#9/24, provided by T. G. Kingan, RRID: AB_2314457) was raised in rabbit against conjugates of GABA-glutaraldehyde-keyhole limpet hemocyanin (KLH; Hoskins et al., 1986). The antisem has been used to characterize GABA-ir neurons in many insect species, including the cockroach \(R.\ maderae\) (Homberg et al., 2018). On brain sections of the sphinx moth \(Manduca\ sexta\), immunostaining was abolished by preadsorption of the diluted antiserum with 24 nmol l\(^{-1}\) GABA-KLH conjugates but staining was not reduced by preadsorption with conjugates of L-glutamic acid, L-glutamine, taurine, or \(\beta\)-alanine (Hoskins et al., 1986).

The second anti-GABA antiserum (Sigma-Aldrich Cat# A2052, RRID: AB_477652) was raised in rabbit against GABA conjugated to bovine serum albumin (BSA; Sigma Aldrich; cat# A2052). The pattern of immunostaining obtained with this antiserum on brain sections of \(R.\ maderae\) was identical to that obtained with the #9/24 antiserum. Liquid-phase preadsorption of the diluted antiserum with 10 µmol l\(^{-1}\) GABA-BSA conjugate abolished all immunostaining on brain sections of \(R.\ maderae\) (Giese et al., 2018a).

The anti-GAD antiserum (RRID: AB_477019, Sigma-Aldrich, cat# GS163) was raised in rabbit against the highly conserved C-terminal region of the 65- and 67-kDa isoforms of human GAD. On locust brain sections, the staining pattern of anti-GAD matched that reported for GABA staining (Homberg et al., 2020; Stern, 2009), and in Western blots of locust brains, the GAD antiserum labeled a double band close to 50 kDa (Stern, 2009), which is in the range of molecular weights for the two GAD subunits (Stapleton et al., 1989).

The monoclonal mouse antibody against PDF (RRID: AB_760350, Developmental Studies Hybridoma Bank) was obtained from hybridoma cell cultures (Cyran et al., 2005). The antibody was generated against amidated PDF peptide (NSELINSLLSPKNNMDA-NH\(_2\)) from Drosophila and was used in several studies analyzing the circadian system of \(R.\ maderae\) (Arendt et al., 2016; Schulze et al., 2012; Soehler et al., 2011; Wei & Stengel, 2011). Liquid-phase preadsorption of the diluted antibody with 10 µmol l\(^{-1}\) of Rhm-PDF abolished all immunostaining on brain sections of \(R.\ maderae\) (Schulze et al., 2012).

The anti-histamine antiserum was raised in rabbit against histamine conjugated to KLH with EDAC (RRID: AB_177540; Merck Millipore). The antiserum has been used to label histamine-immunoreactive photoreceptors in the butterfly \(Papilio\ xuthus\) (Hamanaka et al., 2012) and \(R.\ maderae\) (Arendt et al., 2016, 2017). On \(R.\ maderae\) brain sections, immunostaining was abolished following liquid-phase preadsorption of the diluted antiserum with 100 µmol l\(^{-1}\) of histamine–BSA conjugate (Arendt et al., 2016).

2.6 | Confocal imaging and evaluation

Confocal laser scanning microscopy (TCS SP5; Leica) was employed to evaluate the fluorescently labeled brain sections. Sections were scanned with an HCX PL apochromate 20x/0.7 multi-immersion objective at 1024 x 1024 pixels per stack in z-direction with a step size of 0.5–1 µm and were merged by using the maximum projection option. Cy2 fluorophores were excited with an argon laser at 488 nm and detected at 490–530 nm, Alexa 647 fluorophores were excited with a helium/neon (He-Ne) laser at 633 nm and detected at 650–690 nm, and Cy3 was excited with the 543 nm line of a He-Ne laser and detected at 560–590 nm. All signals were sequentially scanned to avoid false-positive signals of the fluorophores. Images were imported to Fiji Image software and arranged with Corel DRAW Graphics Suite X7 (Corel, Ottawa, ON, Canada) or the open source software Inkscape vector graphic editor (version 0.91). Image processing of PAP staining was done with Adobe Photoshop CS5 (San Jose, CA, USA). Reconstruction of immunolabeled cell bodies was done from consecutive frontal sections with a Leitz compound microscope (SM-LUX, Leica) equipped with a camera lucida attachment. A Zeiss microscope (Jena, Germany) equipped with CCD camera (ProRes C5; Jenoptik, Jena, Germany) and objectives with 10x (A Plan Ph1; Zeiss) or 20x (ECPlan: Neofluar Ph2M27; Zeiss) were used to count GABA immunostained somata. Somata counted from sections were either labeled individually using the cell count plugin of ImageJ Fiji to avoid double counting of cells on adjacent sections, or crude counts were corrected using the Abercrombie correction factor (Abercrombie, 1946).

2.7 | Diurnal and circadian variation in GABA levels

To investigate diurnal and circadian variations of GABA levels, the intensity of GABA immunostaining in the distal tract was analyzed at different zeitgeber times, and GABA levels in the optic lobe were investigated using ELISA and mass spectrometry.

2.7.1 | Quantification of GABA immunostaining

For quantification of GABA immunostaining in the distal tract, we used a protocol modified from that of Hermann-Luibl et al. (2014). Animals were taken out of the colonies at 6h intervals (ZT 00, ZT 06, ZT 12, and ZT 18). Brains were dissected, immersed in fixative, and embedded in gelatin/albunin as described above. To exclude
staining differences based on variations in the immunostaining procedure, brains at ZT 00 and ZT 12 and brains at ZT 06 and ZT 18, taken from the two colonies with inverse L:D rhythm, were processed together. Brains were sliced at 100 µm thickness. Brain sections were incubated in primary antibody solution for 4 days and in secondary antibody solution for 3 days. Antibody concentrations and procedures were identical to procedures described for GABA immunofluorescence above.

The optic lobes of all brains were scanned with identical laser settings. Sections were scanned with an HCX PL apochromate 20x/0.7 multi-immersion objective at 1024 x 1024 pixels per stack in z-direction with a step size of 0.64 µm. Ten distal tracts from five brains were analyzed for each time point. Image stacks were imported to ImageJ (Fiji) and saved as grayscale formats within an 8-bit range. The average pixel intensity of the maximum projection of the stacks within the region of interest (ROI, 70 × 45 µm²) which only contained the distal tract was determined. Pixel intensities from 0 to 100, corresponding to background noise, were excluded prior to averaging. To do this, areas without staining were identified in the image stack. These areas had pixel intensities that did not exceed 100. Pixel intensities of the ROI from 10 distal tracts for each time point were plotted as box plots relative to maximal possible intensity.

2.7.2 Sample preparation for ELISA

Tissue preparation and ELISA largely followed the procedures outlined in Schendzielorz et al. (2014). Male, adult cockroaches were taken from the colonies at 4-h intervals (ZT times 00, 04, 08, 12, 16, and 20). Animals were cold-anesthetized and all preparation steps performed on ice. Optic lobes were dissected in insect saline (128 mmol l⁻¹ NaCl, 2.7 mmol l⁻¹ KCl, 2 mmol l⁻¹ CaCl₂, 1.2 mmol l⁻¹ NaHCO₃, dissolved in double distilled water, pH 7.25). For each time point, the optic lobes of two animals (= 4 optic lobes) were transferred to iced Eppendorf cups containing 400 µl PBS (137 mmol l⁻¹ NaCl, 8.1 mmol l⁻¹ Na₂HPO₄ x 2 H₂O, 2.7 mmol l⁻¹ KCl, 1.47 mmol l⁻¹ KH₂PO₄; pH 7.4), including 2% perchloric acid and 10 mg of 1 mm diameter glass beads (Biospec Products, cat# 11079110). Samples were homogenized in a refrigerated Mini-Bead Beater (Biospec Products, Bartlesville, OK, USA), and the mixture was centrifuged (Heraeus Fresco 17, Thermo Scientific, Schwerte, Germany) at 900 g for 15 min at 4°C to remove cells and tissues debris. For neutralization, 300 µl supernatant was mixed with 300 µl chloroform/trietylamine solution (1:1) and centrifuged at 500 g for 5 min at 4°C. Two hundred microliters of supernatant was taken and stored at −80°C until further measurement.

To investigate circadian variations in GABA levels, animals taken at ZT 00, 04, 08, 12, 16, and 20 from the colony were isolated at the end of the night in separate vessels for 48 h in constant darkness. Optic lobes were dissected on the second day of constant darkness under darkness at the respective circadian time window. Each sample contained four optic lobes (= 2 animals). Sample processing was done as described above.

2.7.3 Competitive ELISA

Microtiter plates (Nunc, Maxisorp, C-Bottom, Thermo Fisher Scientific, Denmark) were coated with 125 µl (15 µg/ml) goat-anti rabbit IgG (Dianova, Hamburg, Germany), dissolved in PBS overnight at 4°C with agitation. Microplates were protected against evaporation with self-adhesive foil. The next day, wells were washed with 250 µl washing buffer (WB; 0.05% Tween® 20 in PBS). Nonspecific binding sites were blocked by incubating each well in 250 µl of blocking buffer (BB; 1% BSA in WB) for 1 h at 37°C. Afterward, wells were rinsed three times with BB. Samples were diluted 1:10 in PBS and 25 µl of each pipetted in the wells in a quadruple determination, followed by 25 µl of rabbit-anti GABA antiserum (Sigma-Aldrich, cat# A2052, RRID: AB_477652) at a final concentration of 1:20,000 in BB. GABA standards were determined in concentrations ranging from 2.5 to 80 pmol/25 µl dissolved in PBS, followed by 25 µl of rabbit-anti GABA antiserum at a final concentration of 1:20000 in BB. Four wells were each loaded with 25 µl of PBS and GABA antiserum for the maximum signal (B0) determination and four other wells with 25 µl PBS and 25 µl BB without GABA antiserum for the determination of nonspecific binding (NSB). Microplates were covered with self-adhesive foil and incubated overnight at 4°C with agitation. The following day, the competitive reaction was carried out by quickly pipetting 25 µl of GABA conjugated to horseradish peroxidase (Carter, 1996) at a final concentration of 1:600 in BB. After 30 min incubation time at 4°C without shaking, plate was emptied, and wells were washed three times with WB. For detection, 25 µl of development solution I (0.02% H₂O₂, 0.3% H₃PO₄ dissolved in 100 mmol l⁻¹ citrate buffer, pH 5) and 50 µl of development solution II (420 mmol l⁻¹ 3,3',5,5'-tetramethylbenzidine, 0.7% dimethylsulfoxide, 0.1% H₂PO₄, dissolved in H₂O) were added to each well and developed at RT for 30 min on the shaker. The reaction was stopped by the addition of 25 µl 2 mol l⁻¹ sulfuric acid. The photometric analysis was carried out at 450 nm using a microplate reader (POLARStar, BMG Labtech, Ortenberg, Germany). To calculate a standard curve and GABA concentrations, the NSB value was subtracted from each value. For calculation of a standard curve and GABA concentrations, the NSB value was subtracted by NSB, and logit transformation (logit [standard/B0]; y-axis) was plotted versus log concentration of standards for linearization. The coefficient for determination of the regression fit was always above 0.95.

