# The Circadian System of the Cockroach *Leucophaea maderae*: Role of the Neuropeptide Orcokinin and Light Entrainment

(Das circadiane System der Schabe *Leucophaea maderae*: Die Rolle des Neuropeptides Orcokinin und die Synchronisation der Inneren Uhr durch Licht)

Dissertation zur Erlangung des Doktorgrades
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Erstgutachter: Prof. Dr. Uwe Homberg
Zweitgutachter: Prof. Dr. Paul Galland

Tag der mündlichen Prüfung am 2004.

Meinen Eltern.

# Contents

# Erklärung: Eigene Beiträge und zur Veröffentlichung vorgesehene Teile der Arbeit

Laut Promotionsordnung der Philipps-Universität Marburg vom 29.11.1989 (idF. vom 12.4.2000) müssen bei den Teilen der Dissertation, die aus gemeinsamer Forschungsarbeit entstanden, die individuellen Leistungen des Doktoranden deutlich abgrenzbar sein. Diese Beiträge werden im folgenden näher erläutert:

**Kapitel I: Comparative distribution of orcokinin immunostaining in the brains of selected dicondylian insects**

- Ausarbeitung, Durchführung und Auswertung der meisten Experimente durch die Autorin. Immuncytochemische Färbungen mit dem Antiserum gegen Orcokinin im Gehirn der Heuschrecke *Schistocerca gregaria* wurden zum Teil von Stud. Biol. Stanley Heinze im Rahmen eines Laborpraktikums durchgeführt. Die Präparate wurden für die Auswertung herangezogen und Abbildungen hiervon in die Arbeit übernommen. Weiterhin wurde die biochemische Charakterisierung des Peptides Orcokinin in der Schabe *Leucophaea maderae* und der Heuschrecke *S. gregaria* von Herrn Dr. Heinrich Dircksen übernommen. Alle weiteren Versuche und Auswertungen wurden von der Autorin vorgenommen.
- Verfassen der Veröffentlichung in Zusammenarbeit mit Prof. Dr. Uwe Homberg und Prof. Dr. Heinrich Dircksen. Die Arbeit ist bei „Journal of Comparative Neurology“ eingereicht worden und befindet sich derzeit in Revision. Das Manuskript in der vorliegenden Arbeit entspricht, bis auf den noch einzufügenden biochemischen Teil von Dr. Heinrich Dircksen, der einzureichenden Version, nachdem die Vorschläge der Gutachter eingearbeitet wurden.

**Kapitel II: Orcokinin immunoreactivity in the accessory medulla of the cockroach *Leucophaea maderae***

- Ausarbeitung, Durchführung und Auswertung aller Experimente durch die Autorin.
- Verfassen einer Veröffentlichung in Zusammenarbeit mit Prof. Dr. Uwe Homberg. Die Arbeit wird demnächst bei „Cell and Tissue Reseach“ eingereicht. Das vorliegende Kapitel entspricht der demnächst einzureichenden Version.

**Kapitel III: Evidence for a role of orcokinin-related peptides in the circadian clock of the cockroach *Leucophaea maderae***

- Ausarbeitung, Durchführung und Auswertung aller Experimente durch die Autorin.
- Verfassen einer Veröffentlichung in Zusammenarbeit mit Prof. Dr. Uwe Homberg. Die Arbeit wird demnächst bei „Journal of Experimental Biology“ eingereicht. Das vorliegende Kapitel entspricht der demnächst einzureichenden Version.

**Kapitel IV: Extraretinal photoreceptors in the cockroach *Leucophaea maderae*: involvement of cryptochrome and UV-opsin in light entrainment?**

- Ausarbeitung, Durchführung und Auswertung aller Experimente durch die Autorin.

**Kapitel V: Involvement of the ocelli of the cockroach *Leucophaea maderae* in entrainment of the circadian clock**

- Ausarbeitung, Durchführung und Auswertung aller Experimente durch die Autorin.

Weiterhin ist die Verfasserin Co-Autorin der Arbeiten:

- Homberg U, Hofer S, Mappes M, Vitzthum H, Pfeiffer K, Gebhardt S, Müller M, Paech A (2004) Neurobiology of polarization vision in the locust *Schistocerca gregaria*. Acta Biol Hung 55:81–89
- Homberg U, Hofer S, Pfeiffer K, Gebhardt S (2003) Organization and neural connections of the anterior optic tubercle in the brain of the locust, *Schistocerca gregaria*. J Comp Neurol 462:415–430

Die Verfassung der Dissertation in englischer Sprache wurde vom Dekan der Fachbereichs Biologie am 31.08.2004 genehmigt.

# Zusammenfassung

Seit sich vor mehr als einer Milliarde Jahren innere tagesrhythmische Taktgeber in einzelligen Organismen entwickelten, hat die Evolution unterschiedlichste Innere Uhren in fast jedem Organ hervorgebracht. Innere Uhren, die das Verhalten und komplexe physiologische Abläufe regulieren, sind jedoch zumeist im Zentralnervensystem untergebracht. Vom frühen 18. Jahrhundert bis heute gewann man immer mehr Kenntnisse über die Bedeutung und Funktion Innerer Uhren in verschiedensten Lebewesen. Da Innere Uhren keinen exakt 24-stündigen Rhythmus produzieren, müssen die Uhren ständig an den realen Tagesablauf synchronisiert werden („entrainment"). Soziale Kontakte, Temperaturen und Futtergabe vermögen die Uhren zu synchronisieren, jedoch ist Licht bzw. der tägliche Wechsel von Hell und Dunkel der zuverlässigste und damit auch wichtigste Synchronisationsfaktor.

Durch Läsionsexperimente in den 60er und 70er Jahren konnte der circadiane Oszillator, der das Laufverhalten der Schabe *Leucophaea maderae* steuert, in den optischen Loben lokalisiert werden. Erst kürzlich war es aber möglich, den circadianen Schrittmacher der Schabe in der akzessorischen Medulla (AMe), einem kleinen Neuropil in der Medulla des optischen Lobus, zu orten. Die AMe wird von etwa 250 Neuronen, die sich in verschiedenen, abgrenzbaren Zellgruppen der AMe befinden, innerviert. Bisherige Ergebnisse zeigen, daß sowohl klassische Neurotransmitter, als auch Peptide bei der Aufrechterhaltung, Steuerung und Synchronisation der Inneren Uhr der Schabe eine bedeutende Rolle spielen. Die Identifizierung und das funktionelle Charakterisieren von Neuropeptiden der AMe leisten daher einen wichtigen Beitrag zur Aufklärung der Funktion des Uhrwerks in der AMe von *L. maderae*.

Die vorliegende Dissertation hatte vor allem zum Ziel, ein in der AMe der Schabe neu gefundenes Peptid, das aus der Familie der Orcokinine stammt, zu charakterisieren. Da das Peptid bisher bei Insekten vollkommen unbekannt war, wurde zuerst untersucht, ob es auch bei anderen Insektenarten zu finden ist und inwiefern sich die Verteilung von immunmarkierten Neuronen in verschiedenen Arten unterscheidet. Bei allen Insekten, die Orcokinin-ähnliche Immunfärbung aufwiesen, waren unter anderem auch einige Neurone der AMe gefärbt, was eine Funktion dieses Peptids in der Inneren Uhr nahe legte. Daher wurden diese Neurone bei *L. maderae* detailliert morphologisch charakterisiert, wobei auch Doppelfärbungen mit anti-Orcokinin Antiserum und Antiseren gegen bisher bekannte Transmitter und Peptide der AMe durchgeführt wurden. Im nächsten Schritt wurde die physiologische Rolle von Orcokinin in der Inneren Uhr mittels Peptidinjektionen an circadian freilaufenden Schaben untersucht, wobei sich herausstellte, daß Orcokinin eine Rolle in der Lichtsynchronisation des circadianen Schrittmachers der Schabe *L. maderae* zu spielen scheint.

Neben der Charakterisierung von Orcokinin wurden weitere Arbeiten zum Lichtsynchronisationsweg der Schabe durchgeführt. Im optischen Lobus der Schabe befinden sich

zwei Strukturen, das Lamina- und das Lobulaorgan, die als mögliche extraretinale Photorezeptoren an der Lichtsynchronisation beteiligt sein könnten. Diese Annahme wurde durch immuncytochemische und proteinbiochemische Untersuchungen von dort vorkommenden Photorezeptormolekülen weiter untermauert. In einem weiteren Projekt konnten schließlich Hinweise dafür gefunden werden, daß die Ocellen der Schabe entgegen bisheriger Vermutungen eine modulierende Rolle bei der Lichtsynchronisation der Schabe spielen könnten. Die Inhalte der einzelnen Kapitel der Arbeit werden im folgenden näher zusammengefaßt:

**Kapitel I: Comparative distribution of orcokinin immunostaining in the brains of selected dicondylian insects**

Orcokinine wurden bei den verschiedensten Crustaceen identifiziert, den ursprünglichen Namen jedoch haben diese Neuropeptide von dem Flußkrebs *Orconectes limosus*. Alle bisherigen biochemischen und immuncytochemischen Untersuchungen wurden ausschließlich an decapoden Crustaceen durchgeführt. Da dieses Peptid sowohl im Nervensystem, als auch in der Hämolymphe präsent ist, wirkt es bei diesen Tieren offenbar sowohl als Neuromodulator als auch als Hormon. Da Insekten mit Crustaceen eng verwandt sind, lag es nahe, ein Antiserum gegen $Asn^{13}$-Orcokinin in immuncytochemischen Färbungen im Oberschlundganglion (Gehirn) von Insekten zu testen und bei verschiedenen Arten zu vergleichen. Um eine größere Bandbreite unterschiedlicher Insektengruppen vergleichen zu können, wurden Vertreter hemimetaboler (Zygentoma: Silberfisch *Lepisma saccharina*; Blattaria: Schabe *Leucophaea maderae*; Orthoptera: Heuschrecken *Locusta migratoria* und *Schistocerca gregaria*) als auch holometaboler Insektengruppen (Hymenoptera: Honigbiene *Apis mellifera*; Lepidoptera: Tabakschwärmer *Manduca sexta*; Diptera: Taufliege *Drosophila melanogaster*) herangezogen. Interessanterweise wurde Orcokinin-ähnliche Immunreaktivität nur in Neuronen der hemimetabolen Arten gefunden, wobei die gefärbten Neurone in den jeweiligen Gehirnen sehr ähnlich verteilt waren. Die optischen Loben, vor allem die Medulla und die AMe, wiesen sehr prominente Immunmarkierungen auf. Weiterhin waren auch intrinsische und extrinsische Neurone des Pilzkörpers und des Zentralkomplexes sowie weitere Bereiche des Protocerebrum markiert. Ebenso wiesen auch lokale Interneurone des Antennallobus (Deutocerebrum) als auch das Tritocerebrum zahlreiche Orcokinin-immunreaktive (-ir) Neurone auf. Immunmarkierte Fasern von neurosekretorischen Zellen im Gehirn ließen sich bis in die *corpora cardiaca* verfolgen. Die Ergebnisse lassen deshalb vermuten, daß Orcokinin nicht ausschließlich als Neuromodulator, sondern auch als Hormon wirken könnte, wie es auch schon bei den Crustaceen beschrieben wurde. Erste biochemische Charakterisierungen des Peptides in der Schabe und der Heuschrecke bestätigen das Vorhandensein von Orcokinin. Im Gegensatz dazu konnte bei den holometabolen (endopterygoten) Arten keine Immunantwort gefunden werden. Möglicherweise wurde das Peptid entweder von dem verwendeten Antiserum nicht erkannt, da die Peptidsequenz bei den phylogenetisch weiter abgeleiteten Endopterygoten stark verändert ist, oder aber das Peptid ist bei diesen Insekten im Laufe der Evolution verloren gegangen.

**Kapitel II: Orcokinin immunoreactivity in the accessory medulla of the cockroach *Leucophaea maderae***

Immunfärbungen mit dem Antiserum gegen Asn[13]-Orcokinin aus *Orconectes limosus* zeigten zahlreiche Orcokinin-ir Neurone in verschiedenen Neuropilen im Gehirn der Schabe *Leucophaea maderae*, u.a. auch in der AMe. In dieser Arbeit wurde die Verteilung von Orcokinin-ir Neuronen in der AMe, dem circadianen Schrittmacherzentrum der Schabe untersucht. Die AMe ist bilateral angelegt und wird über die anteriore und posteriore optische Kommissur mit der AMe der anderen Gehirnhälfte verbunden. Sie besteht aus dichtem nodulären und umgebendem lockeren internodulären Neuropil. Diese Untereinheiten werden u.a. aus sechs verschiedenen, der AMe benachbarten Neuronengruppen aufgebaut. Dabei werden Eingangsneurone, lokale Interneurone und Ausgangsneurone unterschieden, die den verschiedenen Neuronengruppen zugeordnet werden konnten. Das Verteilungsbild der Orcokinin-ir Neurone unterschied sich von allen bisher durch Peptid- oder Transmitter-Immunreaktivität charakterisierten Neuronen. Dreißig Orcokinin-ir Neurone verteilten sich auf fünf der insgesamt sechs Neuronengruppen der AMe. Von besonderem Interesse waren drei markierte Neurone in der Gruppe der ventromedianen Neurone (VMNe). Ihre Fortsätze ziehen in das internoduläre Neuropil der AMe, verzweigen in einer medianen Schicht der ipsilateralen Medulla, und verbinden über den sogenannten *lobula valley tract* und die posteriore optische Kommissur beide AMae. Diese Neurone wiesen eine sehr große Ähnlichkeit zu bereits bekannten lichtsensitiven Neuronen auf. Dies ist bislang der erste Hinweis für einen möglichen Neuromodulator in dieser Neuronengruppe. Weiterhin waren bis zu 16 ventrale Neurone (VNe) markiert, deren Verzweigungen hier jedoch nicht weiter dargestellt werden konnten. Aus vorherigen Studien ist bekannt, daß bis zu vier Neurone aus dieser Zellgruppe ebenfalls beide AMae verbinden und möglicherweise beide AMae in ihrer Phasenlage koppeln. Zur Charakterisierung möglicher Kotransmitter und -peptide in den markierten Neuronen wurden Doppelmarkierungen mit Asn[13]-Orcokinin-Antiserum und Antiseren gegen bereits in der AMe bekannte Peptide (*pigment dispersing hormone* [PDH], FMRFamid, Mas-Allatotropin, Leucokinin) und dem Neurotransmitter $\gamma$-Aminobuttersäure (GABA) durchgeführt. Kolokalisation von Orcokinin mit PDH, FMRFamid, Mas-Allatotropin und GABA konnte in mehreren Zellgruppen der AMe gefunden werden, nicht jedoch in der anterioren- und posterioren optischen Kommissur. Die morphologischen Daten lassen vermuten, daß Orcokinin Lichtinformation in die kontralaterale AMe übermittelt und/oder die Phasenlage beider Oszillatoren koppelt. Somit scheint Orcokinin eine multifunktionelle Rolle in der AMe von *L. maderae* zu übernehmen.

**Kapitel III: Evidence for a role of orcokinin-related peptides in the circadian clock of the cockroach *Leucophaea maderae***

Die vorgehenden Untersuchungen ließen vermuten, daß Orcokinin-ir Neurone aus zwei Gruppen der AMe (ventromediale Neurone: VMNe und ventrale Neurone: VNe) beide AMae über die posteriore optische Kommissur untereinander verbinden und eventuell Lichtinformation und/oder Information über die Phasenlage beider Oszillatoren übermitteln. Aufgrund der zahlreichen Orcokinin-ir Neurone in der Uhr selbst und zahlreichen Fasern in der anterioren und posterioren optischen Kommissur war es bislang nicht möglich, die tatsächliche Anzahl der Orcokinin-ir Neurone zu ermitteln, die beide

AMae verbinden. Um dies herauszufinden, wurde der Farbstoff Rhodamin-Dextran, der von Neuronen aufgenommen und in die Verzweigungen transportiert wird, in eine AMe injiziert und die Gehirne daraufhin mit anti-Orcokinin Antiserum immuncytochemisch gefärbt. Hierbei wurden ein Orcokinin-ir Neuron aus der Gruppe der VNe und alle drei Orcokinin-ir Neurone der VMNe in der kontralateralen AMe markiert, sowie Fasern in der posterioren optischen Kommissur. Dies bedeutet, daß diese vier Zellen beide AMae direkt verbinden und somit Information zwischen den Schrittmachern austauschen. Um eine mögliche Funktion von Orcokinin in der Inneren Uhr nachzuweisen, wurde $Asn^{13}$-Orcokinin in die Nähe einer AMe injiziert und die Phasenlage und Periodenlänge des circadianen Laufverhaltens vor und nach der Injektion verglichen. Phasenverschiebungen in Abhängigkeit von der injizierten Zeit ergaben eine dosisabhängige, lichtähnliche Phasen-Antwort-Kurve. Diese Daten unterstützen die aus den morphologischen Beobachtungen hergeleitete Annahme, daß Orcokinin eine wichtige Rolle im Lichteingang zur AMe der Schabe *L. maderae* über das kontralaterale Auge spielt. Diese Studie zeigte zum erstenmal eine mögliche physiologische Rolle von Orcokinin bei Insekten.

**Kapitel IV: Extraretinal photoreceptors in the cockroach *Leucophaea maderae*: involvement of cryptochrome and UV-opsin in light entrainment?**

Viele Vertebraten, aber auch Invertebraten, haben zusätzlich zu den retinalen auch extraretinale Photorezeptoren, die in den Lichteingang der Inneren Uhr involviert sind. Vor kurzem wurden im optischen Lobus der Schabe *L. maderae* zwei Photorezeptor-ähnliche Strukturen gefunden, das Lamina- und das Lobulaorgan. Das Laminaorgan liegt nahe der Lamina anterior des ersten optischen Chiasma, das Lobulaorgan anterior zwischen Lobula und Medulla nahe der AMe. Morphologische Untersuchungen ergaben in beiden Organen mikrovilläre Strukturen und das Vorhandensein von Desmosomen, was zusammen charakteristisch für Photorezeptoren ist. Immuncytochemische Evidenzen für das Vorhandensein des Blaulichtrezeptors Cryptochrom (CRY) in beiden Organen untermauerten die Annahme, daß es sich um Photorezeptoren handelt. Die immuncytochemischen Experimente wurden in dieser Arbeit mit Antiseren gegen CRY aus *Drosophila* und aus der Ackerwinde *Arabidopsis thaliana* wiederholt und weitergeführt. Nachdem das Antiserum gegen CRY aus *Drosophila* an *Drosopila*-Embryonen präadsorbiert wurde, ließen sich nicht nur Strukturen im Lamina- und Lobulaorgan, sondern auch im Zentralhirn anfärben, was eine weitere Verbreitung von CRY im Gehirn der Schabe, als bisher angenommen, vermuten läßt.

Mit Hilfe der Protein-Immunoblot („Western-blot“)-Technik konnte eine tageszeitliche Schwankung von CRY im Gehirn gezeigt werden. Wie in den Immunfärbungen gezeigt, wurde auch in den Western-blots CRY in verschiedenen Gehirnbereichen inklusive der AMe nachgewiesen. Diese Daten widersprechen bisher veröffentlichten Resultaten bei *L. maderae*, jedoch zeigen sie Ähnlichkeit zu den Verhältnissen bei *Drosophila*. Um aber CRY in der Schabe zweifelsfrei nachweisen und charakterisieren zu können ist es nötig, das Gen zu sequenzieren und mit bekannten Cryptochromen zu vergleichen. Voruntersuchungen dieser Art erbrachten erste direkte Hinweise auf das Vorhandensein von CRY in *L. maderae*.

Weiterhin wurde untersucht, ob neben CRY noch weitere Photorezeptormoleküle im Lamina- und Lobulaorgan vorhanden sind. Dazu wurden eine Reihe Antiseren getestet,

die gegen verschiedene Proteine in der Phototransduktionskaskade gerichtet waren. In diesen Versuchen konnten mit einem Antiserum gegen UV-Opsin Strukturen im Lamina- und Lobulaorgan markiert werden. Diese Untersuchungen lassen vermuten, daß es sich beim Lamina- und Lobulaorgan um extraretinale Photorezeptoren handelt, die vor allem für kurze Wellenlängen sensitiv sind.

**Kapitel V: Involvement of the ocelli of the cockroach *Leucophaea maderae* in entrainment of the circadian clock**

Viele Insekten besitzen zusätzlich zu den Komplexaugen einfacher gebaute Augen, die Ocellen. Im Grundbauplan der Insekten sind drei Ocellen vorhanden, die aber bei verschiedenen Gruppen teilweise oder völlig reduziert sein können. Im allgemeinen sind die Ocellen besonders lichtsensitiv und sind wegen eines hierfür ungeeigneten dioptrischen Apparates zum Bildsehen nicht geeignet. Schaben besitzen zwei Ocellen nahe der Antennenbasis, die möglicherweise die größten und auch lichtempfindlichtesten Ocellen im Insektenreich darstellen und morphologisch gut untersucht sind. Funktionell ist über diese Augen jedoch wenig bekannt. In den letzten Jahren fand man bei der Schabe Interneurone, die die Ocellarnerven mit der Medulla sowie mit der AMe verbinden. Bisher konnte für die Ocellen nur bei der Fliege (*Drosophila)* und der Grille (*Teleogryllus commodus*) eine Rolle im Lichteingang in die Innere Uhr vermutet werden. Bei der Schabe ergab sich bei den bisher untersuchten relativ hohen Lichtstärken kein Hinweis darauf. Um bei der Schabe *L. maderae* eine mögliche Beteiligung der Ocellen am Lichteingang in den Oszillator bei niedrigen Lichtstärken zu untersuchen, wurde zunächst die Lichtintensität ermittelt, bei der 50 % der Tiere noch an die vorherrschenden 12:12 h Licht-Dunkel (LD) Bedingungen ankoppeln (50 %-Schwelle). Für diese Versuche wurde Weißlicht verwendet, das zu Beginn und Ende eines jeden Lichtzyklus langsam erhellt bzw. abgedunkelt wurde, um so eine Dämmerung zu simulieren. Die Lichtintensität variierte von $5{,}8 \times 10^{-6}$ bis hinunter zu $3{,}6 \times 10^{-12}\,\mathrm{W/cm^2}$. Nach jeder Lichtreduktion folgte auch eine Phasenverschiebung im LD Zyklus von 6 h, und es wurde untersucht, ob sich die Tiere auf die neue Phasenlage des Zeitgebers einstellen (synchronisieren). Dabei stellte sich heraus, daß die 50 %-Schwelle zwischen $1{,}5 \times 10^{-10}\,\mathrm{W/cm^2}$ (73 % der Tiere) und $3{,}6 \times 10^{-12}\,\mathrm{W/cm^2}$ (32 % der Tiere) lag. Die 50 %-Schwelle wurde durch lineare Interpolation bei etwa $7{,}7 \times 10^{-11}\,\mathrm{W/cm^2}$ bestimmt. Nachdem den Schaben die Ocellen entfernt wurden, konnten weiterhin 77 % der Tiere an Lichtintensitäten von $1{,}5 \times 10^{-10}\,\mathrm{W/cm^2}$ ankoppeln, jedoch keines der operierten Tiere an $3{,}6 \times 10^{-12}\,\mathrm{W/cm^2}$. Die Daten weisen zum ersten Mal auf eine direkte oder indirekte Rolle der Ocellen bei der Synchronisation der circadianen Uhr der Schabe *Leucophaea maderae* hin.

# Introduction

## Circadian rhythms

Most organisms on earth are submitted to the daily changes of day and night with a periodicity of 24 hours caused by the rotation of the earth around its geographical axis. This daily solar cycle causes periodic changes of light and temperature, which provide an ordered temporal structure to each day. Animals and plants had to adapt to this temporal order and developed species-specific preferences for the right timing of physiological and behavioral events, thereby forming and inhabiting temporal ecological niches, in addition to spatial niches. To maintain this temporal order, it became advantageous very early in the evolution of life not to react passively to changes of the surrounding world, but to develop internal clocks that allow for anticipation of periodic events. These clocks provide organisms with information about the current time of the day, but since their periods are not exactly identical to 24 h (and are, therefore, called circadian rhythms, from the Latin *circa*: about, and *dies*: a day), they have to be steadily synchronized to the real time. The importance of our internal clocks becomes obvious if clock synchronization and function is disturbed due to exposure to jetlag, shift work, and short winter days in northern latitudes that may cause severe health problems to affected people.

Nevertheless, the existence of self-sustained internal clocks was for long times not accepted or not taken into consideration at all. Just in 1729, the French astronomer Jean-Jacques de Mairan observed daily leaf movements in the plant *Mimosa pudica*. These movements continued every day under light-dark (LD) cycle and also in constant darkness. De Mairan's conclusion was: "... the plant thus senses the sun with out seeing it in any way". Until that time it was universally assumed that such rhythms only persist in the cycle of light and dark. During the 1920s Erwin Bünning (1935) confirmed that plants housed in constant light showed rhythmic movements with a cycle length that slightly deviated from 24 h, and were not dependent on external cues.

Not only in plants, also in animals were scientists interested in the function of the circadian clock. Circadian rhythms in mammals were already observed in the 1920ies while observing locomotor activity in rats (Richter 1922). In 1935, the deviation of the period of animal rhythm from 24 h in constant darkness was established in the fruitfly *Drosophila* by Kalmus and Bünning. Also Johnson in 1939 assumed that the origin of the rhythm must be sought within the organism itself, because he kept mice in constant conditions and noted that the periodicities differed from 24 h. Therefore, Johnson concluded that "... the animal has an exceptional substantial and durable self-winding and self-regulating physiological clock...". In 1950, Aschoff (1960) raised chickens under constant conditions and found that they express normal daily rhythms, indicating that a rhythmic environment is not essential for the organism to develop a fully functional rhythm-generating

system. But at that time none of the scientists understood the underlying mechanism of the endogenous clock.

Several years later, in the cockroach *Leucophaea maderae* the optic lobes of the brain were recognized as a circadian clock locus for the daily activity/rest cycle (Nishiitsutsuji-Uwo and Pittendrigh 1968). In these night-active animals, arrhythmic activity was induced by complete ablation of the optic lobes of the cockroach. The locus of the clock could be more precisely determined when Roberts (1974) found that surgical lesions of the proximal medulla resulted in loss of rhythmic locomotor activity. By means of precise electrolytic lesions Sokolove (1975) narrowed down the clock location to an area in the ventral part of the optic lobe between medulla and lobula. In 1978, Page transected the optic lobes of the cockroach, thus causing arrhythmicity, but when he left the lobes in situ for about 40 days, all of the arrhythmic animals became rhythmic again. The period of the restored rhythmicity was similar to the original rhythm. Moreover, after exchange of optic lobes between cockroaches with different endogenous periods, the host animals regained a circadian rhythm with the periods of the donors (Page 1982). These results strongly confirmed that the oscillator is situated in the optic lobe and demonstrated that it continues to function after being separated from the brain. The data further showed that neuronal output mechanisms must convey signals from the pacemaker to driven output structures in the brain. Several years later, the accessory medulla (AMe), a small neuropil at the anterior ventroproximal edge of the medulla was proposed as a clock locus by Homberg et al. (1991). Immunocytochemical studies with an antiserum against the crustacean octadecapeptide pigment dispersing hormone (PDH) in several orthopteroid insect species including cockroaches revealed three to four immunostained neuron groups in the optic lobe. One or, in cockroaches, two of these PDH-immunoreactive (-ir) neuron groups are situated near the anterior proximal margin of the medulla (PDH-ir medulla neurons, PDHMe) and densely innervate the AMe. These neurons also arborize in the medulla, lamina, in several regions of the midbrain, and some of them have contralateral projections. They fulfilled all anatomical properties predicted for pacemaker neurons in insects.

The search for the molecular mechanism of the circadian clock also started in an insect, namely the fruitfly *Drosophila melanogaster* (Konopka and Benzer 1971). Benzer's and Konopka's mutant fly strains showed an aperiodic behavior with no signs of an overt circadian rhythm. When they tested further mutants in constant conditions (constant darkness), one of them had a short (19 h), another a longer (29 h) period in circadian behavior, respectively. A search for the genetic defect revealed that all these animals had a mutation in the *per* (*period*) locus, which appeared to function in the circadian clock mechanism: the mutant alleles $per^{01}$ (*aperiodic*), $per^{s}$ (s*hort*) and $per^{l}$ (*long*; Konopka and Benzer 1971). Up to now cloning and molecular analyses of clock genes combined with behavioral studies revealed novel rhythm variants. In summary, it is now widely accepted that two transcriptional and translational feedback loops, which house a variety of molecules (e.g., PERIOD, TIMELESS, CLOCK, and CYCLE), are interwoven to generate a stable circadian rhythm (Stanewsky 2002).

Self sustained endogenous oscillators in organisms manage not only to reinforce the regularity of rhythmic cycles, but have the great advantage to allow for anticipating environmental conditions. For instance, it might be vital for a rodent to reach its burrow

before dawn to avoid day-active predators. An internal clock allows the organism to prepare right before a new phase begins. Nearly all physiological and behavioral functions in organisms, including humans, occur on a rhythmic basis. The knowledge about circadian rhythms and oscillator function in humans has a steadily growing impact on medicine. Many adverse effects of disrupted circadian rhythms may be linked to disturbance in the sleep-wake cycles and to seasonal affective disorder, as well as to problems with shiftwork and jetlag. An enhanced understanding of our internal clock allows for a better appreciation of the importance of a normal temporal organization for human health and disease. Therapeutic strategies considering chronobiological insights might be able to influence time dependent illness (heart attack and strokes show time-of-day variation). The range of applications of chronobiological thinking to medical care has just begun to be explored.

### Influence of light on the circadian clock: light entrainment

The most obvious daily cycle in our world is the change of day and night. The length of the light phase changes with the season, except for the Equator, and the intensity of light at any time of the day changes with the weather, but the period of the illumination cycle is constant at 24 hours. Many other environmental factors vary with the daily changes of illumination, such as temperature and humidity, but these variations are far less reliable than the light cycle. It is therefore not surprising that for most organisms the daily cycle of light and dark (and perhaps spectral quality of light at dawn and dusk), is the dominant Zeitgeber for their daily rhythm. If a biological clock is synchronized by the environmental changes of, e.g., illumination, this process is called entrainment. Synchronization of the clock by the daily cycle of light and dark is termed photoentrainment. It is important to understand that light cycles are not imposing circadian rhythmicity, but circadian rhythms in organisms are generated internally in both constant conditions and under light cycles. The function of light cycles is to slightly modify the period ($\tau$) of the underlying oscillator that in constant conditions would differ slightly from 24 h, so that it is adjusted precisely to 24 h. Individuals of different species can have different freerunning periods that range from very short (21 h) to very long (27 h; Pittendrigh 1960). Furthermore, there are variations in $\tau$ within species, depending on age, hormone status, and previous light conditions. How can illumination cycles synchronize different periods to the same 24 h period of the day?

If an animal is kept in constant conditions (e.g., constant darkness, DD), the circadian rhythm freeruns with a species-specific period length (Pittendrigh 1993). In diurnal animals, the freerunning period is often slightly longer than 24 h, and in nocturnal animals shorter than 24 h (Pittendrigh 1960). In animals such as hamsters, different behavioral and physiological parameters that are circadianly driven can be monitored, e.g., by using running wheels. Their rotations are automatically counted to record locomotor activity (Fig. 1). If the animal is kept in constant darkness and complete temporal isolation, it does not experience an external cue for real day or night. Instead, the behavior indicates that the animal experiences a subjective day and night, in which the time is not equal to real time, but is expressed as circadian time (CT; Fig. 1A). The time when the animal behaves as it would do during the real day is termed the subjective day; when it acts as in the real night, the animal is in its subjective night. The period from one so-called

phase reference point (e.g., begin of the activity phase) to the next under freerunning conditions is called the circadian day. Since the hamster is a nocturnal species and is active at night, it starts to run at the beginning of its subjective night, which is defined as CT 12 h (Fig. 1A).

The exposure of a hamster freerunning under constant darkness to 10 minutes light-pulses at different circadian times during the circadian cycle reveals strikingly different results depending on the circadian time at which the pulse occurred (Pittendrigh and Daan 1976; Ralph and Menaker 1988). Light-pulses that occur during the subjective day cause little or no effects (Fig. 1B). In contrast, light-pulses that fall early in the subjective night cause phase delays (the subsequent freerun starts from a phase later than predicted by the extrapolation of the pre-pulse freerun), light-pulses in the late subjective night cause phase advances (the freerun starts from a phase earlier than predicted by the previous freerun; Fig. 1C, D). These phase shifts can be interpreted insofar as single brief pulses of light lengthen or shorten the freerunning period for one cycle, respectively, by slowing down (delaying) or accelerating (advancing) the oscillator. The rhythm thereby shifts away from the time expected if the freerun had continued undisturbed. These phase shifts are additionally dependent on the intensity and duration of the light-pulses.

Phase response curves (PRCs) can be created, if the amplitude and direction of phase shifts resulting after light-pulses that are equal in duration and intensity, but are applied at different circadian times, are plotted against the respective circadian time (Fig. 1E). When comparing the PRCs obtained with light-pulses between nocturnal and diurnal organisms, it turned out that they all were strikingly similar in shape, with phase delays in the early and phase advances in the late subjective night (Daan and Pittendrigh 1976; Pittendrigh 1993). These similarities, but also the remaining differences between PRCs of different species, allow deep insights into the function of circadian clocks.

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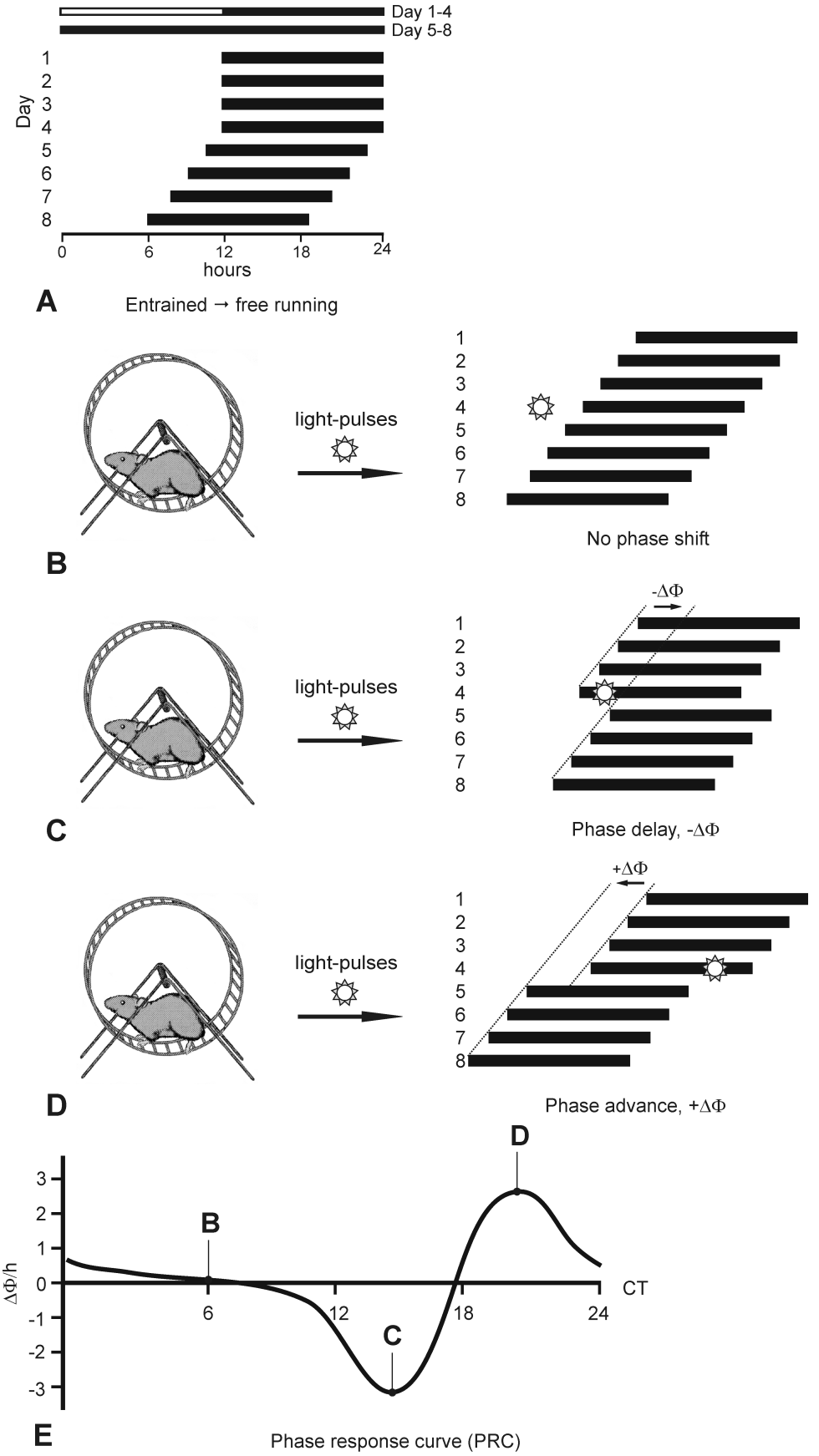
**Fig. 1A–E** (next page) Schematic outline of an experimental strategy to study the circadian clock, e.g., in hamsters. A hamster is placed in a running wheel and the circadian locomotor activity is monitored. **A** The activity phases of the animal appear as black bars in the actogram plot. The bars above the actogram indicate a light-dark (LD) cycle of 12:12 h during day 1–4 and constant darkness from day 5–8. In the first four days the animal entrains to the LD regime (lights on at Zeitgeber time (ZT) 0, and lights off at ZT 12) and in this condition the endogenous period ($\tau$) was exactly 24 h. The hamster is entrained to the LD cycle, i.e., every day the animal starts its activity phase at ZT 12. At day 5 the light is turned off. In constant darkness, the circadian clock of the hamster freeruns, i.e., the activity phase of every day starts earlier and $\tau$ is shorter than 24 h, and the activity phase of the animal begins at circadian time (CT) 12 h. CT 0–12 h is called the subjective day, CT 12–24 h is called the subjective night. **B** A hamster is placed in a running wheel in constant darkness. Under these conditions it shows a freerunning circadian rhythm shorter than 24 h. When a short light-pulse is applied at the middle of the subjective day at day 4, no phase shift occurs. **C** The animal receives a light-pulse in the early subjective night at day 4. The following day activity onset is delayed with respect to the expected activity onset (*dotted line*). The phase delay is expressed as negative values of $\Delta\phi$, i.e., expected activity onset is calculated to the actual activity onset. **D** A light-pulse given in the late subjective night at day 4 advances onset of the subsequent activity cycle resulting in a phase advance, characterized by a positive $\Delta\phi$. **E** The resulting phase shifts can be plotted against the circadian time when the light-pulses were applied. The result is a phase response curve (PRC). The PRC for light-pulses has a typical biphasic shape. Light-pulses at the subjective day result in no or minor phase shifts, at the early subjective night phase delays and at the late subjective night phase advances are observed. Figures modified after Albrecht and Eichele 2003.