2.7.4 GABA quantification by mass spectrometry

GABA concentration in the optic lobes of individual brains was determined by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). For quantification experiments, six sample sets consisting of 3 x 2 optic lobes at ZT 06 and 3 x 2 optic lobes at ZT 12 were dissected in ice cold dissection saline as described above. Each sample set was collected in 20 µl 50% MeOH containing 1% aqueous formaldehyde on ice. Tissue samples were homogenized using an ultrasonic bath (Transonic 660/H; Elma Schmidbauer) for 3 h on ice and in darkness. Afterward, the samples
were centrifuged for 15 min at 13,000 rpm at 4°C. The supernatants were separated and stored at −20°C until use. Endogenous GABA was derivatized with benzoyl chloride and analyzed by LC-MS/MS using a procedure previously described in Wong et al. (2016) with several modifications: 15 µl of extract was mixed with 10 µl of a 10 µmol l⁻¹ solution of the internal standard d₆-GABA (Sigma-Aldrich, Taufkirchen, Germany) in Milli-Q water. Endogenous GABA and the internal standard were derivatized by adding 10 µl of freshly prepared 2% benzoyl chloride in acetonitrile and 10 µl of 0.1 mol l⁻¹ sodium carbonate in water and thorough mixing. After addition of 50 µl of Milli-Q water and centrifugation (16,100 RCF, 5 min, 4°C), 80 µl of supernatant was transferred to autoinjector vials.

LC-MS/MS analysis was performed by injection of 5 µl of sample onto a Core-Shell Kinetex C18 column (100 mm × 2.1 mm ID, 2.6 µm particle size, 100 Å pore size, Phenomenex) and detection using a QTRAP 6500 triple quadrupole/linear ion trap mass spectrometer (SCIEX). The LC (Nexera X2 UHPLC System, Shimadzu) was operated at 30°C and at a flow rate of 0.4 ml/min with a mobile phase of 1 mmol l⁻¹ ammonium formate and 0.15% formic acid in water (solvent A) and acetonitrile (solvent B). Benzoylated GABA was eluted with the following gradient: initial, 10% B; 0.01 min, 15% B; 0.5 min, 17% B; 14 min, 55% B; 14.5 min, 100% B; 16.5 min, 100% B; 16.6 min, 15% B; and 20 min, 10% B.

Benzoylated endogenous GABA and d₆-GABA were monitored in the positive ion mode using their specific multiple reaction monitoring (MRM) transitions m/z 208.1 to 105.1 (GABA) and m/z 214.1 to 105.1 (d₆-GABA). The instrument settings for nebulizer gas (Gas 1), turbogas (Gas 2), curtain gas, and collision gas were 50, 70, 40 psi, and medium, respectively. The interface heater was on, the Turbo V ESI source temperature was 500°C, and the ion spray voltage was 5.5 kV. For both MRM transitions, the values for declustering potential, entrance potential, cell exit potential, and collision energy were 80, 10, 10, and 10 V, respectively (Wong et al., 2016). The LC chromatogram peaks of benzoylated endogenous GABA and d₆-GABA were integrated using the MultiQuant 3.0.2 software (SCIEX). The peak area of benzoylated endogenous GABA was normalized to the peak area of the internal standard d₆-GABA. Each sample was analyzed twice.

2.8 Statistical analysis

Statistical data analysis was done using Prism6 software (GraphPad Software, San Diego, CA, USA). Bar graphs generated with Prism6 were edited with Inkscape vector graphic editor (version 0.91). The Shapiro-Wilk normality test was used to analyze the distribution of data sets; nonparametric tests were applied for data sets that were not normally distributed. Six different ZTs or CTs per day were analyzed to determine GABA concentrations by using the Kruskal–Wallis test followed by Dunn’s post hoc test. To obtain a single estimate for GABA levels during the day and during the night, the data from ZT (resp. CT) 04–08 and ZT (resp. CT) 16–20 were pooled. For statistical analysis of quantitative mass spectrometrical data, linear ranges, accuracy, relative standard deviations (RSD), and linearity (R²) were calculated with Microsoft Excel 2010 and/or R 3.1.3 (R Development Core Team). To compare GABA titers between different ZTs, data points were tested for normal distribution using a one-sample Kolmogorov–Smirnov test and were either analyzed by Student’s t test.

3 | RESULTS

3.1 | GABA immunostaining in the central brain

In the central brain of R. maderae, about 3000 (2771–3205, n = 4) neurons are GABA immunoreactive (GABA-ir) (Figure 1). While some of these neurons occur as isolated cell bodies in the soma rind, most somata are concentrated in cell clusters that send their primary neurites in a common fiber fascicle into various central brain neuropils. Thirteen bilaterally paired GABA-ir cell clusters were numbered G1–G13 (Figure 1). An additional stained cluster of lateral neurons of the antennal lobe was termed LC (Figures 1a, 2a,b, and 3a). Most central brain neuropils involved in sensory processing and premotor control show dense immunostaining (Figures 2 and 3). Mass staining in general did not allow for identification of projection patterns of individual neurons or cell groups (Figure 2a–c). In contrast, the lobes and pedunculi of the mushroom bodies are only sparsely supplied by stained processes (Figures 2a,b and 3a–d). Some GABA-ir fibers invade the pedunculus divide and ascend the pedunculus (Figures 2a,b and 3a–c). Their cell bodies likely belong to soma cluster G2. Likewise, small bands in the medial lobe (Figure 3b) and vertical lobe, as well as the tip of the vertical lobe are sparsely innervated by GABA-ir processes (Figure 3a,b). The calyces of the mushroom bodies are innervated by axonal processes of four GABA-ir giant neurons (Figures 2a,b and 3b–e). Their large cell bodies belong to cluster G12 in the lateral soma rind posterior to the optic stalk (Figures 1c and 2c). Some neurons of soma cluster G11 in the superior lateral soma rind lateral from the calyces, likewise, send processes into the calyces (Figures 2c and 3e). All calyx projections are confined to the outer layer of the calyces where inputs arrive, while the inner layer is free of immunostaining. The large G2 group consists of at least three distinct clusters of soma anterior and medial to the crepine, the neuropil anterior-frontally to the mushroom body lobes (Figures 1a, 2a, and 3a). Apparently, some of these cells innervate the pedunculus divide. The large neighboring G3 group is located between the antennal lobe and the medial lobe of the mushroom body (Figures 1a and 3b,c). Its neurons innervate the bulbs and the lower division of the central body (Figure 3c) (Homberg et al., 2018). The protocerebral bridge as well as the upper division of the central body are almost free of GABA immunostaining (Figures 2b and 3c,d). In the antennal lobe, all glomeruli are densely supplied by neurons of the large LC cluster, which consists of about 310 (236–393, n = 5) labeled somata (Figures 1a, 2a,b, and 3a,d). Immunoreactivity is largely concentrated in the core of each glomerulus. The medial antennal lobe tract is free of immunostaining. However, GABA-ir fibers run in a medio-lateral tract, which probably corresponds to the antennal-cerebral tracts II or III of...
Figure 1  Distribution of GABA-ir cell bodies in the central brain of R. maderae. Cell body clusters, labeled G1 (most anterior) to G13 (most posterior) are illustrated in frontal planes at anterior (a), intermediate (b), and posterior (c) levels of the brain. A prominent cell cluster of the antennal lobe (AL) is labeled LC (lateral cluster). Arrows in (a) point to single immunoreactive cells in the anterior pars intercerebralis, arrows in (c) mark cell bodies of giant G12 neurons that innervate the calyces (CA) of the mushroom body (MB). Abbreviations: CBL, lower division of the central body; ML, medial lobe; PED, pedunculus; VL, vertical lobe. Scale bar = 200 µm