Day 1-4
Day 5-8
Day
1
2
3
4
5
6
7
8
0
6
12
18
24
hours
A
Entrained → free running
light-pulses
No phase shift
B
light-pulses
-ΔΦ
Phase delay, -ΔΦ
C
light-pulses
+ΔΦ
Phase advance, +ΔΦ
D
D
B
C
3
2
1
0
-1
-2
-3
ΔΦ/h
6
12
18
24
CT
E
Phase response curve (PRC)

Light signals reach the oscillator of a circadian clock via photoreceptors and certain neuronal or molecular pathways, called entrainment pathways. Many animals have, in addition to their retinal photoreceptors (located in their principal eyes), extraretinal photoreceptors (in the brain or elsewhere in the body). Extraretinal photoreceptors are widespread in the animal kingdom and often play a critical role in the regulation of biological clocks. In non-mammalian vertebrates deep brain and pineal photoreceptors have been shown to play a fundamental part in circadian clock entrainment (reviewed by Vigh et al. 2002).

In contrast, mammalian vertebrates have apparently no extraretinal photoreceptors. They use their eyes for the regulation of time-dependent physiological events and for the light entrainment (Zordan et al. 2001; Foster and Helfrich-Förster 2001). Photic information from the retina is conveyed directly to the SCN by the retinohypothalamic tract and indirectly by the geniculohypothalamic tract. Interestingly, many blind humans show normally entrained circadian rhythms. However, in individuals that later had their eyes removed (sometimes done for cosmetic reasons), light entrainment was not possible anymore (Czeisler et al. 1995; Lockley et al. 1997). It was quite recently one of the most astonishing discoveries in chronobiological research that the mammalian eye has, despite of the well-known rods and cones, additional photoreceptive cells (reviewed by Bellingham and Foster 2002). These are a subtype of retinal ganglion cells, which do not only employ the ancient melanopsin as photoreceptor molecule, but also carry the blue light photopigment cryptochrome. Cryptochrome may thus function as a light sensor in the retina integrated in the mammalian photoentrainment pathway (Miyamoto and Sancar 1998).

In insects, especially in *Drosophila*, different ocular and extraocular photoreceptors were determined that entrain the circadian clock to environmental light cycles (Helfrich-Förster et al. 2001). Possible candidates for clock-synchronizing photoreceptors are the compound eye, the Hofbauer-Buchner eyelet, the ocelli, and even the clock neurons, the $LN_v$s themselves, which contain cryptochrome as a photoreceptive molecule (Rieger et al. 2003; Helfrich-Förster et al. 2001). Only after total elimination of all of these photoreceptive structures the *Drosophila* clock is blind to external LD cycles.

## The clock of the cockroach *Leucophaea maderae*

The circadian systems of some hemimetabolic insects, such as cockroaches or crickets, are valuable models to study the mechanisms of circadian time keeping. Although the fruitfly *Drosophila melanogaster* is a well established model to unravel the molecular function of circadian pacemakers, pharmacological, surgical, or physiological experiments are nearly impossible in this insect. In contrast, the cockroach *L. maderae* (Fig. 2A–C) contributed extensively to studies on the physiology of circadian clock function, owing to its large size enabling easy surgical and physiological manipulations, and due to its clear circadian locomotor activity pattern that is easy to record with relatively simple techniques. Thereby, lesion and transplantation studies as well as electrophysiological experiments could locate the circadian pacemaker driving circadian activity rhythms in the optic lobe between the medulla and lobula (Nishiitsutsuji-Uwo and Pittendrigh 1968; Roberts 1974;

Sokolove 1975; Page 1978; Colwell and Page 1990). In this area, a small neuropil called accessory medulla (AMe) was found (Fig. 2C), which appeared as a promising candidate for the circadian clock location in the cockroach brain (Homberg et al. 1991). From the AMe, time information is transmitted via neuronal pathways to the midbrain and to different target areas in the optic lobe. Recent lesion and transplantation experiments on adult *L. maderae* provided indeed compelling evidence for a circadian pacemaker function of the AMe (Reischig and Stengl 2003a). Anatomical analyses revealed that the AMe is a distinct, non-retinotopic neuropil with a nodular structure that is surrounded and interwoven with coarse neuropil (Reischig and Stengl 1996). Nodular and internodular structures in the AMe may serve for different functions in the circadian clockwork of the cockroach. The nodular neuropil of the AMe appears to be the main target for ipsilateral photic input, while contralateral light input appears to enter the AMe preferentially via the internodular neuropil (Petri et al. 2002; Reischig and Stengl 2003b).

Six morphologically distinguishable groups of neuronal somata are situated in the cell cortex around the AMe (Reischig and Stengl 2003b) and have differential arborizations in the nodular, internodular and shell neuropil of the AMe. These AMe neurons may include pacemaker neurons, pacemaker coupling neurons, neurons delivering entrainment information, neurons mediating phase information to other brain centers, and interneurons connecting all these cell types (Homberg et al. 2003).

### The role of neuroactive substances in the clock of *L. maderae*

To establish a stable circadian rhythm and phase relationship between the bilateral pacemakers, and to provide stable light entrainment, a large variety of neuroactive substances contribute to circadian clock function. In particular, neuropeptides appear to be involved in the orchestration of pacemaker functions in the AMe (Homberg et al. 2003).

To date, together with orcokinin in this current study, twelve different neuroactive substances were detected in the AMe of *L. maderae* by means of immunocytochemical techniques: the neuropeptides allatostatin, allatotropin, baratin, corazonin, FMRFamide, gastrin, leucokinin, pigment dispersing hormone (PDH), and the neurotransmitters $\gamma$-aminobutyric acid (GABA), histamin, and serotonin (Petri et al. 1995, 2002; Loesel and Homberg 1999; Nässel et al. 2000).

The most prominent and well documented peptide in the AMe is PDH. In *D. melanogaster,* the PDH-ir neurons express the circadian clock proteins PERIOD, TIMELESS, CLOCK, and CYCLE; they are thought to be pacemaker cells and clock outputs in the fly and the cockroach (Homberg et al. 1991; Stengl and Homberg 1994; Helfrich-Förster et al. 1998; Renn et al. 1999; Taghert et al. 2001; Reischig and Stengl 2003a, b; Stanewsky 2002; Homberg et al. 2003). Anatomical studies and microinjections of PDH into the AMe of *L. maderae* suggest that PDH transmits coupling information to the contralateral AMe and output information to the midbrain (Petri and Stengl 1997; Reischig et al. 2004).

The most prominent classical transmitter in the AMe is GABA. Anatomical studies with an antiserum against GABA and microinjections of GABA into the AMe suggest

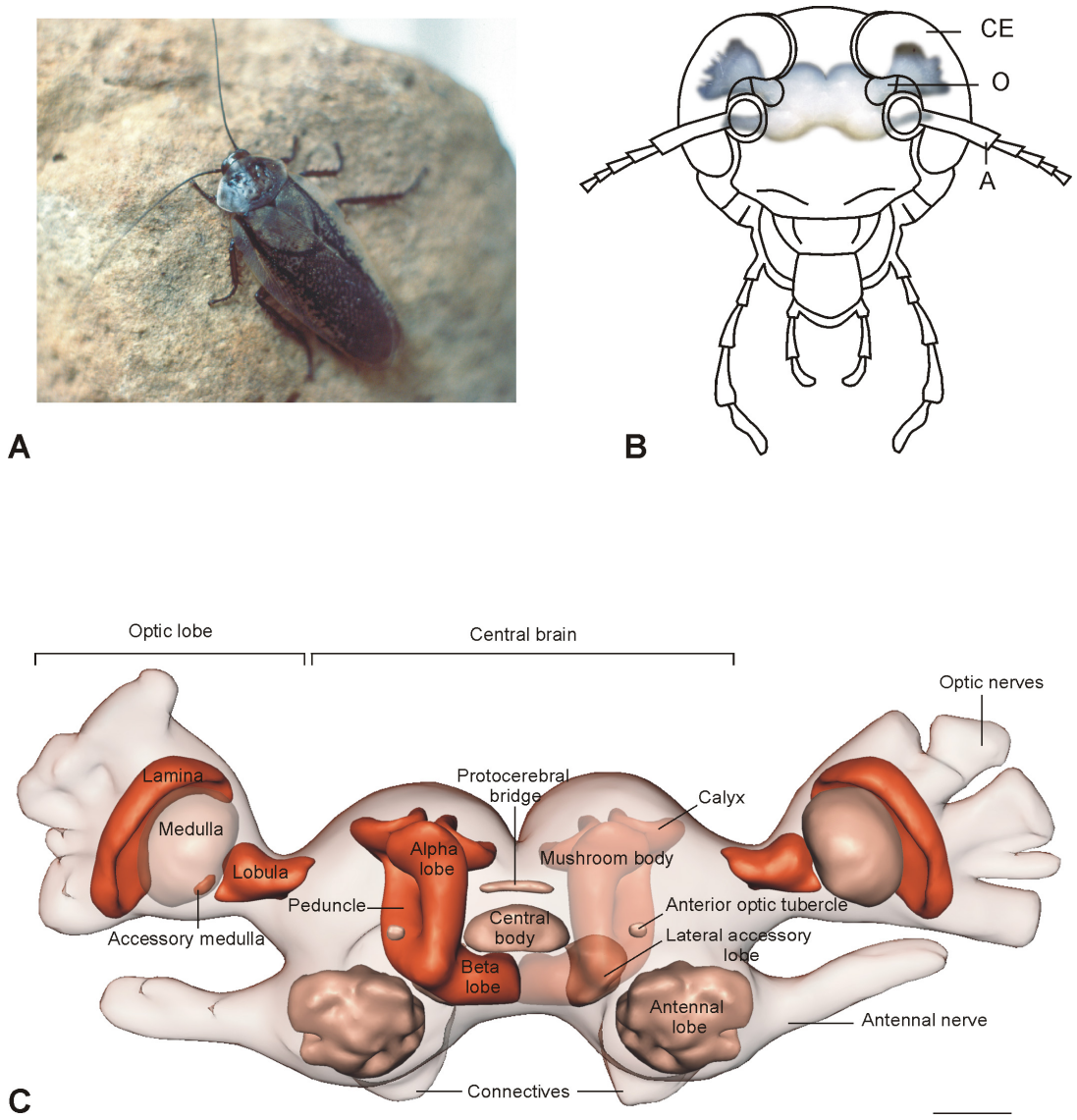



**Fig. 2A–C** Images of the cockroach *Leucophaea maderae* and its brain. **A** Dorsal view of *L. maderae*, sitting on a stone. **B** Diagram of the cockroach head with an image of the brain inside. *CE* compound eye, *O* ocellus, *A* antenna. **C** Three-dimensional reconstruction of the cockroach brain. Modified after Reischig and Stengl 2003b. *Scale bar* 200 $\mu$m

that this transmitter plays a role in the light entrainment pathway into the pacemaker (Petri et al. 2002).

## Light entrainment of the circadian clock of the cockroach

Light entrainment pathways and some of their underlying mechanisms are well investigated in the fruitfly *Drosophila melanogaster*. In contrast, in the cockroach relatively little is known about the light entrainment mechanisms. Roberts (1965), Nishiitsutsuji-Uwo and Pittendrigh (1968), and Page (1983) proposed that both pacemakers are only be entrained via photoreceptors of the compound eyes. However, no direct inputs from

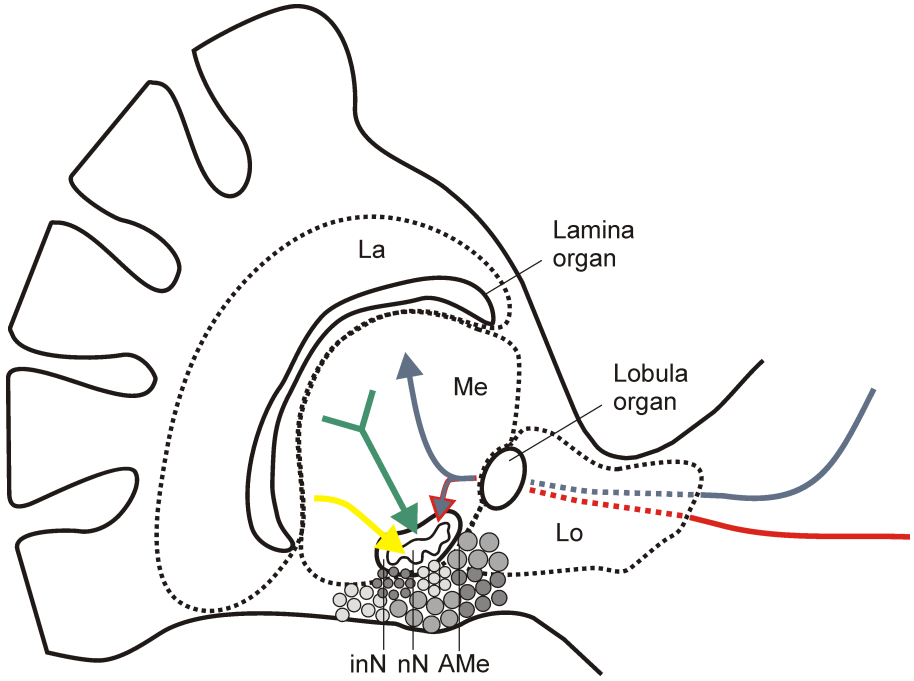



**Fig. 3** Diagram of the optic lobe of the cockroach and light entrainment pathways to the circadian clock, the accessory medulla (*AMe*). Neurons associated with the AMe have somata in six distinct groups shown in different shades of gray. Light entrainment to the clock occurs via the medulla (*Me*) through the distal tract (*yellow*) into the nodular neuropil (*nN*) of the AMe. Several fibers from the medulla (*green*) enter the internodular neuropil (*inN*) of the AMe and form an additional pathway. Light entrainment from the contralateral compound eye enters the *inN* via the posterior optic commissure and through the lobula valley tract (*red*). Light sensitive neurons from the ocellus arborize in the inN of the AMe and in the medulla (*blue*). *La* lamina, *Lo* lobula

ommatidial photoreceptors into the AMe have been found in the cockroach (Loesel and Homberg 1999). In the AMe, a prominent tract has been described, which connects the medulla with the AMe, the so-called distal tract of the AMe (Fig. 3; Reischig and Stengl 1996). It later turned out that most if not all fibers of this tract are GABA-ir, and that these fibers form terminal arborizations in the nodular neuropil of the AMe. Microinjections of GABA in the vicinity to the AMe at different circadian times in cockroaches freerunning in constant darkness lead to phase shifts of the overt circadian rhythm with phase delays at the early and phase advances at the late subjective night. The resulting PRC was very similar to that obtained with light-pulses (Petri et al. 2002). This suggests the involvement of neurons with GABA as neurotransmitter in the light entrainment pathway of the cockroach. Further, electrophysiological recordings in the AMe combined with intracellular staining revealed a neuron with a morphology very similar to that obtained with anti-GABA staining (Loesel and Homberg 2001; Petri et al. 2002). Since the medulla, from which the GABA-ir distal tract originates, receives direct as well as indirect input from the ommatidial photoreceptors, it is most likely that the distal tract provides the main light input pathway from the ipsilateral eye to the clock. In addition, several parallel pathways could be involved in the entrainment mechanism, since intracellular recordings from the AMe revealed light-sensitive neurons connecting the ipsilateral medulla with the AMe (Fig. 3; Loesel and Homberg 2001).

### The role of the compound eyes for the light entrainment of the cockroach

The composition of wavelengths emitted by the sun, and the scattering and absorption of sunlight by the Earth's atmosphere together create the condition of illumination on our planet's surface. Moreover, these conditions fluctuate through the course of the day. Because of these specific conditions of illumination, specific mechanisms for circadian photoreception have developed during evolution to adapt the organisms to the particular spatial and temporal light conditions of their environment.

In the cockroach, two sets of identified photoreceptors are known, the compound eyes and the ocelli. In 1954, Harker assumed that the locomotor rhythm in the cockroach *Periplaneta americana* is synchronized to LD cycles exclusively by the ocelli and not by the compound eyes. Her data showed that after the ocelli were painted, the cockroaches shifted to diurnal activity, but she did not show a freerunning locomotor rhythm. However, her data have been criticized on various technical reasons and were subsequently disproved by several authors.

Forty years ago, Roberts (1965) postulated that the compound eyes are the only sensory input that channel the information about environmental light to the endogenous circadian system. After surgical ablation of the ocelli the animals entrained well to the imposed LD cycles. Therefore, Roberts excluded an involvement of the ocelli in the light entrainment pathway. Bilateral transection of the optic nerves, which connect the optic lobe to the compound eyes, resulted in freerunning locomotor rhythms independent of the LD cycles (Nishiitsutsuji-Uwo and Pittendrigh 1968). After the authors painted the entire head and prepared a glass window over the protocerebrum, the animals entrained to the light cycles. By following surgical removing of the optic nerves, these animals freeran irrespective of the LD regime. Therefore, it was assumed that light is not absorbed directly by the brain via extraretinal photoreceptors, because it was only effective when it activated ommatidial photoreceptors. In 1977, Page and colleagues repeated experiments on light entrainment in *L. maderae*. They confirmed Robert's conclusions that *L. maderae* lacks any extraocular pathways to the circadian pacemaker. In addition, through lesion experiments the authors showed that light, which passes only one compound eye, can entrain both the ipsilateral and contralateral pacemaker. Therefore, the experimental data required heterolateral neuronal pathways, which transmit light information to each pacemaker (Fig. 3).