Malun et al. (1993) connecting the antennal lobe to the lateral horn (Figure 3d). The glomerular lobe, the target of chemosensory afferents from the maxillary palps, shows sparse beaded GABA immunostaining (Figure 3a). A GABA-ir fiber fascicle connects the glomerular lobe to the antennal lobe (Figure 3a). Medioventrally, the G4 cell cluster sends its neurites in a wide arc posteriorly along the antennal lobe into the protocerebrum (Figures 2c and 3a). Two small cell groups, G1 and G6, have somata in the superior lateral soma rind (Figures 2a,b and 3a). G1 cells target the anterior ventrolateral protocerebrum, but the projections of G6 neurons could not be traced. Two large lateral soma clusters, G7 and G12, extend far into the soma rind along the anterior (G7) and posterior side (G12) of the lobula complex (Figures 2b,c). Several commissures connecting the two brain hemispheres contain GABA-ir fibers, but they could not be traced to their respective somata (Figures 2c and 3a-d). In contrast to most other cell clusters, neurons of the G5 group in the pars intercerebralis send their neurites in several fascicles into the superior neuropil suggesting that they originate from more than one neuroblast. Some of these neurons may be descending cells as judged by the soma positions of descending cells analyzed in the cockroach Periplaneta americana (Okada et al., 2003). The antennal mechanosensory and motor center of the deutocerebrum is innervated by neurons with somata in two large lateral cell groups, G8 and G9 (Figures 2c and 3f). The large G8 cluster marks the
FIGURE 3  Frontal sections through the central brain of the cockroach showing GABA immunostaining in different brain regions. (a–c) The vertical (VL) and medial (ML) lobes of the mushroom bodies largely lack GABA immunoreactivity. The peduncular divide (PEDD; arrowheads in a and b) and a small band in the VL (arrow in b) are innervated by GABA-ir extrinsic mushroom body neurons apparently belonging to G2. Local interneurons from a lateral cell cluster (LC) give rise to dense GABA staining in all glomeruli of the antennal lobe (AL), while a small GABA-immunoreactive (−ir) fascicle (open arrowhead in a) of unknown origin connects the AL with the glomerular lobe (GLO) of the tritocerebrum. The superior lateral protocerebrum (SLP) and the superior medial protocerebrum (SMP) are stained, at least partly due to innervation via G1 neurons that innervate the superior protocerebrum, and G5 neurons of the pars intercerebralis that send fibers to the SMP and clamp, neuropil around the PED and VL. (b) Several stained commissural fibers connect the SMPs of both hemispheres (open arrowheads). (c) The lower division of the central body (CBL) and bulbs (BU) are strongly GABA-ir, due to innervation via G3 and G2 neurons. Several GABA-ir commissural fibers connect the two brain hemispheres below the VLs of the mushroom bodies (arrows). (d) GABA immunostaining is prominent in the mediolateral antennal lobe tract (mlALT) connecting the AL to the lateral horn (LH) of the lateral protocerebrum. White arrowhead points to four large immunolabeled fibers from giant neurons in cluster G12 (see Figure 1c) entering the calyces (CA). (e) Strongly GABA-ir neurons reside in the dorsal lateral protocerebrum (G11). A small cluster of these neurons (filled arrowhead) contributes to beaded ramifications throughout the outer layer of the calyces (OCA). The axons of other GABA-ir somata of G11 (open arrowhead) innervate the SLP. The lateral protocerebrum is invaded by a fascicle of neurites from somata in the G7 cluster (double open arrowhead). (f) The antennal mechanosensory and motor center (AMMC) posterior from the AL is diffusely invaded by GABA-ir processes. They partly originate from neurons with somata in GABA-ir cell cluster G8 and G9 (double open arrowheads). GABA-ir somata G10 are located in the tritocerebrum. Abbreviations: CBU, upper division of the central body; ICA, inner layer of the calyces. Scale bars = 100 µm e
3.2 | GABA immunoreactivity in the optic lobe

All neuropils of the optic lobe, including the lamina, medulla, and lobula complex, are GABA-ir with strongest immunoreactivity in the medulla and AME (Petri et al., 2002; Rosner et al., 2017). Based on soma position and trajectory of cell body fibers, six groups of neurons, termed M1–M6, could be distinguished in the cell cortex of the medulla (Figure 4). Neither photoreceptors nor lamina monopolar cells with cell bodies between the lamina and retina were GABA-ir. However, prominently labeled soma groups associated with the medulla and AME connect the lamina, medulla, and AME via the first optic chiasma (Figure 5a,b), the medulla and lobula complex via the second optic chiasma (Figure 5a,c), and the optic lobe with central brain neuropils via the lobula valley tract (Figures 4b and 5c,d).

About 430 columnar M1 neurons (388–498, n = 7) with cell bodies in the soma rind posterior to the most proximal layer 10 (ME10) of the medulla are organized retinotopically (Figures 4a,b and 5a–e). They appear to connect corresponding columns of the medulla and lamina in retinotopic order. It could not be revealed which specific medulla layers were innervated by these neurons, but immunostained fibers in the first optic chiasma connecting the medulla and lamina largely originate from these neurons indicating that M1 neurons are columnar centrifugal neurons with axonal terminals in the lamina.

M2 like M1 neurons are organized retinotopically and consist of about 880 (780–1050, n = 7) cells distributed along the distal face of the medulla (Figures 4a,b and 5a,b,d,e). The neurons are most likely columnar transmedullary neurons. A common characteristic of these neurons is the orientation of their main fibers perpendicular to the medulla layering and parallel to the columns of the medulla. Owing to dense staining, the medulla layers innervated by these neurons could not be determined. Their axonal fibers project through the second optic chiasma to the distal medulla cell group 2 (M2, blue) are located along the anterior dorsal to anterior ventral face of the ME. Long axonal processes from M2 neurons travel along the proximal edge of the ME and enter the lobula (LO) or continue to the lateral protocerebrum through the second optic chiasma and the lobula valley tract (LOVT). (b) Somata of M3 (green) lie posterior-laterally to the ME and send processes to ME layers 7–9. Somata of M4 (magenta) are clustered close to the ventral edge of the ME and innervate the accessory medulla (AME). A group of large somata (M5) ventrally between the proximal ME and the distal LO sends long neurites centripetally and distally along the outer face of the LO. Their fibers project via the LOVT to central brain regions. Some M4 (magenta) and M5 (orange) neurons send neurites via the anterior fiber fan (AFF, magenta) to the dorsal and ventral accessory laminae (vALa) and to the posterior LA. A ventral-proximal group of M6 cells (mustard) sends processes toward ME layer 4. Abbreviations: DT, distal tract; MLFT, median layer fiber tract, asterisk: largest M4 soma. Scale bar = 50 μm.

All GABA-ir neurons that send their primary neurites to the AME were assigned to the M4 group (Figures 4b and 5d,f) although they are morphologically heterogeneous (Figure 6). Strongest GABA immunoreactivity occurred in the glomerular core of the AME (Figure 5a). The interglomerular regions and parts of the shell regions of the AME show sparser but homogenous GABA immunostaining. A total of about 33 (25–43, n = 7) GABA-ir M4 neurons were counted, which could be assigned to six AME soma groups (Figure 6), as distinguished by Reischig and Stengl (1996). Most of the GABA-ir neurons belong to the ventral neurons (VNe); mean: 20; min–max: 17–23). Other immunolabeled neurons were identified as medial neurons (MNes; mean: 5; min–max: 4–6), distal fronto-ventral neurons (DFVNe; mean: 4; min–max: 3–5), ventromedian neurons (VMNes; mean: 3; min–max: 2–5), and ventro-posterior neurons (VPNe; mean: 3; min–max: 1–5). Because all these neurons are closely packed next to each other,
FIGURE 5  Horizontal vibratome sections showing GABA immunoreactivity in the optic lobe. (a) Overview. All neuropils, including the lamina (LA), medulla (ME), accessory medulla (AME), and lobula (LO), exhibit GABA immunostaining. Numbers of layers in the LA and ME are adapted from Arnold et al. (2020). Asterisk indicates largest soma of the M4 group of somata of AME neurons. (b) The inner layer 3 of the LA (arrowheads) and the accessory laminae (ALA), small distinct neuropils at the proximal border of the LA, are prominently labeled. GABA-ir fibers in the first optic chiasma (OCH1) originating from medulla centrifugal neurons (M1) connect the ME to the LA. (c) Large soma located anteriorly to the distal face of the LO (M5) send thick fibers around the LO to the central brain, along with other fibers in the lobula valley tract (LOVT). (d) At a more ventral level, GABA-ir fibers in the distal tract (DT) bifurcate near the AME and densely innervate the glomeruli of the AME. Large GABA-ir processes in the median-layer fiber tract (MLFT) connect the AME and ME layer 4 (ME4). (e) Horizontal section at a plane ventral from (d). A cluster of cell bodies (M6) sends processes toward ME4. Abbreviations: AFF, anterior fiber fan; OCH2, second optic chiasma. Scale bars 100 µm

usually, individual projection patterns could not be resolved. However, GABA-ir MNes, which had the largest soma, send their primary neurites to the dorsal glomerulus (Figure 6a,b). Among these is the largest GABA-labeled neuron of the AME (asterisk, Figure 5a), reconstructed by Petri et al. (2002). It is a centrifugal neuron of the AME innervating the lamina and accessory laminae (Figure 5a,b) via fibers of the anterior fiber fan, a system of fiber projections covering the anterior face of the medulla (Figure 6a). The somata of GABA-ir VPNes and VMNes send long neurites to the ventro-proximal region of the AME (Figure 6b,c). Neurobiotin backfills (n = 5) from the contralateral optic stalk combined with GABA immunofluorescence revealed double labeling of at least one VMNe (not shown), two VNes, and one MNe (Figure 6d–l), indicating that these neurons have projections in the contralateral optic lobe. Optic stalk backfills by Reischig et al. (2004) and Soehl et al. (2011) indicated that most, if not all VMNes are contralaterally projecting neurons, suggesting that all four GABA-immunolabeled VMNes, at least two VNes, and at least one MNe are commissural neurons interconnecting the two optic lobes and probably both AMEs. As reported previously (Arnold et al., 2020; Petri et al., 2002), a distinct fiber fascicle associated with the AME, the distal tract, exhibits prominent GABA immunostaining (Figures 5d, 6b, and 7a). The distal tract densely innervates the glomerular core of the AME (Figure 6b). It passes ventrally along the distal face of the medulla toward the lamina but does not contact the lamina. Instead, along the surface of the distal medulla, the tract gives off small fiber fascicles at right angle that dive deep into the medulla, at least to medulla layer 4 (Figure 6b,d), with the last fascicle entering the medulla at its posterior distal edge (Petri et al., 2002). The somata belonging to distal tract fibers could not be identified.

A group of neurons near the anterior face of the lobula, M5, consists of about eight somata (4–12, n = 6) close to M4 cells with some of the largest GABA-ir somata in the optic lobe, suggesting that they have extensive arbors (Figures 4b and 5c). Most of these cells appear to be tangential neurons of the medulla. Processes from these neurons enter the medulla via the median-layer fiber tract (MLFT; Figure 5d) and give rise to dense immunostaining in layers 2–5, while axonal fibers project centrally via the lobula valley tract. Other M5 neurons do not invade the medulla but send their cell body neurites into the lobula valley tract. Some neurons with fibers in the lobula valley tract invade the anterior lobe of the lobula (not shown), others project to various areas in the central brain or via commissures contralaterally.
FIGURE 6  (a–c) Six of seven soma groups associated with the accessory medulla (AME, encircled) are GABA-immunoreactive (-ir). Frontal sections of the left optic lobe cut from most anterior (a) to posterior (b and c). A total of 33 (25–43) GABA-ir neurons were assigned to median neurons (MNe), ventral neurons (VNe), medial-frontoventral neurons (MFVNe), distal-frontoventral neuron (DFVNe), ventromedian (VMNe), and ventro-posterior neurons (VPNe) that innervate the AME. (a) One GABA-ir MNe innervates the dorsal glomerulus (black arrowhead) of the AME. (b) GABA-ir fibers of the distal tract (DT) bifurcate and ramify in the glomeruli of the AME (arrowheads). (c) The shell of the AME is connected by some GABA-ir fibers via the lobula valley tract (LOVT) to the central brain. (d–l) Maximum projections of confocal images from three adjacent fronto-horizontal sections through the AME showing a Neurobiotin-backfill from the contralateral optic stalk (green) combined with GABA immunostaining (magenta). Neurobiotin and GABA are colocalized in one MNe (f, arrowhead) and two VNes (i and l; double arrowheads) of the AME. Prominent Neurobiotin-labeled arborizations overlap with GABA-ir fibers in the median-layer fiber system (MLF) of the medulla (ME). Abbreviations: AFF, anterior fiber fan; DT, distal tract; MLFT, median-layer fiber tract. Scale bars = 50 µm

3.3 Colocalization of GABA with GAD staining

To provide further evidence for the specificity of the GABA antisera, raised against GABA-KLH conjugates, a second antisera raised against GABA-BSA conjugates was tested and compared with immunostaining for GAD, the GABA-synthesizing enzyme. Both GABA antisera revealed identical staining patterns throughout the brain. Moreover, double-label immunostaining with anti-GABA-BSA and anti-GAD antisera, likewise, resulted in identical staining patterns in the cockroach brain (Figure 7). However, the distribution of relative staining intensity differs with both antisera. Overall, GABA staining is relatively stronger in cell bodies and GAD immunostaining, relatively
Figure 7  Double immunofluorescent staining for GABA (a–c) and glutamic acid decarboxylase (GAD, a’–c’) in selected brain areas. The pattern of immunolabeling with both antisera is identical throughout the brain. (a and a’) Frontal section through the optic lobe showing GABA- (a) and GAD (a’) immunoreactivity in the medulla (ME), lobula (LO), accessory medulla (AME), and distal tract (DT). (b and b’) Prominent GABA- (b) and GAD (b’) immunoreactivity in the lower division of the central body (CBL), the bulb (BU), and the superior medial protocerebrum (SMP). GABA- and GAD-ir cell cluster G5 projects to the SMP. (c and c’) Interneurons with somata in the lateral cluster (LC) of the antennal lobe show both GABA (c) and GAD (c’) immunostaining. The antennal lobe hub (ALH) as well as the antennal lobe glomeruli (GL) exhibit strong GABA- (c) and GAD (c’) immunoreactivity. Images are maximum projections from stacks of 7–12 optical sections (z-distance between single sections = 0.63 µm). Abbreviation: CBU, upper division of the central body. Scale bars = 100 µm

3.4  Triple-label immunofluorescence for GABA, PDF, and histamine

To examine, whether GABA-ir processes colocalize PDF or contact histaminergic photoreceptors of the compound eye, triple-label immunostainings were performed with anti-GABA, anti-histamine, and anti-PDF antisera (Figure 8). No colocalization exists between GABA and histamine immunoreactivities in the optic lobe (Figure 8a–b). In the proximal lamina, the termination sites of short photoreceptor axons overlap with GABA- and PDF-ir processes. However, the accessory laminae are devoid of histamine staining, while they exhibit relatively strong GABA- and PDF immunoreactivity. In the medulla, histamine immunostaining is characterized by a loose fiber network in medulla layers 6 and 7. This staining originates from a single histamine-ir neuron with soma in the lateral cell body rind of the central brain (Arnold et al., 2020; Loesel & Homberg, 1999). Its centrifugal axon runs together with PDF- and GABA-ir fibers in the lobula valley tract. In addition, the medulla shows dense histamine immunoreactivity in layer 2, which is the termination site of long photoreceptor axons of the compound eyes, overlapping with GABA staining, but not with PDF-ir branches (Figure 8a’–d). GABA immunoreactivity overlaps also with arborizations of the histamine-ir centrifugal neuron in medulla layer 6, but apparently not in layer 7 (Figure 8a’–d). Very dense GABA-immunoreactivity
FIGURE 8  Horizontal vibratome sections through the optic lobe showing triple immunofluorescent labeling for histamine (a and a’, green), GABA (b and b’, magenta), and PDF (c and c’, cyan). Overlays of all stainings are shown in (d) and (d’). (a–a’) Histamine-ir processes of photoreceptor neurons terminate in the lamina (LA) and in layer 2 (ME2) of the medulla (ME, filled double arrowhead). Histamine immunoreactivity in ME layers ME4, ME6, and ME7 originates from a neuron in the protocerebrum that enters via the lobula valley tract (LOVT). Several histamine-ir side branches from that neuron extend to the termination sites of the long photoreceptor axons in layer 2 (open double arrowheads). The accessory laminae (ALA) are free of histamine staining. (b–b’) The AME is densely innervated by the GABA-ir distal tract (DT) and by the median-layer fiber tract (MLFT). Open arrows mark GABA-ir fibers that branch off from the DT, extending perpendicular to the ME layers toward layers 2 and 4. GABA-ir fibers of the anterior fiber fan (AFF) connect the AME to the posterior LA, where they overlap with histamine-labeled terminals of short photoreceptor neurons. (b’) A branch of the GABA-ir AFF arborizes in the ALA. (c–c’) PDF immunoreactivity in the optic lobe is prominent in the proximal LA3, ALA, and AFF. Layer 2 of the ME is free of PDF staining (filled double arrowhead). (d–d’) The overlays show no colocalization of histamine, GABA, and PDF immunoreactivities. However, in the proximal LA3, histamine-ir terminals of short photoreceptor neurons overlap with GABA immunostaining as well as with PDF-ir fibers. In ME2 GABA-ir processes overlap with histamine-ir termination sites of long photoreceptor axons, while in ME4, GABA- and PDF-ir branches overlap. All images are maximum projections of image stacks. Scale bars = 50 µm
FIGURE 9  Confocal laser images obtained from frontal sections through the central brain of the cockroach showing overlap but no colocalization of GABA- (a–d) and PDF immunofluorescence (a’–d’). (a’–d’) Overlays of both staining patterns. Boxed areas in the schematic overview of the brain indicate brain areas shown in a–d. (a–a’) The superior medial protocerebrum (SMP) is innervated by a dense network of GABA-ir fibers. Several GABA-ir commissural fibers connect the SMP of both brain hemispheres (arrowheads). PDF immunoreactivity is confined to a dense fiber network of area 2 (a2), which encircles GABA immunostaining in the SMP. The vertical (VL) and medial (ML) lobes of the mushroom body are free of GABA and PDF staining. (b–b’, c–c’) GABA and PDF immunoreactivities in the superior lateral (SLP), ventrolateral (VLP), and anterior ventrolateral protocerebrum (AVLP). PDF-ir projections in the anterior fiber plexus (AFP, open arrowheads) overlap with GABA-ir fibers, especially where GABA-ir fiber bundles originating from cell clusters G1 ramify in the AVLP. Double arrowheads point to GABA-ir commissural fibers. (d–d’) GABA- and PDF-ir fibers in the lobula valley tract (LOVT) run in parallel between the medulla and central brain regions. Neurites from GABA-ir cell cluster 11 (G11) ramify in the region of the PDF-ir plexus 2 (open arrowheads, p2). Abbreviations: p1, p3, plexus 1 and 3 of PDF-ir processes along the superior optic tract; SOC, PDF-ir fibers in the superior optic commissure. Images are maximum projections from stacks of 16 to 22 optical sections (z-distance between single sections = 0.63 µm). Scale bars = 100 µm.

in medulla layer 4 overlaps with PDF- (not shown), but not with histamine immunoreactivity (Figure 8a–d).

Double- and triple-label immunofluorescence showed no colocalization between GABA and PDF immunoreactivity in any brain area of the cockroach R. maderae (Figures 8 and 9). However, neurons expressing PDF and GABA immunoreactivities were often close to each other both in the optic lobe (Figure 8) and in the central brain (Figure 9). In the optic lobe, GABA- and PDF-ir fibers in the lobula valley tract connecting the medulla and central brain run in close neighborhood to each other (Figures 8b–d and 9d–d’). Likewise, PDF- and GABA-ir
FIGURE 10  GABA immunoreactivity cycles daytime dependently in the distal tract (DT). (a–d) Staining intensity in the distal tract and all GABA-immunoreactive cells of the optic lobe is lower at ZT 12 compared to other ZTs (c). Confocal laser images obtained from frontal sections of the optic lobe at different zeitgeber times in ZT 12:12 with ZT 00–ZT 12 light phase and ZT 12–ZT 00 dark phase (a, ZT 00; b, ZT 06; c, ZT 12; and d, ZT 18). Abbreviations: AME, accessory medulla; ME, medulla. Scale bars = 50 µm

Centrally projecting fibers of PDF-ir neurons of the AME target several areas in the central brain of the cockroach that have been mapped in detail by Wei et al. (2010). Among these areas is a densely innervated region in the superior medial protocerebrum, termed area 2 (a2, Figure 9a–a’), a wide meshwork of PDF-ir processes extending from the superior lateral protocerebrum to the ventrolateral protocerebrum, termed anterior fiber plexus (AFP, Figure 9b–c’), small and more focused terminals along the lobula valley tract and superior optic commissure, termed plexus 1 and 3 (p1, p3, Figure 9d–d’), and an additional larger plexus p2 in the ventrolateral protocerebrum (Figure 9d–d’). The superior optic commissure has previously been termed anterior optic commissure (Wei et al., 2010) but is renamed here for consistency with other insect species. All these target areas of PDF-ir central projections are also densely supplied by GABA-immunolabeled profiles. PDF-ir fibers in the superior optic commissure, which connects both AMEs, as well as the fiber network of area 2 are in close vicinity to GABA-ir fibers in the superior medial protocerebrum (Figure 9a–a’). Projections of PDF-ir processes in the anterior fiber plexus overlap with GABA-ir arborizations in the superior lateral protocerebrum. GABA-ir fibers from G1 somata run in parallel, near the PDF-ir fibers of the anterior fiber plexus (Figure 9b–c’). Neurons of GABA-ir cell cluster G11 arborize in the ventrolateral protocerebrum, where the PDF-ir plexus 2 (p2) is located (Figure 9d–d’). Also, in the anterior and posterior ventrolateral protocerebrum, PDF- and GABA-ir fibers branch close to each other, however, without expressing colocalized immunoreactivity.