### The role of extraretinal photoreceptors

In different holometabolic insects, light entrainment of the circadian clock is managed by several photoreceptor structures (Diptera: Hofbauer and Buchner 1989; Lepidoptera: Ichikawa 1991; Mecoptera: Bierbrodt 1942; Trichoptera: Hagberg 1986). A few years ago, Fleissner and colleagues (2001) found two types of photoreceptor-like organs in the cockroach brain, the so-called lamina and lobula organs, which showed striking similarities to the known lamina and lobula organs of the beetle *Pachymorpha sexguttata* (Fleissner et al. 1993). In both insects, these organs are localized next to the lamina and lobula, respectively, frontally in the optic lobe (Fig. 3). The putative photoreceptive nature of

these organs can be derived so far only from indirect evidence. Behavioral or electrophysiological evidence for this hypothesis is missing until now. Nevertheless, the ultrastructure of these organs showed a rhabdom-like, microvillar structure as is typical for photoreceptive organs. Axonal processes from cells forming the organs ran along their outer surface and to neighboring optic neuropils (Fleissner et al. 2001). These characteristics suggest that both the lamina and lobula organs are photoreceptive structures. Additionally, both organs showed immunostaining associated with their central lumen with an antiserum against *Arabidopsis* cryptochrome (Fleissner et al. 2001). In *Drosophila*, cryptochrome has been proposed to act as an extraretinal photoreceptor in photic entrainment of the circadian system (reviewed by Stanewsky 2002). This finding underlines the putative photoreceptive function of the lamina and lobula organs in the cockroach. Up to know, the presence and specific characteristics of these organs are the only indication for a putative extraretinal photoreceptor in the cockroach.

### The role of the ocelli in the light entrainment pathway

Most insects, including the cockroach, have three or, in cockroaches, two ocelli, in addition to the compound eyes. The cockroach ocelli are the largest among all insects (Mizunami 1995). The cornea is flat and therefore not useful as a dioptric apparatus. In contrast to the compound eyes, there are no pigment cells in the ocelli of the cockroach (Weber and Renner 1976). A large number of photoreceptors of the ocellus converge onto four second-order neurons in the ocellar plexus. In the ocellar tract neuropil, these interneurons form synaptic connections to a large number of third order neurons. The third order ocellar neurons project into different neuropil areas, including visual (optic lobe; Fig. 3), olfactory (antennal lobe) and mechanosensory (deutocerebrum and tritocerebrum) areas (Mizunami 1995). Weber and Renner (1976) studied the ocelli in detail and suggested that they were designed to improve light-gathering power, possibly as an adaptation to a low light habitat. The first link between the circadian clock of the cockroach and the ocellar tract was shown by Loesel and Homberg (2001). They found a light-sensitive ocellar neuron with projections to the AMe of the cockroach *L. maderae* (Fig. 3).

As discussed above, Roberts (1965) excluded an involvement of the ocelli in the light entrainment of circadian rhythms. He showed that ocelli-less animals normally entrain to the imposed light regime. However, it was not demonstrated whether the ocelli play a role in low light conditions, which seems to be plausible since the ocellar apparatus is highly light sensitive.

## Goals of this study

As described above, peptide-releasing in the AMe plays an important role in clock functioning. Hence, revealing the anatomical distribution and physiological function of peptides in the clock neuropil is important to understand the neuronal network of the circadian system. After Homberg et al. (1991) provided first evidence for the occurrence of the crustacean PDH in insects and ascribed this peptide an important role in clock function, Petri and colleagues found more peptides in the AMe of the cockroach, which apparently

have different functional relevance (Petri et al. 1995). Hence, identifying all neuroactive substances together with the characterization of newly discovered and known peptides in the circadian clock of the cockroach are important steps toward an understanding of the neuronal network of this system.

Therefore, the first aim of this study was to examine the role of orcokinin-related peptides in the circadian pacemaker system of the cockroach *Leucophaea maderae*. Like PDH, this peptide family was first described in crustaceans; its name is derived from the name of the crayfish *Orconectes limosus*, from which it was first purified (Stangier et al. 1992). In insects, it was completely unknown so far, until U. Homberg and S. Heinze in pilot experiments tested an antiserum against the *O. limosus* $Asn^{13}$-orcokinin on brain sections of locusts, where it stained the AMe among other brain structures. Despite extensive biochemical characterization of the crustacean orcokinins, relatively little is known about their physiological roles in these animals. The presence of orcokinin-like peptides in neurons as well as in the hemolymph of several crustacean species suggests that orcokinins act both as hormones and as locally acting neuromodulators or cotransmitters in these species (Stangier et al. 1992; Bungart et al. 1995; Dircksen et al. 2000; Li et al. 2002). To investigate whether an orcokinin-related peptide is indeed present in the brains of insects, and to characterize its distribution among the neurons, I used an antiserum against $Asn^{13}$-orcokinin in several dicondylian insects including the silverfish *Lepisma saccharina*, three polyneopteran species (the cockroach *L. maderae* and the two locusts *Schistocerca gregaria* and *Locusta migratoria*), and three endopterygote (holometabolous) species (the sphinx moth *Manduca sexta*, the honeybee *Apis mellifera*, and the fruitfly *Drosophila melanogaster*).

Orcokinin immunostaining was widespread and occurred in similar patterns in the brain, including the AMe in the silverfish and the polyneopteran species. In contrast, orcokinin immunostaining was completely absent in the brains of the honeybee, the fruitfly, and the sphinx moth. This indicates that orcokinins are either modified considerably or may be completely absent in endopterygote insects. Further, recent biochemical characterizations suggest that peptides with close similarity to crustacean orcokinins are, indeed, present in the nervous systems of various insect species (H. Dircksen, personal communication).

Because of the dense innervation of the AMe by orcokinin-ir neurons in *L. maderae*, the question was raised whether an orcokinin-related peptide plays a role in the circadian system. To investigate the role of this peptide in the pacemaker system, I employed several immunocytochemical and pharmacological experiments.

- I first analyzed the distribution of orcokinin-ir neurons in the brains of different insect groups, as described above. The distribution of orcokinin-ir neurons in the brain of the three non-holometabolous insects showed a typical but common pattern (Chapter I).

- Further, detailed anatomical analysis of orcokinin-ir neurons together with colocalization studies employing several peptide and neurotransmitter antisera in the AMe of *L. maderae* suggest that orcokinin plays a multifunctional role in the circadian system (Chapter II).

- Finally, I was interested in a possible circadian function of orcokinin in the cockroach. Therefore, I injected $Asn^{13}$-orcokinin solution into the vicinity of the AMe at different circadian times (Chapter III). Together with the anatomical data the results suggest that orcokinin-related peptides play an important role in light entrainment pathways to the circadian clock via the contralateral compound eye.

The second goal was to investigate a possible photoreceptive function of the lobula- and lamina organ, respectively. Therefore, I employed lesion experiments and immunocytochemical experiments with different antisera against proteins involved in the phototransduction cascade, and against cryptochrome.

- Several antisera against substances of the phototransduction cascade (arrestins and opsins) were tested. An antiserum against *M. sexta* UV-sensitive opsin revealed prominent immunostaining in both putative photoreceptive organs, thus underlining the assumption of a photoreceptive function of these organs (Chapter IV).
- To get insights into the role of cryptochrome in the circadian system of the cockroach, immunocytochemical and Western blot techniques were used (Chapter IV). The daily fluctuations of cryptochrome in the cockroach were similar to the concentration changes observed in *Drosophila*.
- In search for behavioral evidence for a role of the lamina and the lobula organs in light entrainment, the optic nerves of the compound eyes of cockroaches were cut, and the locomotor activities of these animals were monitored in an LD regime with twilight simulation (Chapter V). However, none of the eyeless animals entrained to the imposed light regime, suggesting that the putative extraocular photoreceptor structures play at most a subordinate role in light entrainment.

The third goal was to reinvestigate the role of the ocelli in the light entrainment pathway of the circadian clock of the cockroach. Therefore, the threshold sensitivity of the circadian clock for white light was determined for ocelli-less and control animals. In these experiments the light conditions were modified; instead of a simple lights on/off situation (Roberts 1965), a twilight simulation was employed to obtain more realistic light conditions. After surgical removal of the ocelli, significantly fewer animals entrained to a low light regime with a dawn and dusk simulation. These results are the first evidences that the ocelli play a role in the direct or indirect light entrainment of the circadian clock of the cockroach (Chapter V).

## References


Albrecht U, Eichele G (2003) The mammalian circadian clock. Curr Opin Genet Dev 13:271–277

Aschoff J (1960) Exogenous and endogenous components in circadian rhythms. Cold Spring Harbor Symp Quant Biol 25:11–28

Bellingham J, Foster R (2002) Opsins and mammalian photoentrainment. Cell Tissue Res 309:57–71

Bierbrodt E (1942) Der Larvenkopf von *Panorpa communis* L. und seine Verwandlung, mit besonderer Berücksichtigung des Gehirns und der Augen. Zool Jahrb (Anat) 68:49–136

Bünning E (1935) Zur Kenntnis der endonomen Tagesrhythmik bei Insekten und bei Pflanzen. Berichte Deut Bot Gesell 53:594–623

Bungart D, Kegel G, Burdzik S, Keller R (1995) Structure-activity relationships of the crustacean myotropic neuropeptide orcokinin. Peptides 16:199–204

Colwell CS, Page TL (1990) A cicadian rhythm in neural activity can be recorded from the central nervous system of the cockroach. J Comp Physiol A 166:643–649

Czeisler CA, Shanahan TL, Klerman EB, Martens H, Brotman DJ, Emens JS, Klein T, Rizzo JF (1995) Suppression of melatonin secretion in some blind patients by exposure to bright light. New Engl J Med 332:6–11

Daan S, Pittendrigh CS (1976) A functional analysis of circadian pacemakers in nocturnal rodents. II. The variability of phase response curves. J Comp Physiol 106:253–266

De Mairan M (1729) Observation botanique. Histoire de l'Academie royale des Sciences Paris, pp 35–36

Dircksen H, Burdzik S, Sauter A, Keller R (2000) Two orcokinins and the novel octapeptide orcomyotropin in the hindgut of the crayfish *Orconectes limosus*: identified myostimulatory neuropeptides originating together in neurones of the terminal abdominal ganglion. J Exp Biol 18:2807–2818

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 beetles. J Comp Neurol 433:401–414

Fleissner Ge, Fleissner Gü, Frisch B (1993) A new type of putative non-visual photoreceptors in the optic lobe of beetles. Cell Tissue Res 273:435–445

Foster RG, Helfrich-Förster C (2001) The regulation of circadian clocks by light in fruitflies and mice. Philos Trans R Soc Lond B Biol Sci 356:1779–1789

Hagberg M (1986) Ultrastructure and central projections of extraocular photoreceptors in caddiesflies. Cell Tissue Res 245:643–648

Harker JE (1956) Factors controling the diurnal rhythm of activity in *Periplaneta americana*. J Exp Biol 33:224–234

Helfrich-Förster C, Stengl M, Homberg U (1998) Organization of the circadian system in insects. Chronobiol Int 15:567–594

Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 30:249–261

Hofbauer A, Buchner E (1989) Does *Drosophila* have seven eyes? Naturwissenschaften 76:335–336

Homberg U, Reischig T, Stengl M (2003) Neural organization of the circadian system of the cockroach *Leucophaea maderae*. Chronobiol Int 20:577–591

Homberg U, Würden S, Dircksen H, Rao KR (1991) Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. Cell Tissue Res 266:343–357

Ichikawa T (1991) Brain photoreceptors in the adult and pupal butterfly: fate of larval ocelli. Zool Sci 8:471–476

Johnson MS (1939) Effect of continuous light on periodic spontaneous activity of white-footed mice (*Peromyscus*). J Exp Zool 82:315–328

Kalmus H (1935) Periodizität und Autochronie (= Ideochronie) als zeitregelnde Eigenschaften der Organismen. Biol Gen 11:93–114

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci USA 68:2112–2116

Li L, Pulver SR, Kelley WP, Thirumalai V, Sweedler JV, Marder E (2002) Orcokinin peptides in developing and adult crustacean stomatogastric nervous systems and pericardial organs. J Comp Neurol 444:227–244

Lockley SW, Skene DJ, Arendt J, Tabandeh H, Bird AC, Defrance R (1997) Relationship between melatonin rhythms and visual loss in the blind. J Clin Endocrinol Metab 82:3763–3770

Loesel R, Homberg U (1999) Histamine-immunoreactive neurons in the brain of the cockroach *Leucophaea maderae*. Brain Res 842:408–418

Loesel R, Homberg U (2001) Anatomy and physiology of neurons with processes in the accessory medulla of the cockroach *Leucophaea maderae*. J Comp Neurol 439:193–207

Miyamoto Y, Sancar A (1998) Vitamin $B_2$-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clocks in mammals. Proc Natl Acad Sci USA 95:6097–6102

Mizunami M (1995) Morphology of higher-order ocellar interneurons in the cockroach brain. J Comp Neurol 362:293–304

Nässel DR, Persson MG, Muren JE (2000) Baratin, a nonamidated neurostimulating neuropeptide, isolated from cockroach brain: distribution and actions in the cockroach and locust nervous systems. J Comp Neurol 422:267–286

Nishiitsutsuji-Uwo J, Pittendrigh CS (1968) Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? Z Vgl Physiol 58:14–46

Page TL (1978) Interactions between bilaterally paired components of the cockroach circadian system. J Comp Physiol A 124:225–236

Page TL (1982) Transplantation of the cockroach circadian pacemaker. Science 216:73–75

Page TL (1983) Regeneration of the optic tracts and the circadian pacemaker activity in the cockroach *Leucophaea maderae*. J Comp Physiol 152:231–240

Page TL, Caldarola PC, Pittendrigh CS (1977) Mutual entrainment of bilaterally distributed circadian pacemakers. Proc Natl Acad Sci USA 74:1277–1281

Petri B, Homberg U, Loesel R, Stengl M (2002) Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. J Exp Biol 205:1459–1469

Petri B, Stengl M (1997) Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. J Neurosci 17:4087–4093

Petri B, Stengl M, Würden S, Homberg U (1995) Immunocytochemical characterization of the accessory medulla in the cockroach *Leucophaea maderae*. Cell Tissue Res 282:3–19

Pittendrigh CS (1960) Circadian rhythm and circadian organization of living systems. In Cold Spring Harbor Symposia on Quantitative Biology: Vol XXV. Biological Clocks. Cold Spring Harbor Press, New York, pp 159–182

Pittendrigh CS (1993) Temporal organisation: Reflections of a Darwinian clockwatcher. Annu Rev Physiol 55:17–54

Pittendrigh CS, Daan S (1976) A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. J Comp Physiol A 106:223–252

Ralph MR, Menaker M (1988) A mutation of the circadian system in golden hamsters. Science 241:1225–1227

Reischig T, Stengl M (1996) Morphology and pigment-dispersing hormone immunocytochemistry of the accessory medulla, the presumptive circadian pacemaker of the cockroach *Leucophaea maderae*: a light- and electron-microscopic study. Cell Tissue Res 285:305–319

Reischig T, Stengl M (2003a) Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). J Exp Biol 206:1877–1886

Reischig T, Stengl M (2003b) Ultrastructure of pigment-dispersing hormone-immunoreactive neurons in a three-dimensional model of the accessory medulla of the cockroach *Leucophaea maderae*. Cell Tissue Res 314:421–435

Reischig T, Petri B, Stengl M (2004) Pigment-dispersing hormone (PDH)-immunoreactive neurons form a direct coupling pathway between the bilaterally symmetric circadian pacemakers of the cockroach *Leucophaea maderae*. Cell Tissue Res (in press)

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802

Richter CP (1922) A behavioristic study of the activity of the rat. Comp Psych Monographs 1:1–55

Rieger D, Stanewsky R, Helfrich-Förster C (2003) Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. J Biol Rhythms 18:377–391

Roberts SK (1965) Photoreception and entrainment of cockroach activity rhythms. Science 148:958–959

Roberts SK (1974) Circadian rhythms in cockroaches: effects of optic lobe lesions. J Comp Physiol 88:21–30

Sokolove PG (1975) Localization of the cockroach optic lobe circadian pacemaker with microlesions. Brain Res 87:13–21

Stanewsky R (2002) Clock mechanisms in *Drosophila*. Cell Tissue Res 309:11–26

Stangier J, Hilbich C, Burdzik S, Keller R (1992) Orcokinin: a novel myotropic peptide from the nervous system of the crayfish, *Orconectes limosus*. Peptides 13:859–864

Stengl M, Homberg U (1994) Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. J Comp Physiol A 175:203–213

Taghert PH, Hewes RS, Park JH, O'Brien MA, Han M, Peck ME (2001) Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in *Drosophila*. J Neurosci 21:6673–6686

Vigh B, Manzano MJ, Zadori A, Frank CL, Lukats A, Rohlich P, Szel A, David C (2002) Nonvisual photoreceptors of the deep brain, pineal organs and retina. Histol Histopathol 17:555–590

Weber G, Renner M (1976) The ocellus of the cockroach, *Periplaneta americana* (Blattariae): receptory area. Cell Tissue Res 168:209–222

Zordan MA, Rosato E, Piccin A, Foster R (2001) Photic entrainment of the circadian clock: from *Drosophila* to mammals. Semin Cell Dev Biol 12:317–328

Chapter I. Comparative distribution of orcokinin immunostaining in the brains of selected dicondylian insects

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# Chapter I

# Comparative Distribution of Orcokinin Immunostaining in the Brains of Selected Dicondylian Insects

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## Abstract

Orcokinins are a family of myotropic neuropeptides that were identified in various decapod crustaceans. Their presence in the nervous system and in the hemolymph suggests that they act as hormones and as locally acting neuromodulators or cotransmitters. To investigate whether orcokinin-related peptides are also present in insects, we used an antiserum against Asn$^{13}$-orcokinin in dicondylian insects including a silverfish, three polyneopteran species (a cockroach, two locusts), and three endopterygote species (a moth, a bee, and a fly). Orcokinin immunostaining was widespread and occurred in similar patterns in the brain of the silverfish and the polyneopteran species. In the optic lobe, most prominent immunostaining was seen in the medulla (tangential and columnar neurons) and in the accessory medulla. In the antennal lobe, small groups of local interneurons showed immunostaining. Immunolabeling in the mushroom bodies was associated with certain groups of extrinsic and intrinsic neurons. All parts of the central complex and other areas of the proto- deuto-, and tritocerebrum were densely stained. In the silverfish, the cockroach, and the locusts, processes in the corpora cardiaca showed orcokinin immunoreactivity, which in the polyneopteran species originated from brain neurosecretory cells. This suggests that orcokinin is not only present in the nervous system, but also serves a hormonal role. In contrast, orcokinin immunostaining was completely absent in the brains of the honeybee, fruitfly and sphinx moth. This indicates that orcokinins are either considerably modified or are completely absent in endopterygote insects.