3.5  Daily and circadian variation in GABA levels in the optic lobe

McCay et al. (1996) reported daily and circadian fluctuations of GABA levels in the brain of the Madeira cockroach with highest levels occurring at midday. To investigate possible changes of GABA levels in neurons innervating the AME over the course of the day, GABA immunostaining of adult male cockroach brains was performed at 6-h time intervals during the 12:12 light-dark cycle. Staining intensity was quantified in the distal tract that innervates all glomeruli of the AME. Staining of the tract was strong at the beginning of the light phase (ZT 00) and in the middle of the light phase (ZT 06) but decreased significantly at ZT 12 (end of the day/beginning of the night; Figures 10 and 11). A slight increase, however, insignificant, occurred toward the middle of the dark phase (ZT 18).

To determine whether GABA content in the optic lobe shows daily variation, GABA concentrations in the optic lobe were determined at 4-h intervals using a competitive ELISA. Under LD conditions, no significant differences were observed in GABA content at different ZTs (Figure 12a; Kruskal–Wallis test followed by Dunn’s post-hoc test, p = .0085).
FIGURE 12  Box plots illustrate minimal GABA concentration per optic lobe (OL) at ZT 12, the beginning of the night. Animals in (a) were kept in 12:12 light dark cycles, animals in (b) were kept in constant darkness (DD). Accordingly, time intervals are plotted as zeitgeber time (ZT) in (a) and as circadian time (CT) in (b). Boxes show median value, range of first to third quartile of data, minimum and maximum data points. Significant differences were only found in DD between CT 04–08 and CT 12 (asterisks, Kruskal–Wallis test followed by Dunn’s post-hoc test: \( p = .0104 \)).

Abbreviation: \( n \), number of optic lobes, obtained from \( N = n/2 \) cockroaches. Lights on (white bar), lights off (black bar), in DD: subjective day (gray).

FIGURE 13  Statistical comparison of GABA titers in optic lobes (OLs) from cockroach brains (\( N = 3 \)) hinted a slightly higher GABA titer at ZT 06 (4.501 µmol l\(^{-1}/\)2OLs) as compared to ZT 12 (4.242 µmol l\(^{-1}/\)2OLs, Kruskal–Wallis test (\( p = .127; n = 6 \) measurements, with two measurements per two pooled OLs). Crosses represent mean values of GABA titers from six OLs at the respective Zeitgeber time (h).

Abbreviations: \( h \), hour; OL, optic lobes; ZT, zeitgeber time

\( p = .1168 \). When analyzing GABA concentrations at day 2 after transfer to constant darkness, however, GABA levels during the subjective day (CT 04–08) were significantly higher than GABA concentrations at the beginning of the subjective night (CT 12; Figure 12b; Kruskal–Wallis test followed by Dunn’s post-hoc test, \( p = .0104 \)). These data are consistent with differences in the intensity of GABA immunostaining in the distal tract between ZT 06 and ZT 12 (Figure 11). Furthermore, employing sensitive quantitative mass spectrometry (Figure 13) of optic lobes hinted at a slightly higher GABA titer in the optic lobes detected from individual brains (\( n = 3; n = 6 \) measurements) at ZT 06 (4.50 µmol l\(^{-1}/\)2 optic lobes) as compared to ZT 12 (4.24 µmol l\(^{-1}/\)2 optic lobes, Kruskal–Wallis test, \( p = .127 \)). Thus, while there is more variability during light dark cycles, GABA levels show circadian regulation in optic lobes of the Madeira cockroach, confirming previous measurement in whole brains (McCay et al., 1996).

4 | DISCUSSION

Cockroaches are prominent model organisms for studies on the neural control of locomotion (Varga et al., 2017), olfactory processing and odor learning (e.g., Takahashi et al., 2019; Watanabe et al., 2017), as well as circadian rhythms (Homberg et al., 2003; Stengl & Arendt, 2016; Stengl et al., 2015). As in other insect species, GABA is a widespread neurotransmitter in the cockroach brain involved in all of these functions. Accordingly, the distribution of GABA has been studied in the central complex, a motor control area (\( P. americana \), \( R. maderae \), \( Blaberus discoidalis \): Homberg et al., 2018), the antennal lobe (\( P. americana \): Distler, 1989) and mushroom body involved in olfactory processing, learning, and memory (\( P. americana \): Li & Strausfeld, 1999; Strausfeld & Li, 1999; Yamazaki et al., 1998), and in the visual (\( P. americana \): Füller et al., 1989; \( R. maderae \): Rosner et al., 2017) and circadian system (\( R. maderae \): Arnold et al., 2020; Giese et al., 2018a, b; Petri et al., 2002; Schendzielorz & Stengl, 2014). We complemented these studies of specific brain areas with a comprehensive analysis of GABA immunostaining throughout the brain of the Madeira cockroach \( R. maderae \), focusing on the circadian system. We show that GABA-ir neurons in the cockroach brain occur in large numbers of cells with somata in distinct clusters and, around the medulla in two cell types with regularly spaced cell bodies. Identical staining patterns for GABA, and the GABA synthesizing enzyme GAD and mass spectrometry confirmed the specificity of staining for the presence of GABA. The prominent GABA immunoreactivity in the circadian system as well as the circadian changes in GABA levels in the optic lobes support multiple roles of GABA in the circadian system of the cockroach.

4.1 | Immunostaining in the central brain of \( R. maderae \)

The widespread distribution of GABA immunostaining in the central brain of \( R. maderae \) suggests a prominent function of GABA in regulating different neuronal and physiological processes. Similar abundance
of GABA immunostaining as reported here has been demonstrated for the brain of other insects, including the honeybee Apis mellifera (Schäfer & Bicker, 1986), the sphinx moth M. sexta (Homberg et al., 1987), and the cockroach P. americana (Blechschmidt et al., 1990). In M. sexta, about 2800 neurons were GABA-ir in the central brain (Homberg et al., 1987), which is slightly less than the number estimated here for the cockroach. In both species, neurons have cell bodies largely organized in distinct cell clusters in the cell body rind, suggesting clonal relationship. While the physiological role of GABA in most brain areas remains to be explored, the cellular identity and functions of GABAergic neurons have been studied in some detail in the antennal lobes, mushroom bodies, and central complex. In the antennal lobe of R. maderae, a large cluster of lateral neurons gives rise to immunostaining in all glomeruli and in a medio-lateral tract connecting the antennal lobe to the protocerebrum. Highly similar staining patterns were found in the cockroach P. americana (Distler, 1989), the moths Heliothis virescens (Berg et al., 2009), M. sexta (Homberg et al., 1987), and Bombyx mori (Iwano & Kanzaki, 2005), the honeybee A. mellifera, and the fruit fly D. melanogaster (Okada et al., 2009; Sinakevitch et al., 2013; Wilson & Laurent, 2005). In P. americana, D. melanogaster, M. sexta, B. mori, and A. mellifera, the immunostained neurons were identified as large numbers of local interneurons interconnecting all glomeruli of the antennal lobe and a smaller number of antennal-lobe projection neurons in the medio-lateral antennal lobe tract targeting the lateral horn and adjacent areas in the brain (Distler, 1989; Hoskins et al., 1986; Okada et al., 2009; Wilson & Laurent, 2005). Their cell bodies are, like in R. maderae, invariably clustered laterally from the antennal lobe neuropil. Pharmacological experiments in M. sexta (Christensen et al., 1998; Waldrop et al., 1987), P. americana (Boeckh & Tolbert, 1993), A. mellifera (Sachse & Galizia, 2002; Stopfer et al., 1997), and D. melanogaster (Olsen & Wilson, 2008; Wilson & Laurent, 2005) revealed that GABAergic local interneurons of the antennal lobe inhibit projection neurons via pre- and postsynaptic mechanisms through GABA_A and GABA_B receptors. This leads to sharpening of odor profiles, improves spatial resolution in antennal lobe output neurons, and promotes oscillatory synchrony in projection neurons. GABA immunostaining in the glomerular lobe, a target of gustatory afferents from the maxillary palps (Ernst et al., 1977), has not been described before. The glomerular lobe exists only in apterygote and hemimetabolous insects (Farris, 2008). Neurons of the glomerular lobe project to the mushroom body calyces and lateral protocerebrum via the tritocerebral tract (Farris, 2008). So far, no functional data on signal processing within the glomerular lobe exist.

GABA immunoreactivity in the mushroom bodies, centers for olfactory learning, of R. maderae closely resembles the staining pattern observed in P. americana (Li & Strausfeld, 1999; Straussfeld & Li, 1999; Yamazaki et al., 1998). Similar to R. maderae, the calyces are innervated by four giant GABA-ir neurons with somata in the optic stalk and GABA-ir neurons with cell bodies beneath the calyces near the optic stalk. Reports from Yamazaki et al. (1998) that the inputs in the medio-lateral antennal lobe tract originate from ascending neurons from the circumesophageal connective could not be confirmed for R. maderae. In contrast to the honeybee (e.g., Schäfer & Bicker, 1986), sphinx moth (e.g., Homberg et al., 1987), desert locust (Papadopoulou et al., 2011), and fruit fly (Liu & Davis, 2009), GABAergic neurons in cockroaches do not provide direct feedback from the mushroom body lobes to the calyx. However, a recent study demonstrated that GABAergic giant neurons providing input to the calyces of P. americana receive excitatory input from mushroom body output neurons with dendrites in the lobes, indicating that indirect negative feedbacks from the lobes to the calyces exist in the cockroach (Takahashi et al., 2019). The role of this negative feedback is not fully understood. Locusts and fruit flies have only one GABAergic feedback neuron. Physiological data from locust show that this neuron inhibits mushroom body Kenyon cells, thereby regulating their overall firing level (Papadopoulou et al., 2011). In the fly, the neuron, termed APL, suppresses aversive olfactory leaning but is itself inhibited by the trained odor, similar to mushroom body feedback neurons in bees (Grünewald, 1999; Liu & Davis, 2009).

The lower division of the central body shows strong GABA immunostaining in all insects studied (Homberg et al., 2018). In the fruit fly and desert locust, the GABA-ir neurons have been identified as tangential neurons (termed R neurons in flies) providing input from the bulbs to a neural network in the central complex that encodes head direction relative to external and internal cues (Homberg et al., 1999; Pfeiffer & Homberg, 2014; Turner-Evans et al., 2020). The GABAergic tangential neurons inhibit each other as well as postsynaptic columnar cells in a recurrent network called ring attractor, thereby providing a compass-like representation of head directions in the columns of the central complex (Bockhorst & Homberg, 2015; Turner-Evans et al., 2020). Signaling occurs largely via GABA_A receptors but GABA_B receptor-mediated inhibition has also been demonstrated (Buhl et al., 2021; Turner-Evans et al., 2020).

### 4.2 Immunostaining in the optic lobe

The pattern of GABA immunostaining in the optic lobe is highly similar across insect species (cockroaches: Füller et al., 1989; Petri et al., 2002; Rosner et al., 2017; this study; honeybee: Schäfer & Bicker, 1986; sphinx moth: Homberg et al., 1987; swallowtail butterfly: Hamanaka et al., 2012; flies: Kolodziejczyk et al., 2008; Meyer et al., 1986). In all species studied, columnar neurons connecting the medulla to the lamina, transmedulla neurons connecting the medulla to the lobula, medulla tangential neurons with somata at the anterior end of the medulla and centrifugal neurons invading the lobula complex were immunolabeled. In D. melanogaster, eight types of GABAergic neurons were identified in the optic lobe (Davis et al., 2020; Takemura et al., 2020). These include two types of columnar feedback neurons (C2 and C3) from the medulla to the lamina, one type of medulla intrinsic neurons (Mi4), one type of distal (Dm10) and two types of proximal (Pm3 and Pm4) medulla amacrine cells, one type of transmedulla neuron (TmY15), and one type of contralaterally projecting centrifugal tangential cells innervating the medulla and lobula (CT1). A pair of contralaterally motion sensitive GABA-ir neurons with tangential innervation of the medulla has also been described in the desert locust (Stern, 2009), suggesting that similar neurons may be among the GABA-ir tangentials of the cockroach medulla. Targeted genetic manipulations combined
with behavioral assays in D. melanogaster showed that GABAergic C2 and C3 feedback neurons are important for motion vision (Tuthill et al., 2013). Silencing C2 and C3 neurons showed that the fly's responses to asymmetric motion stimuli changed. Therefore, it was proposed that C2 and C3 are responsible for asymmetric filtering of a luminance signal via presynaptic inhibition at the monopolar cell terminals in the medulla (Tuthill et al., 2013). Likewise, C3, Mi4, and CT1 cells provide inhibitory input to T4 cells in the ON-pathway of motion detection (Takemura et al., 2017).

### 4.3 Role of GABA in the circadian timekeeping system of *R. maderae*

The AME, the internal circadian clock of the cockroach (Reischig & Stengl, 2003a), is innervated by about 240 adjacent neurons forming seven distinct cell clusters (Reischig & Stengl, 2003b). Among them, about 33 neurons belonging to six of these clusters are GABA-ir. Thus, while in the suprachiasmatic nucleus, the circadian clock of mammals, about 33 neurons belonging to six of these clusters are GABA-ir. Thus, while in the suprachiasmatic nucleus, the circadian clock of mammals, almost all circadian clock neurons are GABAergic (Maywood, 2018; Ono et al., 2018), in the cockroach, considerably fewer clock cells express GABA. Nevertheless, GABA innervation is very prominent in the cockroach clock and the diversity of GABA-ir cell types of the AME together with light-like phase-shifting effects and circadian fluctuations of GABA levels suggest that GABA plays multiple roles in the circadian network. Accordingly, GABA appears to be involved in photic entrainment, internal synchronization, and coupling of clock neurons, and as an ef f e r e n t signal involved in circadian control of other brain areas (Stengl & Arendt, 2016).

In *R. maderae*, the compound eyes but not ocelli were shown to mediate photic entrainment of circadian locomotor activity (Roberts, 1965). Because histaminergic compound eye photoreceptors do not directly contact the AME, light entrainment of the clock requires intercalated interneurons. GABA injections into the optic lobe delay circadian locomotor activity onset at dusk and advance it at dawn, as light pulses do. This suggests that GABA is involved in two parallel light entrainment pathways of the circadian clock: one that relays advances in the morning, and another that relays delays if light is perceived at dusk (Petri et al., 2002).

It is generally assumed that locomotor activity rhythms in mammals and insects alike are regulated via two antagonistic circadian oscillator circuits, a morning oscillator (M clock) locked to dawn and an evening oscillator (E clock) locked to dusk (Pittendrigh & Daan, 1976; reviews: Helfrich-Förster, 2005; Stengl & Arendt, 2016). In the cockroach, the M and E clock neurons are not identified, yet. However, since phase-response curves of PDF suggest that PDF is released during the day (Petri & Stengl, 1997; Schulze et al., 2013) and since PDF release activates ipsilateral, while inhibiting contralateral projecting clock cells, we hypothesized that ipsilateral AME cells such as the large PDFMEs belong to the M clock promoting sleep and contralaterally projecting clock cells, such as three medium-sized PDFMEs to the E clock that promotes activity (Gestrich et al., 2018; Stengl & Arendt, 2016; Stengl et al., 2015). One likely candidate for an advancing light entrainment pathway to the sleep-promoting M oscillator in the Madeira cockroach is a triple-labeled GABA-, orcokinin-, and corazonin-immunoreactive MNe, because corazonin advances locomotor activity at dawn. It connects the interglomerular and shell neuropils of the AME to medulla layer 4, where only ipsilateral PDF-ir circadian clock neurons arborize. Other peptidergic and GABAergic neurons connect layer 4 to layer 2, where UV-sensitive long photoreceptors of the compound eye terminate (Arendt et al., 2017; Arnold et al., 2020). Therefore, we hypothesize that UV light at dawn activates ipsilateral PDFMEs corazonin/GABA/orcokinin-dependently, thereby advancing the M clock circuit. Additional photic inputs to the M circuit appear to be provided by MNe, like the previously reconstructed GABA-ir MNe (Petri et al., 2002), and similar neurons showing strong responses to light stimuli (Loesel & Homberg, 2001). Furthermore, neurons of the distal tract that densely innervate the glomerular core of the AME as well as tangential neurons connecting the medulla via the median-layer fiber tract to the AME may contribute to light entrainment pathways. Although the complete morphology of distal tract neurons, including the cell body location of these neurons, has not been uncovered, distal tract neurons could be traced to likely input sites in distal medulla layers, including possible inputs from long photoreceptor terminals in medulla layer 2 (Figure 8).

In contrast, candidates for delaying light input to the E clock are neurons that branch in the proximal lamina connecting the green-sensitive short photoreceptors via the anterior fiber fan to the AME, such as a triple-labeled GABA-, orcokinin-, and allatotropin-ir MNe, since allatotropin only delayed locomotor activity rhythms at dusk (Arnold et al., 2020; Schendzielorz & Stengl, 2014; Schulze et al., 2013). Furthermore, it is assumed that the extraoculart lamina and the lobula organs provide light input pathways to the circadian clock, possibly also via GABA-ir neurons connecting the accessory laminae to the AME (Arnold et al., 2020). Also, GABA-ir VNeS that project parallel to PDF-ir clock outputs connecting the AME via the anterior fiber fan to the posterior lamina are hypothesized to regulate circadian sensitivity changes of the compound eye (Arnold et al., 2020).

A role for GABA in internal synchronization of clock cells, as well as gating via ensemble formation has been suggested by Schneider and Stengl (2005). GABA application to excised AMEs leads to phase locking of spiking as well as dose-dependent inhibition of spiking activity in AME neurons mediated by GABA_A receptors (Giese et al., 2018, a, b; Schneider & Stengl, 2005). Backfills combined with immunolabeling showed that at least one GABA-ir VMNe, two VNes, and one MNe project contralaterally and may thus be involved in contralateral photic input and/or bilateral coupling of both pacemakers.

PDF-ir neurons are regarded as circadian pacemaker neurons of the cockroach AME forming inputs as well as clock outputs (Soehler et al., 2008; Stengl & Arendt, 2016). The spatial overlap of GABA immunostaining with PDF-ir terminals in several brain areas, such as the superior lateral, superior medial, and ventrolateral protocerebrum, suggests that GABAergic neuropils in various brain areas are under circadian control, possibly being postsynaptic to PDF neurons. As already reported for the whole brain (McCay et al., 1996), our measurements in the optic lobes, likewise, indicate that GABA levels are under
control of the circadian clock and express a circadian rhythm with maximum abundance during the day, when the nocturnal cockroaches rest. It is important to note that our measurements did not reveal daytime-dependent changes in GABA release but levels of overall GABA content in the optic lobe. In addition to the circadian clock, light appears to directly control GABA levels differentially in different clock circuits. This assumption is supported by the different staining intensity in different cell types (Figure 10), as well as by our ELISAs. Furthermore, also very sensitive mass spectrometry hinted at differences of GABA content in the optic lobes in light dark cycles. Altogether, our findings support the hypothesis that GABAergic AME neurons are circadian pacemaker neurons promoting sleep. Whether specific GABAergic clock cells belong to the M oscillator and are activated by PDF and light during the day needs to be examined in future experiments that can differentiate between different clock cell types.