**Keywords:** Neuropeptides, Immunocytochemistry, Insect brain, *Leucophaea maderae, Schistocerca gregaria, Lepisma saccharina*

## Introduction

Orcokinins are a highly conserved family of myotropic neuropeptides that have been identified in various decapod crustaceans. The first peptide, Asn$^{13}$-orcokinin, was purified from the crayfish *Orconectes limosus* (Stangier et al. 1992). Different orcokinin analogues

were subsequently isolated from the crabs *Carcinus maenas* and *Cancer borealis*, the crayfishes *Procambarus clarkii*, *Cherax destructor*, and the lobster *Homarus americanus* and *Panulirus interruptus* (Bungart et al. 1995; Yasuda-Kamatani and Yasuda 2000; Skiebe et al. 2002; Li et al. 2002; Huybrechts et al. 2003). Several orcokinin isoforms were identified in each of these species that appear to be caused by point mutations at the DNA level (Huybrechts et al. 2003). In addition to [Asn$^{13}$]-orcokinin (NFDEID-RSGFGFN) these are [Val$^{13}$]-orcokinin, [Ser$^{9}$]-orcokinin, [Ala$^{13}$]-orcokinin (Bungart et al. 1994), [Thr$^{8}$-His$^{13}$]-orcokinin, [Ala$^{8}$-Ala$^{13}$]-orcokinin (Skiebe et al. 2002), and [Ser$^{9}$-Val$^{13}$]-orcokinin (Huybrechts et al. 2003). Each crustacean species contains a different combination of these isoforms. In *P. clarkii*, four orcokinins and the more distantly related peptide FDAFTTGFGHS are derived from two precursor proteins, but data from the other species are not yet available (Yasuda-Kamatani and Yasuda 2000).

Despite extensive biochemical characterization of the orcokinins, relatively little is known about their physiological roles. Activity studies showed that orcokinins are potent hindgut-stimulating factors (Stangier et al. 1992; Dircksen et al. 2000). In *H. americanus* application of Ala$^{13}$-orcokinin changes the pyloric rhythm generated by the stomatogastric ganglion through modulation of the excitability of the pyloric neuronal system (Li et al. 2002). Enzyme immunoassays and immunocytochemical studies showed the presence of orcokinins in all parts of the crustacean nervous system including the brain and the ventral nerve cord, the stomatogastric nervous system, and the pericardial organs (Bungart et al. 1994; Skiebe et al. 2002; Li et al. 2002). The presence of orcokinin-like peptides in the hemolymph of several crustacean species suggests that orcokinins act both as hormones and as locally acting neuromodulators or cotransmitters (Stangier et al. 1992; Bungart et al. 1995; Dircksen et al. 2000; Li et al. 2002).

To date, about 40 different myotropic neuropeptides have been described in crustaceans (Dircksen et al. 2000). Many of them belong to peptide families that were revealed by previous investigations in insects, which are by many authors considered to be the closest relatives of the crustaceans (Averof and Akam 1995; Friedrich and Tautz 1995; Nardi et al. 2003). Inspired by the common evolutionary origin of insects and crustaceans, we tested an antiserum against [Asn$^{13}$]-orcokinin for immunostaining in several insect species to investigate the possible presence of orcokinin-related peptides in the insect nervous system. We specifically studied the brains of three polyneopteran species, the cockroach *Leucophaea maderae*, and the locusts *Schistocerca gregaria* and *Locusta migratoria,* the silverfish *Lepisma saccharina* representing the basal Zygentoma, and three holometabolic (=endopterygote) insects (*Manduca sexta, Apis mellifera, Drosophila melanogaster*) from three different orders. We show that orcokinin-related immunostaining is highly abundant and occurs in similar patterns in the brains of the silverfish, the cockroach, and the locusts. In contrast, immunostaining is completely absent in the brains of the three holometabolic species indicating considerable modification or secondary loss of orcokinin-related peptides in these species.

## Materials and methods

### Animals

Adult cockroaches (*Leucophaea maderae*), locusts (*Schistocerca gregaria, Locusta migratoria*), sphinx moths (*Manduca sexta*), and fruitflies (*Drosophila melanogaster*) were taken from laboratory colonies. Cockroaches, desert locusts (*S. gregaria*) and flies were raised in crowded colonies at the University of Marburg. Migratory locusts (*L. migratoria*) were raised in crowded colonies at the University of Bonn. Animals were reared under 12:12 hours light-dark (LD) photoperiod, at about 60 % relative humidity and a temperature of 28 °C. *M. sexta* moths were raised from eggs. Larvae were fed on an artificial diet (modified after Bell and Joachim 1976). The animals were kept under a long-day photoperiod (17:7 LD) at 24–27 °C and 40–60 % relative humidity. Worker honeybees (*A. mellifera*) were captured in the field near Marburg, Germany. Silverfishes *(L. saccharina*) were collected from apartments in Marburg.

### Immunocytochemistry

Immunocytochemical staining for $Asn^{13}$-orcokinin was performed by use of the indirect peroxidase-antiperoxidase (PAP) technique of Sternberger (1979). Animals were anesthetized by cooling to 4 °C and decapitated. Brains were dissected and fixed for 4 h or overnight in 4% paraformaldehyde/7.5% saturated picric acid in sodium phosphate buffer (0.1 M, pH 7.4) at room temperature. The brains of the cockroach, locusts, moth, and honeybee were embedded in gelatine/albumin (4.8 % gelatine and 12 % ovalbumin in demineralized water) and postfixed in 8 % formalin in sodium phosphate buffer (0.1 M, pH 7.4). The brains were sectioned using a vibrating blade microtome (Leica, Nussloch, Germany) in frontal plane at 30 $\mu$m thickness. The free-floating sections were processed according to the PAP-technique (Homberg 1991). Brains were washed in Tris-buffered saline (TBS; 0.1 M Tris-HCL/0.3 M NaCl, pH 7.4) containing 0.1 % Triton X-100 (TrX). They were preincubated in TBS with 0.5 % TrX and 10 % normal goat serum (NGS; DAKO, Hamburg, Germany). Primary antiserum, anti-$Asn^{13}$-orcokinin (Dircksen et al. 2000), was diluted at 1:6000 in TBS containing 0.5 % TrX and 1 % NGS. Secondary antiserum (goat anti rabbit; Sigma, Deisenhofen, Germany) was used at a dilution of 1:40, and rabbit PAP (DAKO, Hamburg, Germany) at a dilution of 1:300 in TBS containing 0.5 % TrX and 1 % NGS. Afterwards the sections were thoroughly washed in sodium phosphate buffer, stained with 0.03 % 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Deisenhofen, Germany) and 0.025 % $H_2O_2$ in sodium phosphate buffer (0.1 M, pH 7.4) for 20–30 minutes, and finally mounted on chromalum/gelatine coated microscope slides. Immunostaining of the fruitfly and silverfish brains was carried out on paraffin sections. Decapitated fly and silverfish heads were fixed for 4 h, dehydrated through a graded series of aqueous ethanol solutions and toluene, and embedded in Paraplast Plus (Monoject Scientific, St. Louis, MO, USA). Serial sections at 10 $\mu$m were cut with a rotary microtome. Incubation steps were performed as described above, except that the incubation was carried out on slide, and the primary antiserum was diluted at 1:1000.

### Specificity controls

The anti-Asn$^{13}$-orcokinin antiserum has been characterized by Bungart et al. (1994), who tested HPLC-fractions of different astacidean crustaceans with an enzyme linked immunosorbent assay (ELISA). On cockroach, locust, and silverfish brain sections, specificity of the antiserum was determined by liquid-phase preadsorption of the diluted primary antiserum with various concentrations ($10^{-4}$ M up to $10^{-11}$ M) of Asn$^{13}$-Orcokinin (NFDEIDRSGFGFN-OH; Stangier et al. 1992; Bachem, Heidelberg, Germany), before adding the combined solution to the preparation. Immunostaining was abolished after preadsorption with 1 nM Asn$^{13}$-orcokinin for 18–20 h at room temperature.

### Evaluation and visualization

Microscopic images were captured with a Zeiss compound microscope equipped with a 2 megapixel digital camera (Polaroid Cambridge, MA, USA). Contrast and brightness of the micrographs were optimized in Adobe Photoshop 6.0. Positional information is given with respect to the body axes of the animals. Figure 2B, F, 3F are superimposed images from two different focal planes taken from single 30 $\mu$m sections. The complete images were achieved in Adobe Photoshop by montaging adjacent areas showing different parts of the neurons in focus.

### 3D-computer modeling of the silverfish brain

A series of frontal paraffin sections of a silverfish brain was photographed with the Polaroid camera and digitally aligned by means of the 3D visualization software AMIRA 3.0 (Indeed-Visual Concepts, Berlin, Germany). Contours of brain structures were manually segmented, and surface models of these structures were calculated. They were simplified to about 10 % of the original triangle quantity and smoothed in AMIRA, and each object was saved as a separate file. A multilayer image file containing all sections of the brain was created in Photoshop 6.0, and soma positions were labeled on additional layers. 2D-images were created from the 3D model and imported into the multilayer image. The reconstructed somata and brain structure models were slightly adjusted to match the proportions of the original preparations. Pseudo-three-dimensionality was achieved by using the transparency functions of Photoshop. The spatial relationships of somata and neuropils were controlled by light microscopy in examination of the original sections. The brain model of the cockroach was kindly provided by T. Reischig (Reischig and Stengl 2002).

### Counting of somata

Multilayer Photoshop image files containing all sections of selected brains of each species were created. The somata of each brain were reconstructed and counted from the digital images. The reconstructed somata of each brain were fit to the brain images as described

above. In addition, the somata were counted directly from the microscopic preparations. The number of cells were corrected using the Abercrombie (1946) correction factor for cell counts. The numbers of cell counts closely matched the numbers obtained with the reconstruction method.

## Results

We have investigated orcokinin immunostaining in the brain of three well-studied polyneopteran insects, the cockroach *L. maderae*, the locusts *S. gregaria* and *L. migratoria*, the silverfish *L. saccharina* representing a basal dicondylian species, and three endopterygote species, the sphinx moth *M. sexta,* the honeybee *A. mellifera,* and the fruitfly *D. melanogaster.* In the cockroach, the locusts and the silverfish, orcokinin-immunostained cell bodies and arborizations were found in all major parts of the brain, including the optic lobes, the median protocerebrum, deutocerebrum, and tritocerebrum. The somata were widely distributed in the cell cortex throughout the brain, and only few defined clusters were found. In contrast, orcokinin immunostaining was not detected in the brains of the three endopterygote insects.

### Orcokinin immunoreactivity in the brains of *L. maderae*

In the brain of the cockroach *L. maderae* about 1400 orcokinin-immunostained somata were counted (Fig. 1A). Most somata were widely dispersed in the cell cortex of the brain.

All neuropils in the optic lobe were invaded by orcokinin-ir neurons. In the lamina, weak orcokinin immunostaining originated from tangential neurons of the medulla (Fig. 2A). In addition, a few efferent orcokinin-ir fibers projected from the medulla through the lamina and the optic nerves toward the retina (Fig. 2A), but their origin in the medulla could not be determined. Immunostaining in the medulla originated from tangential neurons with processes in particular layers and a large number of columnar amacrine cells in the cell cortex around the medulla (Fig. 1A, 2B). The accessory medulla, a small neuropil at the anterior base of the medulla, was densely supplied by orcokinin-ir processes, which originated from a cluster of cell bodies ventrally and medially to the accessory medulla (Fig. 1A, 2B). The third optic neuropil, the lobula, was densely invaded by a diffuse meshwork of immunostained fibers.

In the median protocerebrum, all major neuropils showed orcokinin immunostaining including the central complex, the mushroom bodies, and areas in the superior and inferior protocerebrum (Fig. 2C–E, G). Tangential and columnar neurons gave rise to immunostaining in several layers of the upper and lower division of the central body and in the paired noduli (Fig. 2D, E). The lateral accessory lobes, important projection areas of central body neurons, showed dense immunostaining, and several systems of tangential neurons of the upper and lower division of the central body densely ramified in these areas (Fig. 2C, D). Weak immunostaining in the protocerebral bridge largely originated from a system of columnar neurons of the central complex (Fig. 2D). In the mushroom bodies, varicose orcokinin-ir processes from extrinsic neurons with processes in other brain areas

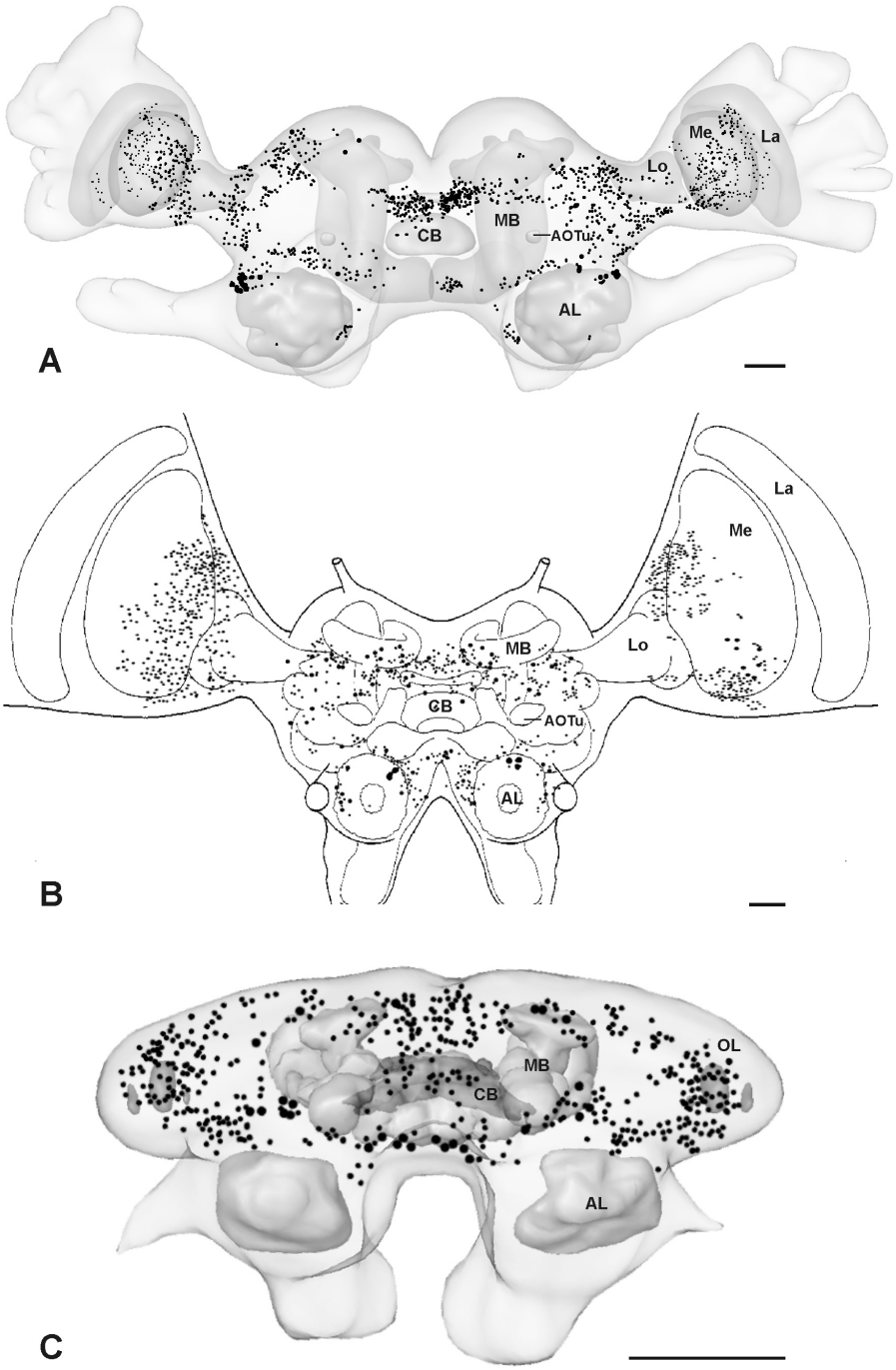



**Fig. 1A–C** Orcokinin-ir cell bodies in the brain of the cockroach *L. maderae* (A), the locust *S. gregaria* (B), and the silverfish *L. saccharina* (C). Only strongly immunostained somata are shown. A, C Surface-reconstruction of contours of brain structures and prominent neuropils (A, provided by T. Reischig). **A** In the brain of the cockroach about 1400 somata shown orcokinin immunostaining. **B** In the brain of the locust about 1300 orcokinin-immunostained somata were counted. **C** In the silverfish brain about 470 somata exhibit orcokinin immunoreactivity. *AL* antennal lobe, *AOTu* anterior optic tubercle, *CB* central body, *La* lamina, *Lo* lobula, *MB* mushroom body, *Me* medulla, *OL* optic lobe. *Scale bars* 200 $\mu$m

were labeled in basal portions of the calyces (Fig. 2C). Immunolabeling of extrinsic neurons, likewise, appeared in several segments of the peduncle, and in the $\alpha$- and $\beta$-lobes of the mushroom bodies (Fig. 2C, G, I). In addition, laminated longitudinally arranged immunostaining was present laterally in the pedunculus and continued to ventral sheets of the $\beta$-lobe and to anterior sheets of the $\alpha$-lobe. Immunostaining was weak in the neck of the pedunculus, but increased in intensity toward the $\alpha$- and $\beta$-lobe. This suggests that the staining is associated with subpopulations of intrinsic Kenyon cell axons of the mushroom body, but corresponding somata in the calyces were not stained. In the anterior protocerebrum, the anterior optic tubercle, a target area of visual interneurons from the optic lobe, showed dense orcokinin immunolabeling (Fig. 2G). Owing to dense immunostaining of the surrounding neuropil, the corresponding somata could not be identified. In the anterior median protocerebrum, small orcokinin-ir axons could be traced through the median bundle and corpora cardiaca nerves I (Fig. 2I–K, NCC I) to the storage lobes of the corpora cardiaca (Fig. 2K).

All areas of the deutocerebrum were invaded by orcokinin-ir processes. Fibers originating from three local interneurons with prominent lateral somata invaded and interconnected all glomeruli of the antennal lobe (Fig. 1A, 2F). Numerous small fibers in the antennal nerves were also stained. Many and perhaps all of them bypassed the antennal lobe and projected to the antennal mechanosensory and motor center of the deutocerebrum. In addition, numerous fine fibers in the tegumentary nerves, probably from mechanosensory receptors of the dorsal head capsule, entered the mechanosensory center of the deutocerebrum (not shown). The glomerular lobe, the projection area of putative gustatory afferents from the subesophageal ganglion, showed dense orcokinin immunoreactivity concentrated in glomerular condensations (Fig. 2H). Immunostaining was finally observed in numerous fibers in the circumesophageal connectives which connect the brain with the subesophageal ganglion, and in the labro-frontal nerve to the labrum and frontal ganglion.