ACKNOWLEDGMENTS
We are grateful to Dr. T.G. Kingan (University of Arizona) for the donation of anti-GABA serum. Funding was obtained from Deutsche Forschungsgemeinschaft, grant number: HO 950/26-1 to U.H., STE donation of anti-GABA serum. Funding was obtained from Deutsche Forschungsgemeinschaft, grant number: HO 950/26-1 to U.H., STE donation of anti-GABA serum. Funding was obtained from Deutsche Forschungsgemeinschaft, grant number: HO 950/26-1 to U.H., STE donation of anti-GABA serum. Funding was obtained from Deutsche Forschungsgemeinschaft, grant number: HO 950/26-1 to U.H., STE donation of anti-GABA serum.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the manuscript: A.M. and U.H.; review and editing: A.M., S.N., U.H., and M.S. Study concept and design: M.S.; acquisition of data A.M., S.N., and S.B.; the authors declare no conflict of interest.

REFERENCES
Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. *Anatomical Record*, 94, 239–247. https://doi.org/10.1002/ar.109040210
Albers, H. E., Walton, J. C., Gamble, K. L., McNeill IV, J. K., & Hummer, D. L. (2017). The dynamics of GABA signaling: Revelations from the circadian pacemaker in the suprachiasmatic nucleus. *Frontiers in Neuroendocrinology*, 44, 35–82. https://doi.org/10.1016/j.yfrne.2016.11.003
Arendt, A., Baz, E.-S., & Stengl, M. (2017). Functions of corazonin and histamine in light entrainment of the circadian pacemaker in the Madeira cockroach, Rhyparobia maderae. *Journal of Comparative Neurology*, 525, 1250–1272. https://doi.org/10.1002/cne.24133
Arendt, A., Neupert, S., Schendzielorz, J., Predel, R., & Stengl, M. (2016). The neuropeptide SiFamide in the brain of three cockroach species. *Journal of Comparative Neurology*, 524, 1337–1360. https://doi.org/10.1002.cne.23910
Arnold, T., Korek, S., Massah, A., Eschtruth, D., & Stengl, M. (2020). Candidate photic entrainment pathways to the circadian clock via optic lobe neuropils in the Madeira cockroach. *Journal of Comparative Neurology*, 528, 1754–1774. https://doi.org/10.1002/cne.24844
Berg, B. G., Schachtner, J., & Homberg, U. (2009). γ-Aminobutyric acid immunostaining in the antennal lobe of the moth *Helothis virescens* and its colocalization with neuropeptides. *Cell and Tissue Research*, 335, 593–605. https://doi.org/10.1007/s00441-008-0744-z
Bleichschmidt, K., Eckert, M., & Penzlin, H. (1990). Distribution of GABA-like immunoreactivity in the central nervous system of the cockroach, *Periplaneta americana* (L.). *Journal of Chemical Neuroanatomy*, 3, 323–336.
Bockhorst, T., & Homberg, U. (2015). Amplitude and dynamics of polarization-plane signaling in the central complex of the locust brain. *Journal of Neurophysiology*, 113, 3291–3311. https://doi.org/10.1152/jn.00742.2014
Boech, J., & Tolbert, L. P. (1993). Synthetic organization and development of the antennal lobe in insects. *Microscopy Research and Technique*, 24, 260–280. https://doi.org/10.1002/jemt.1070240305
Boer, H. H., Schot, L. P. C., Roubos, E. W., ter Maat, A., Lodder, J. C., & Reichelt, D. (1979). ACTH-like immunoreactivity in two electrotonically coupled giant neurons in the pond snail *Lymnaea stagnalis*. *Cell and Tissue Research*, 202, 231–240. https://doi.org/10.1002/jemt.1070240305
Buhl, E., Kottler, B., Hodge, J. J. L., & Hirth, F. (2021). Thermoresponsive motor behavior is mediated by ring neuron circuits in the central complex of *Drosophila*. *Scientific Reports*, 11, 155. https://doi.org/10.1038/s41598-020-80103-9
Carter, J. M. (1996). Conjugation of peptides to carrier proteins via glutaraldehyde. In J. M. Walker (Ed.), The protein protocols handbook (pp. 679–687). Heidelberg, Berlin: Springer.
Christensen, T. A., Waldrop, B. R., & Hildebrand, J. G. (1998). Multitasking of the olfactory system: Context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. *Journal of Neuroscience*, 18, 5999–6008. https://doi.org/10.1523/JNEUROSCI.18-15-05999.1998
Cyran, S. A., Yiannoulos, G., Buchsbaum, A. M., Saez, L., Young, M. W., & Blau, J. (2005). The double-time protein kinase regulates the subcellular localization of the *Drosophila* clock protein period. *Journal of Neuroscience*, 25, 5430–5437. https://doi.org/10.1523/JNEUROSCI.0263-05.2005
Davis, F. P., Nern, A., Picard, S., Reiser, M. B., Rubin, G. M., Eddy, S. R., & Henry, G. L. (2020). A genetic, genomic, and computational resource for exploring neural circuit function. *eLife*, 9, e5091. https://doi.org/10.7554/eLife.5091
Distler, P. (1989). Histochemical demonstration of GABA-like immunoreactivity in coxal labeled neuron individuals in the insect olfactory pathway. *Histochemistry*, 91, 245–249. https://doi.org/10.1007/BF00490139
Ernst, K. D., Boech, J., & Boech, V. (1977). A neuroanatomical study on the organization of the central antennal pathways in insects. II. Deutocerebral connections in *Locusta migratoria* and *Periplaneta americana*. *Cell and Tissue Research*, 176, 285–308. https://doi.org/10.1007/BF00221789
Farris, S. M. (2008). Tritocerebral tract input to the insect mushroom bodies. *Arthropod Structure and Development*, 37, 492–503. https://doi.org/10.1016/j.asd.2008.05.005
Fischbach, K.-F., & Dittrich, A. P. M. (1989). The optic lobe of *Drosophila* melanogaster. I. A Golgi analysis of wild-type structure. *Cell and Tissue Research*, 258, 441–475. https://doi.org/10.1007/BF00218858
Fleissner, G., Loesel, R., Fleissner, G., Waterkamp, M., Kleiner, O., Batschauer, A., & Homberg, U. (2001). Candidates for extraocular photoreceptors in the cockroach suggest homology to the lamina and lobula organs in beetle. Journal of Comparative Neurology, 433, 401–414. https://doi.org/10.1002/cne.1148

Füller, H., Eckert, M., & Blechschmidt, K. (1989). Distribution of GABA-like immunoreactive neurons in the optic lobes of Periplaneta americana. Cell and Tissue Research, 255, 225–233. https://doi.org/10.1007/BF00229085

Gestrich, J., Giese, M., Shen, W., Zhang, Y., Voss, A., Popov, C., & Stengl, M. (2018). Sensitivity to pigment-dispersing factor (PDF) is cell type-specific among PDF-expressing circadian clock neurons in the Madeira cockroach. Journal of Biological Rhythms, 33, 35–51. https://doi.org/10.1177/074873417739471

Giese, M., Wei, H., & Stengl, M. (2018b). Circadian pacemaker neurons of the Madeira cockroach are inhibited and activated by GABA_A and GABA_B receptors. European Journal of Neuroscience, 51, 282–299. https://doi.org/10.1111/ejn.14268

Giese, M., Gestrich, J., Massah, A., Peterle, J., Wei, H., & Stengl, M. (2018a). GABA- and serotonin-expressing neurons take part in inhibitory as well as excitatory input pathways to the circadian clock of the Madeira cockroach Rhyparobia maderae. European Journal of Neuroscience, 47, 1067–1080. https://doi.org/10.1111/ejn.13863

Gillespie, C. F., Mintz, E. M., Marvel, C. L., Huhman, K. L., & Albers, H. E. (1997). GABA_A and GABA_B agonists and antagonists alter the phase shifting effect of light when microinjected into the suprachiasmatic region. Brain Research, 759, 181–189. https://doi.org/10.1016/S0006-8993(97)00235-7

Grünewald, B. (1999). Physiological properties and response modulations of mushroom body feedback neurons during olfactory learning in the honeybee,Apis mellifera. Journal of Comparative Physiology A, 185, 565–576.