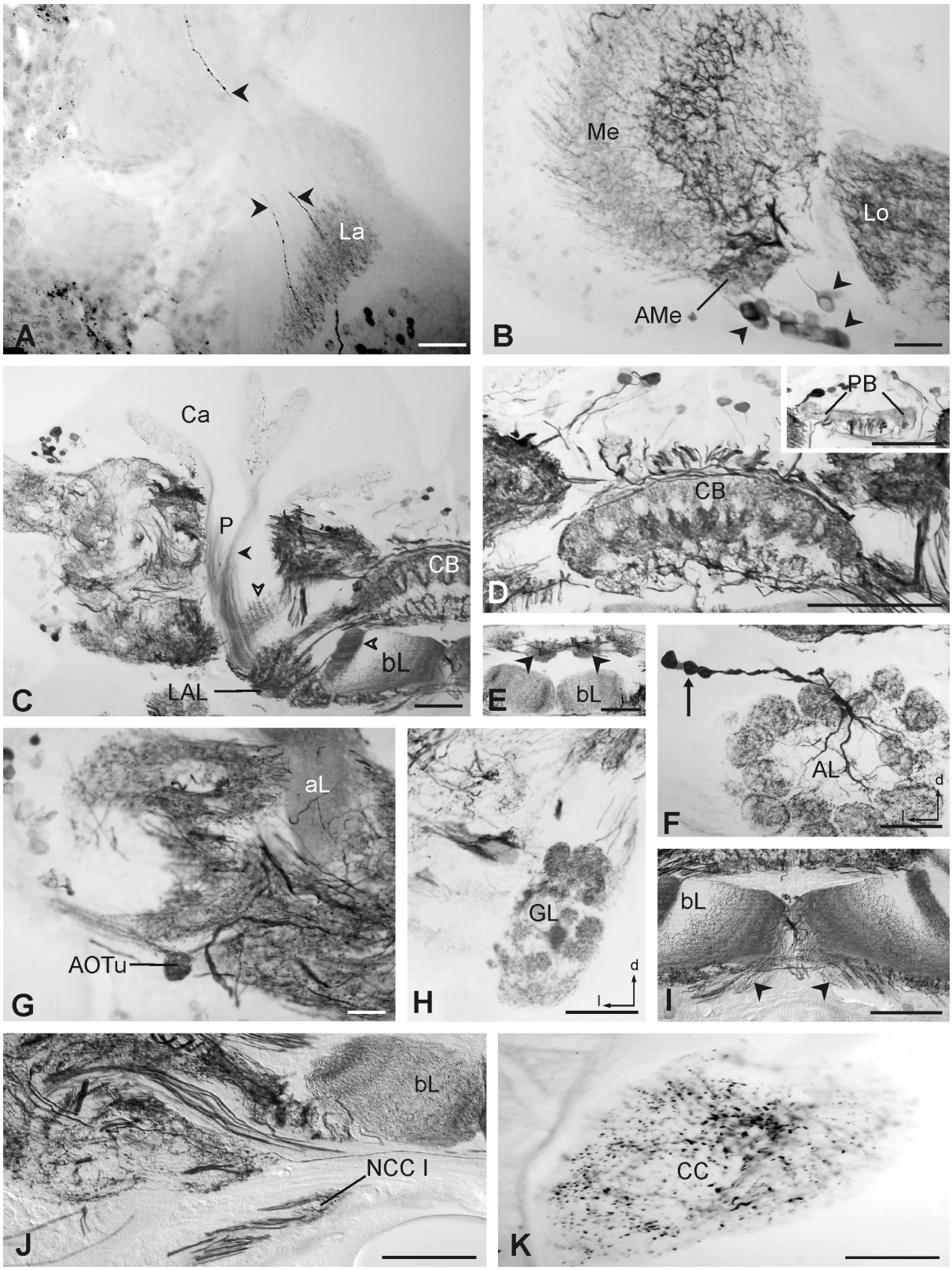
### Orcokinin immunoreactivity in the brains of *Schistocerca gregaria* and *Locusta migratoria*

The patterns of orcokinin-ir neurons in the brain of the two locust species were very similar. In the brain of *S. gregaria* about 1300 orcokinin-immunostained somata were counted (Fig. 1B). As in the cockroach, cell counts only include strongly stained cells, but, in addition, a considerable number of weakly stained cells also occurred in various areas of the brain. The lamina of the locust was sparsely innervated by orcokinin-immunostained processes from the medulla (Fig. 3A). The dorsal rim area of the lamina showed stronger immunostaining, which originated from apparently centrifugal fibers from the densely immunostained area in the dorsal rim of the medulla (Fig. 3A). Immunostaining in the medulla, distributed in several layers, largely originated from columnar neurons with cell bodies in the cell cortex around the medulla (Fig. 1B, 3A). Numerous immunoreactive fibers from these neurons projected through the second optic chiasm and led to strong staining in the ventral hemisphere of the inner lobe of the lobula and in several layers of the outer lobe of the lobula (Fig. 3A, B). The accessory medulla was densely supplied by orcokinin-ir processes, which largely originated from a cluster of cell bodies ventrally to the accessory medulla (Fig. 3C).

In the median protocerebrum, all major neuropils showed orcokinin immunostaining. The anterior optic tubercle was densely supplied by orcokinin-immunolabeled processes from the lobula (Fig. 3D). All subdivisions of the central complex showed immunostaining (Fig. 3E, F). Several types of tangential neurons with cell bodies in the inferior median protocerebrum innervated particular layers of the lower and upper division of the central body. Among them were small groups of TL2 and TL4 neurons (Homberg et al. 1999). TL4 neurons have arborizations in the anterior dorsal shell of the lateral accessory lobe and send axonal projections to the uppermost layer 1 of the lower division of the central body (Müller et al. 1997; Fig. 3E). TL2 neurons have ramifications in the median olive of the lateral accessory lobe (Müller et al. 1997, Fig. 3E). Immunostained neurons with arborizations in the posterior optic tubercle entered the protocerebral bridge tangentially (Fig. 3F). The lower units of the noduli of the central body showed weak staining of undetermined origin (not shown). Immunostaining was also present in the mushroom bodies. An extrinsic neuron entered the calyx of the mushroom body and gave rise to varicose processes throughout the secondary calyx (Fig. 3F). Weak laminated immunolabeling appeared in the periphery of the pedunculi and continued with increasing intensity to the $\alpha$- and $\beta$-lobes of the mushroom body (Fig. 3D). This staining is probably associated with intrinsic Kenyon cell axons. Finally, a dense meshwork of immunostained processes appeared throughout the median, superior, and inferior protocerebrum (Fig. 3F).

In the deutocerebrum, dense orcokinin immunostaining was found in all glomeruli of the antennal lobe (Fig. 3G). Arborizations originating from two to three large median somata and a few smaller cell bodies of apparently local interneurons, invaded and interconnected all glomeruli of the antennal lobe (Fig. 1B, 3G). Small orcokinin-ir fibers in the antennal

**Fig. 2A–K** (next page) Orcokinin immunostaining in the brain of *L. maderae*. All panels show frontal sections labeled with anti-Asn$^{13}$-orcokinin detected with the PAP-technique. **A** Orcokinin immunoreactivity in the lamina (*La*). *Arrowheads* point to centrifugal fibers from the optic ganglia to the retina. **B** Montage of two frontal sections showing orcokinin immunostaining in the medulla (*Me*) and lobula (*Lo*). Diffuse immunostaining extends throughout the lobula. Arborizations from tangential and columnar neurons are stained in the medulla. The accessory medulla (*AMe*) is densely invaded by orcokinin-immunostained processes. The corresponding somata are located ventrally and medially to the AMe (*arrowheads*). **C** Overview of the central brain with orcokinin-ir processes in the central body (*CB*), in the lateral accessory lobe (*LAL*), and in the calyx (*Ca*), peduncle (*P*), and $\beta$-lobe (*bL*) of the mushroom body. *Black arrowhead* points to laminated immunostaining in the peduncle. Immunostaining of extrinsic origin appears in several segments of the peduncle and lobes (*white arrowheads*). **D** Staining in several layers of the central body (*CB*) is derived from sets of tangential and columnar neurons. *Inset:* orcokinin immunostaining in the protocerebral bridge (*PB*). **E** The noduli of the central body (*arrowheads*) show particularly dense immunostaining. *bL* $\beta$-lobe of the mushroom body. **F** Montage of three frontal sections. Three orcokinin-immunostained interneurons (*arrow*) innervate all glomeruli of the antennal lobe (*AL*). *d* dorsal, *l* lateral. **G** The anterior optic tubercle (*AOTu*), the $\alpha$-lobe (*aL*) of the mushroom body, and the surrounding protocerebral neuropil show dense orcokinin immunolabeling. **H** Orcokinin immunostaining in the glomerular lobe (*GL*) is concentrated in glomerular subunits. **I** The $\beta$-lobes (*bL*) of the mushroom bodies show a laminated pattern of immunolabeling. *d* dorsal, *l* lateral. A fine meshwork of extrinsic fibers penetrates the $\beta$-lobe of the mushroom body. *Arrowheads* point to orcokinin-ir fibers of neurosecretory cells in the median bundle. **J** Section posterior to I. Orcokinin-ir processes are present in the $\beta$-lobe (*bL*) and in root of corpora cadiaca nerve I (*NCC I*). **K** In the storage lobe of the corpora cardiaca (*CC*), orcokinin-ir material appears in varicose processes, derived from NCC I. *Scale bars* 100 $\mu$m in A, B, D, F–K; 200 $\mu$m in C; 50 $\mu$m in D inset, E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nerve (Fig. 3G) entered the antennal mechanosensory and motor center. The tritocerebral neuropil was also densely supplied by orcokinin-ir processes, while the glomerular lobe was more sparsely innervated (Fig. 3H, I). In *L. migratoria,* at least three orcokinin-ir axons could be traced through corpora cardiaca nerves II (Fig. 3K) to the glandular lobes of the corpora cardiaca (Fig. 3J, K). In *S. gregaria*, sparse projections in the corpora cardiaca were also immunoreactive, but no staining was detected in NCC I and II (not shown). As in the cockroach orcokinin-ir fibers were also present in the circumesophageal connectives and in the labro-frontal nerves.

### Orcokinin immunoreactivity in the brain of *Lepisma saccharina*

To gain first insights into the ground plan of orcokinin immunostaining in the insects, we studied the silverfish *L. saccharina,* a species representing the basal group of Zygentoma. In the brain of *L. saccharina*, about 470 orcokinin-ir somata were distributed throughout the cell cortex (Fig. 1C). Immunostaining in the optic lobe was completely absent in the lamina, but dense orcokinin-ir staining appeared in the medulla (Fig. 4A). The accessory medulla also showed dense orcokinin immunostaining, which largely originated from a cell cluster dorsally to the accessory medulla (Fig. 4B).

In the median protocerebrum, all major neuropils showed orcokinin immunostaining including the central complex, the mushroom bodies, and neuropil areas in the superior and inferior protocerebrum (Fig. 4A, C, E). The central body was densely supplied by orcokinin-ir processes from tangential and columnar neurons. Only weakly labeled

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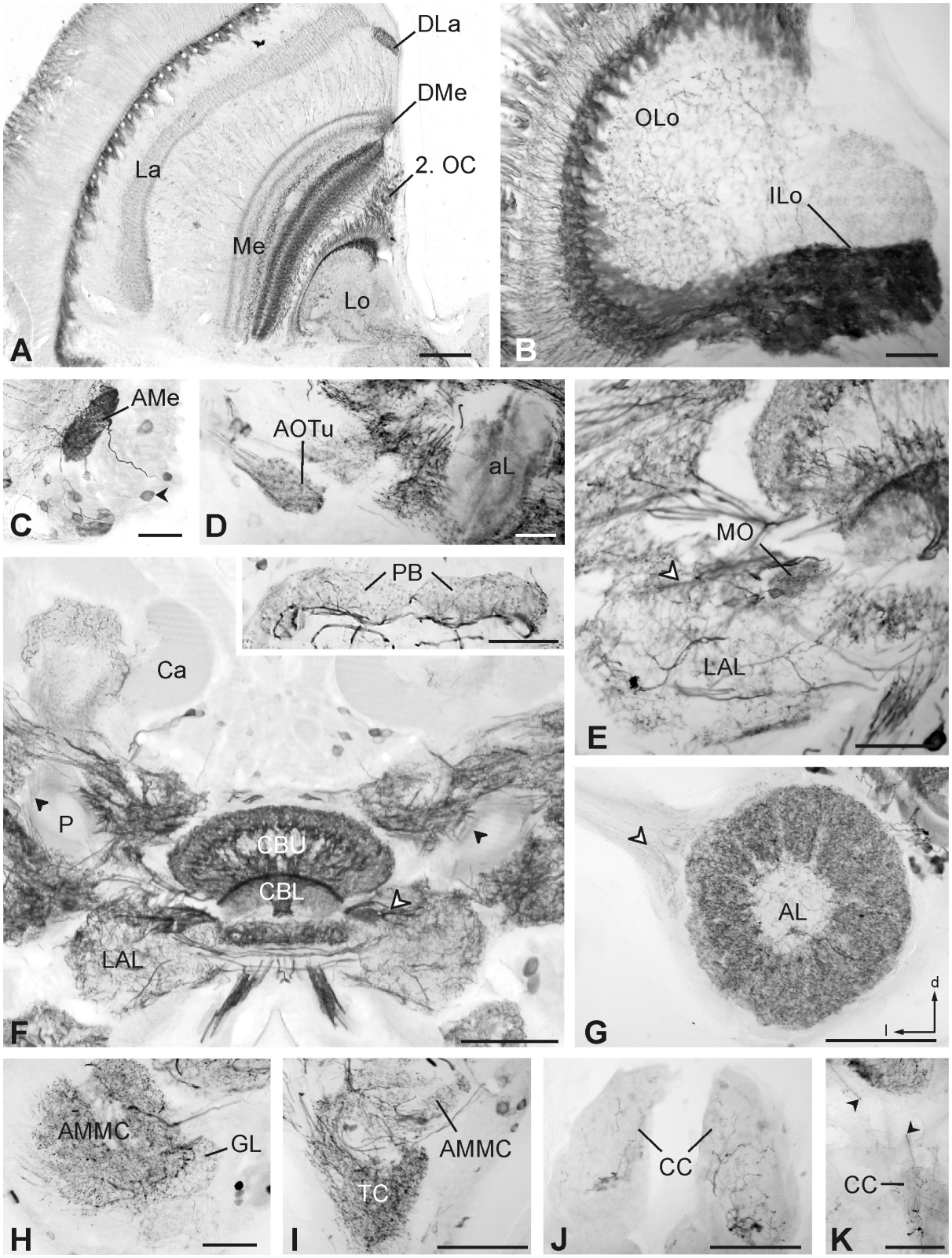
**Fig. 3A–K** (next page) Orcokinin immunostaining in the brain of *S. gregaria* (A–F, H, I) and *L. migratoria* (G, J, K). All panels show frontal sections. **A** Immunostaining in the optic lobe. The lamina (*La*) shows only weak staining except for the dorsal rim area (*DLa*) which is innervated by centrifugal orcokinin-ir fibers from the dorsal rim of the medulla (*DMe*). Immunostaining in several layers of the medulla (*Me*) largely originates from columnar neurons with axonal fibers passing through the second optic chiasm (*2. OC*) to the lobula (*Lo*). **B** Orcokinin-immunostained neurons densely innervate a middle layer in the outer lobe (*OLo*) and the ventral hemisphere of the inner lobe of the lobula (*ILo*). **C** Orcokinin-ir processes densely invade the accessory medulla (*AMe*). The corresponding somata are located ventrally and medially to the AMe (*arrowhead*). **D** The anterior optic tubercle (*AOTu*), the $\alpha$-lobe (*aL*) of the mushroom body, and the surrounding protocerebral neuropil show dense orcokinin immunolabeling. **E** Orcokinin immunostaining in the lateral accessory lobe (*LAL*). The median olive (*MO*) shows particularly dense staining. *Arrowhead* points to tangential neurons in the LAL. **F** Orcokinin immunostaining in the central brain. Several layers in the lower and upper division of the central body (*CBL, CBU*) exhibit orcokinin immunostaining. Orcokinin-ir fibers from TL2 and TL4 neurons project through the isthmus tract (*white arrowhead*) to the CBL. *Inset*: orcokinin staining in the protocerebral bridge (*PB*). An extrinsic neuron enters the calyx (*Ca*) of the mushroom body and gives rise to varicose processes throughout the secondary calyx. Weak laminated immunolabeling appears in the periphery of the pedunculi (*black arrowheads*). *LAL* lateral accessory lobe, *P* peduncle of the mushroom body. **G** Orcokinin-immunostained neurons invade all glomeruli of the antennal lobe (*AL*). Numerous small fibers in the antennal nerve are immunostained (*arrowhead*). *d* dorsal, *l* lateral. **H** Orcokinin immunostaining in the antennal mechanosensory and motor center (*AMMC*) and in the glomerular lobe (*GL*). **I** Orcokinin immunolabeling in the tritocerebrum (*TC*) and in the deep antennal mechanosensory and motor center (*AMMC*). **J** Orcokinin-ir material appears in varicose processes in the glandular lobes of the corpora cardiaca (*CC*). **K** Orcokinin-ir processes invade the corpora cardiaca (*CC*) via the corpora cadiaca nerve II (*arrowheads*). *Scale bars* 200 $\mu$m in A, F; 100 $\mu$m in B–E, F inset, G–K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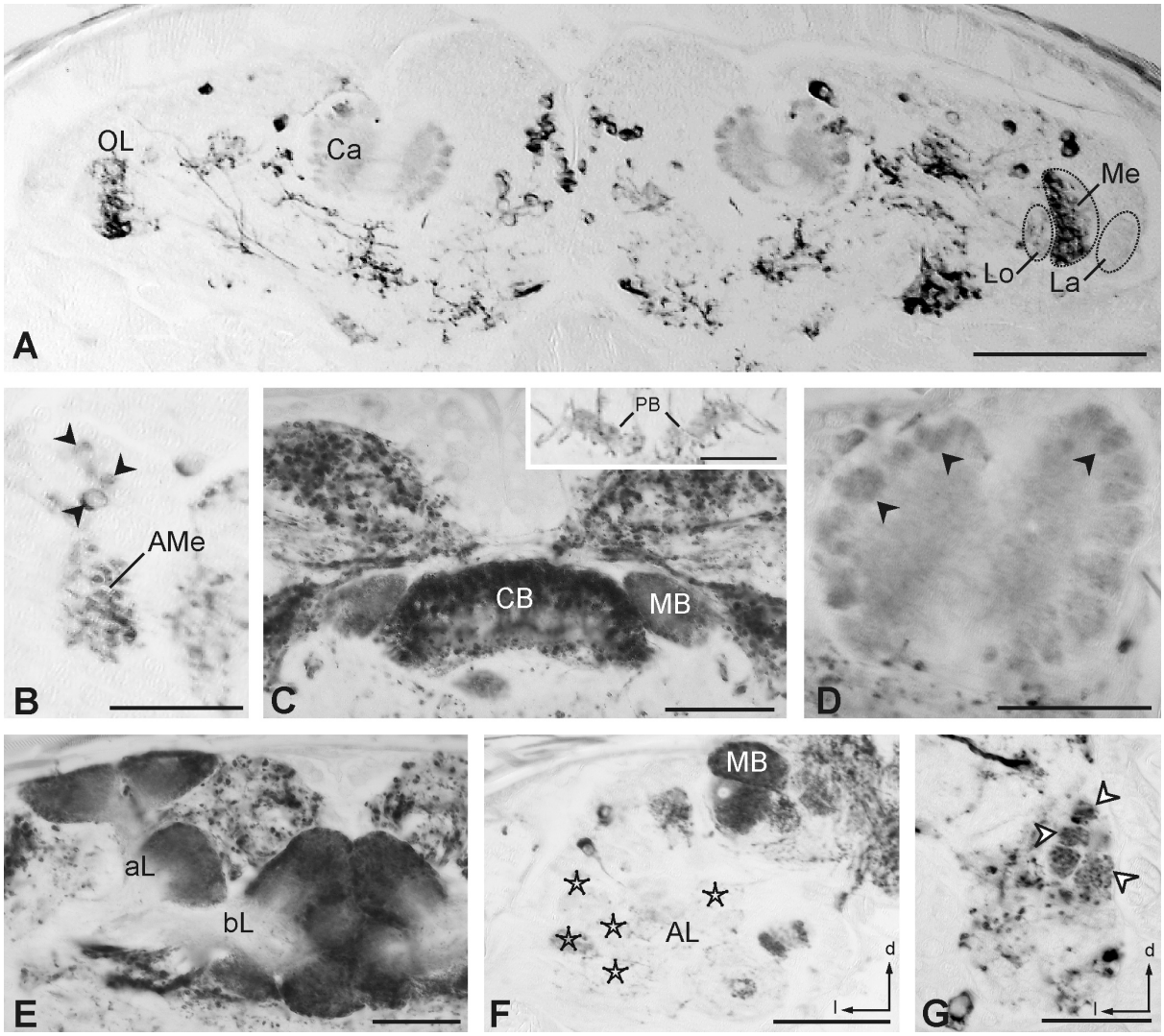