Hamanaka, Y., Kinoshita, M., Homberg, U., & Arikawa, K. (2012). Immunocytochemical identification of neurones identifiable with monoclonal antibodies to GABA. Journal of Comparative Neurology, 519, 181–190. https://doi.org/10.1002/cne.24497

Homberg, U., Humberg, T. H., Seyfarth, J., Bode, K., & Quintero Pérez, M. (2018). GABA immunostaining in the central complex of dicondylin insects. Journal of Comparative Neurology, 526, 2301–2318. https://doi.org/10.1002/cne.24497

Homberg, U., Kingan, T. G., & Hildebrand, J. G. (1987). Immunocytochemistry of GABA in the brain and suboesophageal ganglion of Manduca sexta. Cell and Tissue Research, 248, 1–24. https://doi.org/10.1007/BF01239957

Homborg, U., Reischig, T., & Stengl, M. (2003). Neural organization of the circadian system of the cockroach Leucophaea maderae. Chronobiology International, 20, 577–591. https://doi.org/10.1080/10705711.2002.1082171

Homborg, U., Vitzthum, H., Müller, M., & Binke, U. (1999). Immunocytochemistry of GABA in the central complex of the locust Schistocerca gregaria: Identification of immunoreactive neurons and colocalization with neuropeptides. Journal of Comparative Neurology, 409, 495–507. https://doi.org/10.1002/(SICI)1096-9861(19990705)409:3<495::AID-CNE1213e3.0.CO;2-F

Hoskins, S. G., Homberg, U., Kingan, T. G., Christensen, T. A., & Hildebrand, J. G. (1986). Immunocytochemistry of GABA in the antennal lobes of the sphinx moth Manduca sexta. Cell and Tissue Research, 244, 243–252. https://doi.org/10.1007/BF00219199

Iwano, M., & Kanazi, R. (2005). Immunocytochemical identification of neuroactive substances in the antennal lobe of the male silkworm moth Bombyx mori. Zoological Science, 22, 199–211. https://doi.org/10.2108/jsz.22.199

Kalodziejczyk, A., Sun, X., Meintzshagen, I. A., & Nässel, D. R. (2008). Glutamate, GABA and acetylcholine signaling components in the lamina of the Drosophila visual system. PLoS One, 3, e2110. https://doi.org/10.1371/journal.pone.0002110

Li, Y., & Strausfeld, N. J. (1999). Multimodal efferent and recurrent neurons in the medialis lobes of cockroach mushroom bodies. Journal of Comparative Neurology, 409, 647–663. https://doi.org/10.1002/(SICI)1096-9861(19990712)409:4<647::AID-CNE93e3.0.CO;2-3

Liu, C., & Reppert, S. M. (2000). GABA synchronizes clock cells within the suprachiasmatic circadian clock. Neuron, 25, 123–128. https://doi.org/10.1016/S0896-6273(00)00876-4

Liu, X., & Davis, R. L. (2009). The GABAergic anterior paired lateral neuron suppresses and is suppressed by olfactory learning. Nature Neuroscience, 12, 53–59. https://doi.org/10.1038/nn.2235

Loesel, R., & Homberg, U. (1999). Histamine-immunoreactive neurons in the brain of the cockroach Leucophaea maderae. Brain Research, 842, 408–418. https://doi.org/10.1016/S0006-8993(99)01864-8

Loesel, R., & Homberg, U. (2001). Anatomy and physiology of neurons with processes in the accessory medulla of the cockroach Leucophaea maderae. Journal of Comparative Neurology, 439, 193–207. https://doi.org/10.1002/cne.1342

Malun, D., Waldow, U., Kraus, D., & Boechk, J. (1993). Connections between the deutoecerebrum and the protocerebrum, and neuroanatomy of several classes of deutoecerebral projection neurons in the brain of male Periplaneta americana. Journal of Comparative Neurology, 329, 143–162. https://doi.org/10.1002/cne.903290202

Maywood, E. S. (2018). Synchronization and maintenance of circadian timing in the mammalian circadian clockwork. European Journal of Neuroscience, 51, 229–240. https://doi.org/10.1111/ejn.14279

McCay, J., Romero, K., Gibson, J., Newton, J., Wilson, L., Wright, J., Dahl, D. B., & Ferrell, B. R. (1996). Circadian rhythm in brain gamma aminobutyric acid levels in the cockroach, Leucophaea maderae. Journal of Experimental Zoology, 276, 262–269. https://doi.org/10.1002/(SICI)1097-010X(19961101)276:4<262::AID-JEZ3E3.0.CO;2-M

Meyer, E. P., Matute, C., Streit, P., & Nässel, D. R. (1986). Insect optic lobe neurons identifiable with monoclonal antibodies to GABA. Histochemistry, 84, 207–216. https://doi.org/10.1007/BF00495784

Moore, R. Y., & Speh, J. C. (1993). GABA is the principal neurotransmitter of the circadian system. Neuroscience Letters, 150, 112–116. https://doi.org/10.1016/0304-3940(93)90120-A

Novak, C. M., & Albers, H. E. (2004). Circadian phase alteration by GABA and light differs in diurnal and nocturnal rodents during the day. Behavioral Neuroscience, 118, 498–504. https://doi.org/10.1037/0735-7044.118.3.498
Stengl, M., & Arendt, A. (2016). Peptidergic circadian clock circuits in the Madeira cockroach. Current Opinion in Neurobiology, 41, 44–52. https://doi.org/10.1016/j.conb.2016.07.010

Stengl, M., & Homberg, U. (1994). Pigment-dispersing hormone-immunoreactive neurons in the cockroach Leucophaea maderae share properties with circadian pacemaker neurons. Journal of Comparative Physiology A, 175, 203–213. https://doi.org/10.1007/BF00215116

Stengl, M., Werckenthin, A., & Wei, H. Y. (2015). How does the circadian clock tick in the Madeira cockroach? Current Opinion in Insect Sciences, 12, 38–45. https://doi.org/10.1016/j.cois.2015.09.007

Stengl, M., Werckenthin, A., & Wei, H. Y. (2015). How does the circadian clock tick in the Madeira cockroach? Current Opinion in Insect Sciences, 12, 38–45. https://doi.org/10.1016/j.cois.2015.09.007

Stern, M. (2009). The PM1 neurons, movement sensitive centrifugal visual brain neurons in the locust: Anatomy, physiology, and modulation by identified octopaminergic neurons. Journal of Comparative Physiology A, 195, 123–137. https://doi.org/10.1007/s00359-008-0392-5

Sternberger, L. A. (1979). Immunocytochemistry. New York: Wiley.

Stopfer, M., Bhagavan, S., Smith, B. H., & Laurent, G. (1997). Impaired odour discrimination on desynchronization of odour-encoding neural assemblies. Nature, 390, 70–74. https://doi.org/10.1038/36335

Strausfeld, N. J., & Li, Y. (1999). Organization of olfactory and multimodal afferent neurons supplying the calyx and pedunculus of the cockroach mushroom bodies. Journal of Comparative Neurology, 409, 603–625. https://doi.org/10.1002/(SICI)1096-9861(19990712)409:4<603::AID-CNE7>3.0.CO;2-P

Takahashi, N., Nishino, H., Domae, M., & Mizunami, M. (2019) Separate but interactive parallel olfactory processing streams governed by different types of GABAergic feedback neurons in the mushroom body of a basal insect. Journal of Neuroscience, 39, 8690–8704. https://doi.org/10.1523/JNEUROSCI.0088-19.2019

Takemura, S.-Y., Nern, A., Chklovskii, D. B., Scheffer, L. K., Rubin, G. M., & Meinertzhagen, I. A. (2017). The comprehensive connectome of a biological substrate for ‘ON’ motion detection in Drosophila. eLife, 6, e24394. https://doi.org/10.7554/eLife.24394

Turner-Evans, D. B., Jensen, K. T., Ali, S., Peterson, T., Sheridan, A., Ray, R. P., Wolff, T., Lauritzen, J. S., Rubin, G. M., Bock, D. D., & Jayaraman, V. (2020). The neuroanatomical ultrastructure and function of a biological ring attractor. Neuron, 108, 1–19. https://doi.org/10.1016/j.neuron.2020.08.006

Tuthill, J. C., Nern, A., Holtz, S. L., Rubin, G. M., & Reiser, M. B. (2013). Contributions of the 12 neuron classes in the fly lamina to motion vision. Neuron, 79, 128–140. https://doi.org/10.1016/j.neuron.2013.05.024

Varga, A. G., Kathman, N. D., Martin, J. P., Guo, P., & Ritzmann, R. E. (2017). Spatial navigation and the central complex: Sensory acquisition, orientation, and motor control. Frontiers in Behavioral Neuroscience, 11, 4. https://doi.org/10.3389/fnbeh.2017.00004

Wagner, S., Castel, M., Gainer, H., & Yarom, Y. (1997). GABA in the mammalian suprachiasmatic nucleus and its role in diurnal activity. Nature, 387, 598–603. https://doi.org/10.1038/42468

Waldrop, B., Christensen, T. A., & Hildebrand, J. G. (1987). GABA-mediated synaptic inhibition of projection neurons in the antennal lobes of the sphinx moth, Manduca sexta. Journal of Comparative Physiology A, 161, 23–32. https://doi.org/10.1007/BF0069452

Watanabe, N., Nishino, H., Mizunami, M., & Yokohari, F. (2017). Two parallel olfactory pathways for processing general odors in a cockroach. Frontiers in Neural Circuits, 11, 32. https://doi.org/10.3389/fncir.2017.00032

Wei, H., el Jundi, B., Homberg, U., & Stengl, M. (2010). Implementation of pigment-dispersing factor-immunoreactive neurons in a standardized atlas of the brain of the cockroach Leucophaea maderae. Journal of Comparative Neurology, 518, 4113–4133. https://doi.org/10.1002/cne.22471

Wei H., Stengl M. (2011). Light affects the branching pattern of peptidergic circadian pacemaker neurons in the brain of the cockroach leucophaea maderae. Journal of Biological Rhythms, 26(6), 507–517. http://doi.org/10.1177/0748730411419968

Wilson, R. I., & Laurent, G. (2005). Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the Drosophila antennal lobe. Journal of Neuroscience, 25, 9069–9079. https://doi.org/10.1523/JNEUROSCI.0207-05.2005

Wong, J. M. T., Malec, P. A., Mabrouk, O. S., Ro, J., Dus, M., & Kennedy, R. T. (2016). Benzoyl chloride derivatization with liquid chromatography-mass spectrometry for targeted metabolomics of neurochemicals in biological samples. Journal of Chromatography A, 1446, 78–90. https://doi.org/10.1016/j.chroma.2016.04.006

Yamazaki, Y., Nishikawa, M., & Mizunami, M. (1998). Three classes of GABAergic feedback neurons in the mushroom body of a basal insect. Journal of Comparative Neurology, 390, 78–90. https://doi.org/10.1002/(SICI)1096-9861(19990712)409:4<609::AID-CNE7>3.0.CO;2-P

How to cite this article: Massah, A., Neupert, S., Brodesser, S., Homberg, U., & Stengl, M. (2022). Distribution and daily oscillation of GABA in the circadian system of the cockroach Rhyparobia maderae. Journal of Comparative Neurology, 530, 770–791. https://doi.org/10.1002/cne.25244