**Fig. 4A–G** Frontal paraffin sections showing orcokinin immunostaining in the brain of *L. saccharina*. **A** Overview of immunostaining in brain. In the optic lobe (*OL*), the medulla (*Me*) shows particularly dense staining. The calyx of the mushroom body (*Ca*) shows immunostaining in two hemispheres of glomerular neuropil condensations. *La* lamina, *Lo* lobula. **B** Orcokinin-immunostained processes innervate the accessory medulla (*AMe*). The corresponding somata are located dorsally to the AMe (*arrowheads*). **C** The central body (*CB*), the mushroom body (*MB*), and the surrounding protocerebral neuropil exhibit dense orcokinin immunolabeling. *Inset*: orcokinin immunostaining in the protocerebral bridge (*PB*). **D** Higher magnification of orcokinin immunoreactivity in the calyces showing their glomerular organization (*arrowheads*). **E** Higher magnification of the mushroom body lobes. The multilobed $\alpha$- and $\beta$-lobes (*aL, bL*) show dense orcokinin immunostaining. **F** Orcokinin immunostaining in the antennal lobe (*AL*). The antennal glomeruli (*asterisks*) are sparsely invaded by orcokinin-ir processes. *MB* mushroom body. *d* dorsal, *l* lateral. **G** A neuropil corresponding to the glomerular lobe of polyneopterans exhibits orcokinin immunoreactivity in glomerular subunits (*arrowheads*). *d* dorsal, *l* lateral. *Scale bars* 100 $\mu$ in A; 50 $\mu$m in B–G

fibers were detected in the protocerebral bridge (Fig. 4C). Dense orcokinin immunostaining appeared in the mushroom body. In the calyces, weak staining was associated with glomerular condensations of neuropil arranged in two hemispheres corresponding to the paired calyces in the pterygote insects (Fig. 4A, D). Staining intensity considerably in-

creased toward the multilobed $\alpha$- and $\beta$-lobes (Fig. 4E), as observed in the cockroach and locusts. This suggests, that orcokinin-immunoreactivity is associated with intrinsic Kenyon cells. Kenyon cell bodies, however, were not stained.

In the deutocerebrum, sparsely distributed orcokinin-ir fibers were found in all glomeruli of the antennal lobe (Fig. 4F). The staining intensity was fairly weak and originated from up to three lateral somata of the antennal lobe. Posteriorly and ventrally from the antennal lobe, dense orcokinin immunoreactivity was concentrated in glomerular condensations of a neuropil apparently corresponding to the glomerular lobe (Fig. 4G) of polyneopteran species. As in the cockroach and the locusts, immunostained fibers were present in the labral and frontal nerves, and in the circumesophageal connectives. Immunostaining was present in the retrocerebral complex and the recurrent nerve, but was not detected in NCC I or II.

### Immunoreactivity in the brains of *Manduca sexta*, *Apis mellifera*, and *Drosophila melanogaster*

To determine the presence and distribution of orcokinin immunostaining in phylogenetically more advanced insect groups, we studied the brains of three holometabolic species, the sphinx moth *M. sexta,* the honeybee *A. mellifera,* and the fruitfly *D. melanogaster*. The brains of all three species showed complete lack of immunostaining (Fig. 5). Homogenous background staining was distributed uniformly throughout the brains, but specific staining of neurons was not detected.

## Discussion

To date, orcokinins have been described as a family of closely related myotropic neuropeptides with wide distribution in the nervous system of decapod crustaceans. The present study provides first evidence for the presence of orcokinin-related peptides in insects. As in decapod crustaceans, immunostaining was widely distributed in the brains of a cockroach, two locust species, and a silverfish. The staining patterns in the four species showed striking similarities, and in the cockroach and the locusts the pattern of immunostaining clearly differed from the distribution of all hitherto studied neuropeptides, amines, and classical transmitters (reviewed by Nässel 2000; Homberg 2002; Nässel 2002). The complete block of staining following preadsorption of the antiserum with 1 nM antigen and the detection of orcokinin-ir material in locust brain homogenates (Bungart et al. 1995) further support the presence of orcokinin in the brains of these insects. Therefore, orcokinin-related peptides may be added to the list of candidate substances likely to function as a neuromediator and a hormone in the brain of polyneopteran insects. Interestingly, immunostaining was completely absent in the brains of three endopterygote species including the fruitfly *D. melanogaster*. Although specific for $Asn^{13}$-orcokinin, the antiserum is known to cross-react with orcokinins differing at amino acid position 13 as in the case of $Val^{13}$-orcokinin, but to a lower extent (Dircksen et al. 2000). This suggests that either considerable modification or secondary loss of orcokinins has occurred in these species. A BLAST search of the *D. melanogaster* genome database revealed no

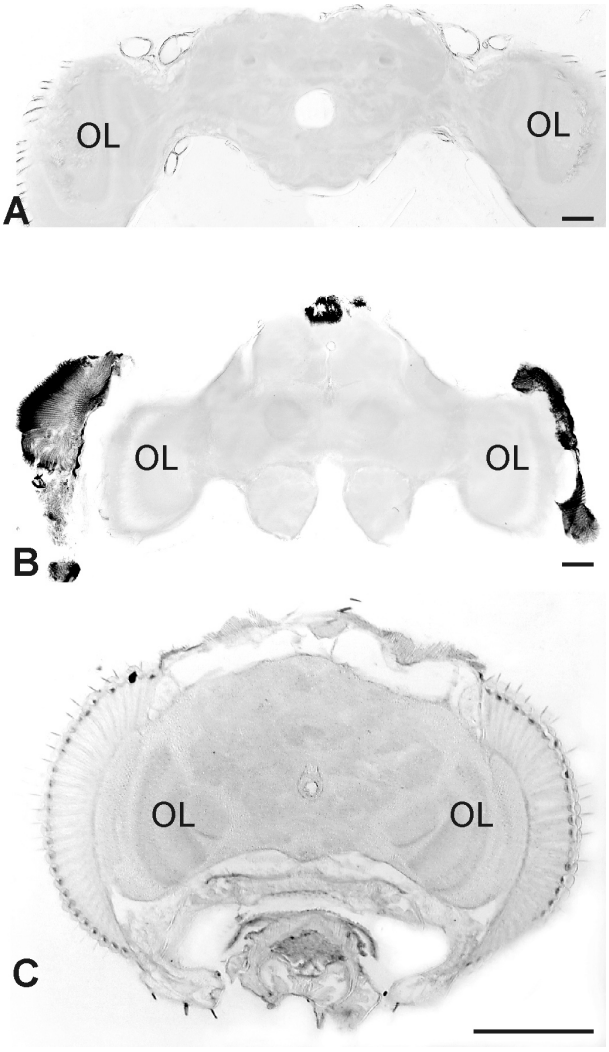


**Fig. 5A–C** Frontal sections through the brains of **A** *M. sexta*, **B** *A. mellifera*, and **C** *D. melanogaster*, treated with anti-Asn$^{13}$-orcokinin antiserum. No orcokinin immunostaining was present in any neuropil structure of the three brains. *OL* optic lobe. *Scale bars* 100 $\mu$m

orcokinin-related sequences, which further underscores the likely absence of orcokinins in endopterygotes.

### Staining patterns in the silverfish, the cockroach, and the locusts

Almost all neuropils were immunostained for orcokinin in the brain of the silverfish, the cockroach, and the locusts. There were conspicuous similarities, but also notable differences in the staining patterns of these animals. Orcokinin immunoreactivity in the optic lobe was present in all three species. Prominent immunostaining appeared in the medulla and accessory medulla. In the cockroach and locusts, the lamina was invaded by centrifugal neurons from the medulla, while in the silverfish the lamina was free of staining. The lobula was supplied by immunostained columnar neurons from the medulla in the locust, but not in the cockroach and silverfish. In all four insects,

orcokinin immunoreactivity was particularly prominent in the accessory medulla (Fig. 2B, 3C, 4B). The accessory medulla has been associated with a circadian pacemaker function in several insects including *L. maderae* (Homberg et al. 2003a). In cockroaches and locusts it contains numerous neuropeptides (Würden and Homberg 1995; Petri et al. 1995), suggesting an exceptional role of neuropeptides in the function of this neuropil. Preliminary studies on the effect of $Asn^{13}$-orcokinin injections on circadian locomotor activity in the Madeira cockroach (Hofer et al. 2003) suggest that orcokinin has a role in light entrainment of the clock. This is the first evidence for a physiological role of orcokinin-related peptides in insects.

In the central brain of the four species, orcokinin immunoreactivity was present in all major neuropils including the central complex and the mushroom bodies. The central complex plays a role in insect navigation and motor coordination (Strauss 2002; Homberg et al. 2003b). In all species, orcokinin immunoreactivity was detected in tangential and columnar neurons of the central body and in the protocerebral bridge. In the locusts, orcokinin-ir neurons of the lower division of the central body have been characterized previously as TL2 and TL4 neurons (Müller et al. 1997). Many and possibly all TL2 neurons are $\gamma$-aminobutyric acid (GABA) immunoreactive (Homberg et al. 1999) and it is, therefore, likely that orcokinins are colocalized with GABA in a subpopulation of TL2 neurons, as shown for Lom-tachykinin- and FMRFamide-related peptides (Vitzthum and Homberg 1998; Homberg et al. 1999). TL2 neurons are, furthermore, part of a neural network in the central complex involved in perception of the sky polarization pattern (Vitzthum et al. 2002). Orcokinin immunostaining in these neurons, therefore, suggests an involvement of orcokinin in polarization vision.

The mushroom bodies of insects are involved in olfactory learning, memory, and other cognitive function (Strausfeld et al. 1998; Heisenberg 2003). They are formed by numerous intrinsic neurons, termed Kenyon cells. Immunostaining along the pedunculus and lobes with increasing staining intensity toward the tips of the $\alpha$- and $\beta$-lobes was found in all insects and is most likely associated with subgroups of Kenyon cell axons. Dendritic ramifications and cell bodies of Kenyon cells were unstained in the cockroach and the locusts, but in the silverfish immunostaining was concentrated in calycal glomeruli arranged in a double hemisphere corresponding to the two calyces in other insect species. In addition, extrinsic processes were also labeled in the basal portion of the calyces and the lobes, most prominently in the locusts and the cockroach (Fig. 2C, 3F).

In all four polyneopteran insects, the deutocerebrum contained orcokinin-immunostained processes. In the cockroach and locusts, at least 2–3 local interneurons interconnected all glomeruli of the antennal lobe, and sparse immunostaining was also present in the silverfish. GABA has been shown to be the principal neurotransmitter of local antennal lobe interneurons in all insects studied so far (Homberg and Müller 1999). Studies in several insects including the cockroach showed that neuropeptides might act as cotransmitters with GABA in subpopulations of these neurons (Homberg and Müller 1999). Double labeling experiments might reveal whether orcokinins are additional candidates for a cotransmitter function in these neurons. Mechanosensory afferents to the deutocerebrum showed orcokinin immunostaining in the cockroach and the locusts, but not in the silverfish, suggesting that this staining may be a more recently evolved feature of polyneopterans.

Immunostaining of the retrocerebral complex was generally present in all species, but differed considerably with respect to cellular origin, even among the two locust species. This suggests that orcokinins generally have a neurohormonal role in all of the four species studied, but may be controlled by considerably different brain circuits. The evolutionary significance of these striking differences will require further analyses of the hormonal roles of orcokinins.

### Evolution of orcokinin immunostaining patterns in hexapods

Although only a few selected species were investigated, certain conclusions can be drawn about evolutionary trends in the patterns of orcokinin immunostaining in insects. From basal Zygentoma to Polyneoptera, imunostaining in the brain was widely distributed and remarkably conserved, as shown by apparently homologous immunoreactive neurons in the medulla and accessory medulla, mushroom body, central body, and antennal lobe. The similarity in immunostaining, moreover, allowed to establish anatomical features in the zygentomatan brain which have recently been claimed to be present only in pterygotes (Strausfeld et al. 1998; Strausfeld 1998). Our observations do not support Strausfeld's (1998) report that a lobula is missing in Zygentoma, but are in full accord with Böttger (1910) and more recently with Sinakevitch et al. (2003) that the optic lobe is, as in pterygote insects, composed of a lamina, a medulla, and a lobula, although of minute size. Unlike reported by Strausfeld et al. (1998), but again in agreement with Böttger (1910), the mushroom body does not lack a calyx but has two well developed calyces strikingly similar to the arrangement in many pterygotes. Our data are also not consistent with the statement of Strausfeld et al. (1998) that the deutocerebrum of the silverfish is, in principle, a mechanosensory neuropil; instead, antennal lobe glomeruli are well developed and, judged by neuroarchitecture shared with pterygote species, most likely serve an olfactory function. The specific staining pattern of a glomerular area near the antennal lobe, which has escaped attention so far, finally, suggests that a glomerular lobe, prominently present in polyneopteran species (Ernst et al. 1977), is present in Zygentoma as well, and likely serves a gustatory role as in the cockroach and locust.

In contrast to the strong conservation of orcokinin immunostaining pattern up to the Polyneoptera, a radical change has occurred in the transition to endopterygotes with complete lack of immunostaining throughout the brain. At present, it remains unclear whether major changes in the sequence of orcokinins have occurred, which render it unrecognizable with our antiserum, or whether orcokinin gene sequences are no longer transcribed or are otherwise lost. To more precisely determine at which stage of insect phylogeny orcokinin-related peptides have been modified or secondarily lost, it would be interesting to study paraneopteran insects (e.g., bugs; sister group of endopterygotes) and, among endopterygotes, neuropteriform species, e.g., coleoptera (sister group of mecopteriformia). A complementation of immunocytochemical data with information on the sequences of orcokinins and their genes will be essential for further conclusions on the evolution of this peptide family in insects and arthropods in general.

## Acknowledgements

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## References

Abercrombie M (1946) Estimation of nuclear population from microtome sections. Anat Rec 94:239–247

Averof M, Akam M (1995) Hox genes and the diversification of insect and crustacean body plans. Nature 376:420–423

Bell RA, Joachim FA (1976) Techniques for rearing laboratory colonies of tabacco hornworm and pink bollworm. Ann Ent Soc Am 69:365–373

Böttger O (1910) Das Gehirn eines niederen Insektes (*Lepisma saccharina* L.). Jen Z Naturwiss 46:801–844

Bungart D, Dircksen H, Keller R (1994) Quantitative determination and distribution of the myotropic neuropeptide orcokinin in the nervous system of astacidean crustaceans. Peptides 15:393–400

Bungart D, Hilbich C, Dircksen H, Keller R (1995) Occurrence of analogues of the myotropic neuropeptide orcokinin in the shore crab, *Carcinus maenas*: evidence for a novel neuropeptide family. Peptides 16:67–72

Dircksen H, Burdzik S, Sauter A, Keller R (2000) Two orcokinins and the novel octapeptide orcomyotropin in the hindgut of the crayfish *Orconectes limosus*: identified myostimulatory neuropeptides originating together in neurones of the terminal abdominal ganglion. J Exp Biol 18:2807–2818

Ernst KD, Boeckh J, Boeckh V (1977) A neuroanatomical study on the organization of the central antennal pathway in insects. Cell Tissue Res 176:285–308

Friedrich M, Tautz D (1995) Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. Nature 376:165–167

Heisenberg M (2003) Mushroom body memoir: from maps to models. Nat Rev Neurosci 4:266–275

Hofer S, Dircksen H, Homberg U (2003) Involvement of a neuropeptide related to orcokinin in light entrainment of the circadian clock of the cockroach. In Elsner N, Zimmermann H, editors. The neurosciences from basic research to therapy. Thieme, Stuttgart, pp 808–809

Homberg U (1991) Neuroarchitecture of the central complex in the brain of the locust *Schistocerca gregaria* and *S. americana* as revealed by serotonin immunocytochemistry. J Comp Neurol 303:245–254

Homberg U (2002) Neurotransmitters and neuropeptides in the brain of the locust. Microsc Res Tech 56:189–209

Homberg U, Müller U (1999) Neuroactive substances in the antennal lobe. In Hanson BS, editor. Insect Olfaction. Springer, Heidelberg, pp 181–206

Homberg U, Vitzthum H, Müller M, Binkle U (1999) Immunocytochemistry of GABA in the central complex of the locust *Schistocerca gregaria*: identification of immunoreactive neurons and colocalization with neuropeptides. J Comp Neurol 409:495–507

Homberg U, Reischig T, Stengl M (2003a) Neural organization of the circadian system of the cockroach *Leucophaea maderae*. Chronobiol Int 20:577–591

Homberg U, Hofer S, Pfeiffer K, Gebhardt S (2003b) Organization and neural connections of the anterior optic tubercle in the brain of the locust, *Schistocerca gregaria*. J Comp Neurol 462:415–430

Huybrechts J, Nusbaum MP, Bosch LV, Baggerman G, De Loof A, Schoofs L (2003) Neuropeptidomic analysis of the brain and thoracic ganglion from the Jonah crab, *Cancer borealis*. Biochem Biophys Res Commun 308:535–544

Li L, Pulver SR, Kelley WP, Thirumalai V, Sweedler JV, Marder E (2002) Orcokinin peptides in developing and adult crustacean stomatogastric nervous systems and pericardial organs. J Comp Neurol 444:227–244

Müller M, Homberg U, Kühn A (1997) Neuroarchitecture of the lower division of the central body in the brain of the locust (*Schistocerca gregaria*). Cell Tissue Res 288:159–176

Nardi F, Spinsanti G, Boore JL, Carapelli A, Dallai R, Frati F (2003) Hexapod origins: monophyletic or paraphyletic? Science 299:1887–1889

Nässel DR (2000) Functional roles of neuropeptides in the insect central nervous system. Naturwissenschaften 87:439–449

Nässel DR (2002) Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. Prog Neurobiol 68:1–84

Petri B, Stengl M, Würden S, Homberg U (1995) Immunocytochemical characterization of the accessory medulla in the cockroach *Leucophaea maderae*. Cell Tissue Res 282:3–19

Reischig T, Stengl M (2002) Optic lobe commissures in a three-dimensional brain model of the cockroach *Leucophaea maderae*: a search for the circadian coupling pathways. J Comp Neurol 443:388–400

Sinakevitch I, Douglass JK, Scholtz G, Loesel R, Strausfeld NJ (2003) Conserved and convergent organization in the optic lobes of insects and isopods, with reference to other crustacean taxa. J Comp Neurol 467:150–172

Skiebe P, Dreger M, Meseke M, Evers JF, Hucho F (2002) Identification of orcokinins in single neurons in the stomatogastric nervous system of the crayfish, *Cherax destructor*. J Comp Neurol 444:245–259

Stangier J, Hilbich C, Burdzik S, Keller R (1992) Orcokinin: a novel myotropic peptide from the nervous system of the crayfish, *Orconectes limosus*. Peptides 13:859–864

Sternberger LA (1979) Immunocytochemistry. John Wiley and Sons, New York

Strausfeld NJ (1998) Crustacean-insect relationships: the use of brain characters to derive phylogeny amongst segmented invertebrates. Brain Behav Evol 52:186–206

Strausfeld NJ, Hansen L, Li Y, Gomez RS, Ito K (1998) Evolution, discovery, and interpretations of arthropod mushroom bodies. Learn Mem 5:11–37

Strauss R (2002) The central complex and the genetic dissection of locomotor behavior. Curr Opin Neurobiol 12:633–638

Vitzthum H, Homberg U (1998) Immunocytochemical demonstration of locustatachykinin-related peptides in the central complex of the locust brain. J Comp Neurol 390:455–469

Vitzthum H, Müller M, Homberg U (2002) Neurons of the central complex of the locust *Schistocerca gregaria* are sensitive to polarized light. J Neurosci 22:1114–1125

Würden S, Homberg U (1995) Immunocytochemical mapping of serotonin and neuropeptides in the accessory medulla of the locust, *Schistocerca gregaria*. J Comp Neurol 362:305–319

Yasuda-Kamatani Y, Yasuda A (2000) Identification of orcokinin gene-related peptides in the brain of the crayfish *Procambarus clarkii* by the combination of MALDI-TOF and on-line capillary HPLC/Q-TOF mass spectrometries and molecular cloning. Gen Comp Endocrinol 118:161–172

Chapter II. Orcokinin immunoreactivity in the accessory medulla of the cockroach *Leucophaea maderae*

Hofer S and Homberg U (2003) Cell Tissue Res (in preparation)

# Chapter II

# Orcokinin Immunoreactivity in the Accessory Medulla of the Cockroach *Leucophaea maderae*

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## Abstract

Orcokinins are a family of highly conserved crustacean neuropeptides, which have mytotropic effects and apparently also act as neuromodulators in the nervous system. Recent biochemical characterization and immunostaining suggest that orcokinins are also present in the nervous systems of various insect species. In this study, we analyzed the distribution of orcokinin immunostaining in the accessory medulla of the cockroach *Leucophaea maderae*. The accessory medulla is the master circadian clock in the brain of the cockroach controlling circadian locomotor activity. Previous studies have shown that a variety of neuropeptides are prominent neuromediators in this brain area. The accessory medulla is densely innervated by about 30 orcokinin-immunoreactive neurons with cell bodies distributed in five of six established cell groups of the accessory medulla. Immunostaining was particularly prominent in three ventromedian neurons. These neurons have processes mainly in the internodular neuropil of the accessory medulla and in a median layer of the medulla, and send axonal fibers via the posterior optic commissure to the contralateral accessory medulla. Double labeling experiments showed colocalization of orcokinin immunostaining with immunoreactivity for pigment dispersing hormone, FMRFamide, Mas-allatotropin, and $\gamma$-aminobutyric acid (GABA) in certain cell groups of the accessory medulla, but not in the ventromedian neurons or in the anterior and posterior optic commissure. Immunostaining in the ventromedian neurons suggests that orcokinin-related peptides play a role in heterolateral transmission of photic input to the pacemaker and/or in coupling of the bilateral pacemakers of the cockroach.

**Keywords:** Peptides, Immunocytochemistry, Insect brain, Circadian rhythms, PDH, cockroach *Leucophaea maderae*

## Introduction

Orcokinins are a family of neuropeptides that were originally purified from the nervous system of the crayfish *Orconectes limosus* (Stangier et al. 1992). Further studies revealed

the occurrence of identical or closely related peptides in various decapod crustaceans (Bungart et al. 1994; Yasuda-Kamatani and Yasuda 2000; Skiebe et al. 2002; Huybrechts et al. 2003). Immuncytochemistry, MALDI-TOF mass spectrometry, and enzyme-linked immunosorbent assays showed that orcokinins are present throughout the nervous system and in the hemolymph of crustaceans (Stangier et al. 1992; Bungart et al. 1995; Dircksen et al. 2000; Li et al. 2002). This suggests that they act as hormones and as locally acting neurotransmitters or modulators. We recently demonstrated immunocytochemically that orcokinin-related peptides are also present in the brains of zygentomid (silverfish) and polyneopteran insects (cockroach, locust), but not in the brains of endopterygote species (sphinx moth, honeybee, fruitfly; Hofer et al. 2004). Immunostaining in the brain and in the retrocerebral complex suggests that orcokinins may function both as hormones and neuromodulators in these insects as described for crustaceans. Additionally, biochemical characterization of this peptide in insects underline the presence of orcokinin in the nervous system (H. Dircksen, personal communication).

In this study, we have analyzed the distribution of orcokinin immunoreactivity in the accessory medulla (AMe) of the cockroach *Leucophaea maderae*. The AMe is a small neuropil at the anterior base of the medulla and has been associated with circadian pacemaker activity in several insects including the fruitfly *Drosophila melanogaster* and the cockroach *L. maderae* (Stanewsky 2002; Homberg et al. 2003). The AMae are bilaterally paired and mutually coupled (Page 1978, 1983). Four categories of neurons have been distinguished in the AMe of the cockroach: photic input elements, local interneurons, output neurons, and at least two types of heterolateral coupling units (Homberg et al. 2003). Most of these neurons have their cell bodies in six anatomically distinguished groups of neuronal somata in the vicinity of the AMe (Reischig and Stengl 2003a). Immunocytochemistry and injection experiments suggest a prominent role of GABA and several neuropeptides in the function of the AMe (Petri et al. 1995, 2002; Petri and Stengl 1997). While GABA and the peptide Mas-allatotropin appear to be involved in photic input pathways (Petri et al. 2002), output neurons show pigment-dispersing hormone (PDH)- and leucokinin-immunostaining, and bilateral coupling elements, PDH- and FMRFamide immunoreactivity (Petri et al. 1995; Petri and Stengl 1997; Reischig et al. 2004).

We examined the distribution of orcokinin-immunoreactive (-ir) somata within the soma groups of the AMe, and compared the pattern of orcokinin immunostaining in the AMe with the distribution of previously mapped neurotransmitters and neuropeptides in *L. maderae* (Petri et al. 1995; Nässel et al. 2000; Reischig and Stengl 2003a). About 30 orcokinin-ir somata were distributed in five of the six soma groups of the AMe and immunoreactive fibers connected both AMae via the posterior optic commissure. To determine whether orcokinin-related peptides are colocalized with previously studied peptides and transmitters in the same AMe neurons, we used antisera against PDH, FMRFamide, GABA, Mas-allatotropin, and leucokinin for double labeling experiments with the orcokinin antiserum. We show that PDH-, FMRFamide-, Mas-allatotropin-, and GABA- immunoreactivities colocalize with orcokinin immunostaining in different soma groups of the AMe, but not in commissural fibers of the brain. Together with additional data, our results suggest that orcokinin-ir neurons constitute a second coupling pathway between both AMae and transmit both light and coupling information to the contralateral pacemaker.

## Materials and methods

### Animals

Adult cockroaches (*Leucophaea maderae*) were taken from crowded colonies at the University of Marburg. Animals were reared under 12:12 hours light-dark (LD) photoperiod, at about 60 % relative humidity and 28 °C.

### Immunocytochemistry

Animals were anesthetized by cooling to 4 °C and decapitated. Brains were dissected at the beginning of the dark phase of the animals and fixed for 4 hours in 4 % paraformaldehyde/7.5 % saturated picric acid in phosphate buffer (0.1 M, pH 7.4) at room temperature. The brains were embedded in gelatine/albumin (4.8 % gelatine and 12 % ovalbumin in demineralized water) and postfixed in 8 % formalin in phosphate buffer (0.1 M, pH 7.4). The brains were sectioned using a vibrating blade microtome (Leica, Nussloch, Germany) in frontal plane at 30 $\mu$m thickness. The free floating sections were immunocytochemically stained for Asn$^{13}$-orcokinin using the indirect peroxidase-antiperoxidase (PAP) technique of Sternberger (1979; see also Homberg 1991). Brains were washed in Tris-buffered saline (TBS; 0.1 M Tris-HCL/0.3 M NaCl, pH 7.4) containing 0.1 % Triton X-100 (TrX) and preincubated in TBS with 0.5 % TrX and 10 % normal goat serum (NGS; DAKO, Hamburg, Germany). Primary antiserum, anti-Asn$^{13}$-orcokinin (provided by Dr. H. Dircksen, Department of Zoology, Stockholm), was diluted at 1:6000 in TBS containing 0.5 % TrX and 1 % NGS. Secondary antiserum (goat anti rabbit; Sigma, Deisenhofen, Germany) was used at a dilution of 1:40, and tertiary antiserum (rabbit PAP; DAKO, Hamburg, Germany) at a dilution of 1:300 in TBS containing 0.5 % TrX and 1 % NGS. Afterwards, the sections were thoroughly washed in TBS containing 0.1 % TrX, stained with 0.03 % 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Deisenhofen, Germany) and 0.025 % $H_2O_2$ in phosphate buffer (0.1 M, pH 7.4) for 20–30 minutes, and then mounted on chromalum/gelatine coated microscope slides. Immunostaining was additionally carried out on paraffin sections. The brains were fixed for 4 h, dehydrated through a graded series of aqueous ethanol solutions and toluene, and embedded in Paraplast Plus (Monoject Scientific, St. Louis, MO, USA). Serial sections at 10 $\mu$m were cut with a rotary microtome. Incubation steps were performed as described above, except that the incubation was carried out on slide, and the primary antiserum was diluted at 1:500. Finally, the sections were counterstained with methylene blue for better visualization of neuropil structures.

### Double labeling experiments

To allow double labeling using the anti-Asn$^{13}$-orcokinin and other antisera that were also raised in rabbits, experiments were performed according to a method modified from that of Negoescu et al. (1994). The brains were fixed and embedded as described above (for GABA immunostaining, 0.1 % glutaraldehyde was added to the fixative). The brains

**Table 1** Antisera, first characterization, and working solution used for double labeling experiments with the antiserum against orcokinin

| Antiserum | Antigen | First characterization | Working dilution (PAP) | Working dilution (fluorescence) |
|---|---|---|---|---|
| Anti-orcokinin | *Orconectes limosus* $Asn^{13}$-orcokinin | D. Bungart (Bungart et al. 1994) | 1:6000 | 1:4000 |
| Anti-$\beta$-PDH | *Uca pugilator* $\beta$-PDH | H. Dircksen (Dircksen et al. 1987) | | 1:10000 |
| Anti-FMRFamide | Synthetic FMRFamide | W.H. Watson (O'Donahue et al. 1984) | | 1:3500 |
| Anti-$\gamma$-aminobutyric acid (GABA) | GABA protein conjugate | S.G. Hoskins (Hoskins et al. 1986) | | 1:10000 |
| Anti-Mas-allatotropin | *Manduca sexta* allatotropin | J. Veenstra (Veenstra and Hagedorn 1993) | | 1:3000 |
| Anti-leucokinin | *Leucophaea maderae* leucokinin | D.R. Nässel (Nässel et al. 1992) | | 1:2000 |

were sectioned using a vibrating blade microtome (Leica, Nussloch, Germany) in frontal plane at 40 $\mu$m thickness. They were washed in TBS containing 0.1 % TrX and were preincubated in TBS with 0.5 % TrX and 5 % normal donkey serum (NDS; DAKO, Hamburg, Germany). Primary antiserum, anti-$Asn^{13}$-orcokinin, was diluted at 1:4000 in TBS containing 0.5 % TrX and 1 % NDS for 18–20 h at room temperature. As secondary antibody, monovalent Fab fragments of goat anti rabbit IgG (Dianova Hamburg, Germany) were used at a dilution of 1:30 and incubated for 4–6 h to block all possible binding sites for the secondary antiserum, following in the second staining cycle. The sections were washed in TBS containing 0.1 % TrX, and incubated with a Cy3-conjugated donkey anti goat antiserum (Dianova Hamburg, Germany, diluted 1:300) in TBS containing 0.5 % TrX and 1 % NDS for 1 h. The sections were then washed as described and incubated with the second primary antiserum (anti-$\beta$PDH, -FMRFamide, -GABA, -leucokinin, or -Mas-allatotropin, see Table 1) overnight. The sections were washed the following day in TBS containing 0.1 % TrX and incubated with Cy2-conjugated donkey anti rabbit antibody (Dianova, Hamburg Germany, diluted 1:300) in TBS containing 0.5 % TrX and 1 % NDS for 1 h. Afterwards, the sections were thoroughly washed and mounted on chromalum/gelatine coated microscope slides. As controls for crossreactivity of the second immunolabeling with the Cy3-labeled anti-orcokinin, the immunostaining protocol was carried out as described above, but the second primary antibody was omitted.

### Specificity controls

The anti-$Asn^{13}$-orcokinin antiserum has been characterized by Bungart et al. (1994), who tested HPLC-fractions of different astacidean crustaceans with an enzyme linked immunosorbent assay. On cockroach brain sections, specificity of the antiserum was determined by liquid-phase preadsorption of the diluted antiserum with various concentrations ($10^{-4}$ M up to $10^{-11}$ M) of $Asn^{13}$-orcokinin (NFDEIDRSGFGFN-OH; Stangier et al. 1992; Bachem, Heidelberg, Germany), before adding the combined solution to the preparation. Immunostaining was abolished after preadsorption with 1 nM $Asn^{13}$-orcokinin for 18–20 h at room temperature.

### Evaluation and visualization

Orcokinin-ir neurons were reconstructed from serial frontal sections using a Leitz compound microscope equipped with a camera lucida attachment. Microscopic images were captured with a Zeiss microscope equipped with a 2 megapixel digital camera (Polaroid, Cambridge, MA, USA). Contrast and brightness of the micrographs were optimized in Adobe Photoshop 6.0. Positional information is given with respect to the body axis of the animals. A confocal laser scan microscope (Leica TCS SP2) equipped with a variable spectrometric emission light detection system was used for double labeling experiments. The scans were performed using a Leica HPX PL apochromate 40x/1.25 oil immersion objective. To exclude crosstalk artefacts, the specimens were always scanned sequentially, and the detection ranges were separated as far as possible. Cy2 fluorescence was excited with a 488 nm argon laser and detected between 505–525 nm. Cy3 fluorescence was excited with the 543 nm line of a helium/neon laser and detected between 585–625 nm.

## Results

We have analyzed orcokinin immunoreactivity in the accessory medulla (AMe), the circadian pacemaker of the cockroach *Leucophaea maderae*. Orcokinin-ir perikarya were found in five of six anatomically distinguishable soma groups of the AMe, comprising about 30 immunostained neurons in total. A comparison of the distribution of orcokinin-ir neurons in the AMe with the distribution of hitherto known peptide- and transmitter immunoreactivities showed that orcokinin immunostaining was different from all previously studied patterns of immunostaining in the AMe. To investigate whether orcokinin plays a role in input, output, or coupling pathways of the AMe, we performed double labeling experiments using the orcokinin antiserum combined with antisera against previously studied neuropeptides and a classical transmitter of the AMe (Table 1).

### Orcokinin immunoreactivity in the AMe

About thirty orcokinin-ir neurons were found in five soma groups of the AMe (Fig. 1A–D, 7A). Six orcokinin-ir neurons were located in the group of the distal frontoventral neurons (DFVNe, nomenclature according to Reischig and Stengl 2003a), which are most frontally associated with the AMe (Fig. 1A). Further, four ventroposterior neurons (VPNe) were orcokinin-ir (Fig. 1B, C, 7A). The VPNe are a heterogeneous group of somata, which send their primary neurites individually to ventrodistal regions of the AMe (Reischig and Stengl 2003a).

Most of the orcokinin-ir neurons (up to 16) belonged to the ventral neurons (VNe) of the AMe (Fig. 1A–C, 7A). At least five of these neurons may belong to a novel soma group of the AMe which had not been described in Reischig and Stengl (2003a). Their somata were slightly smaller than typical VNe somata and lay in a more median position. We included this group among the VNe because of yet unclear discrimination criteria. Four VNe connect both AMae via the posterior (POC) and anterior optic commissure (AOC) (Reischig et al. 2004). Other orcokinin-ir neurons were among the group of the median

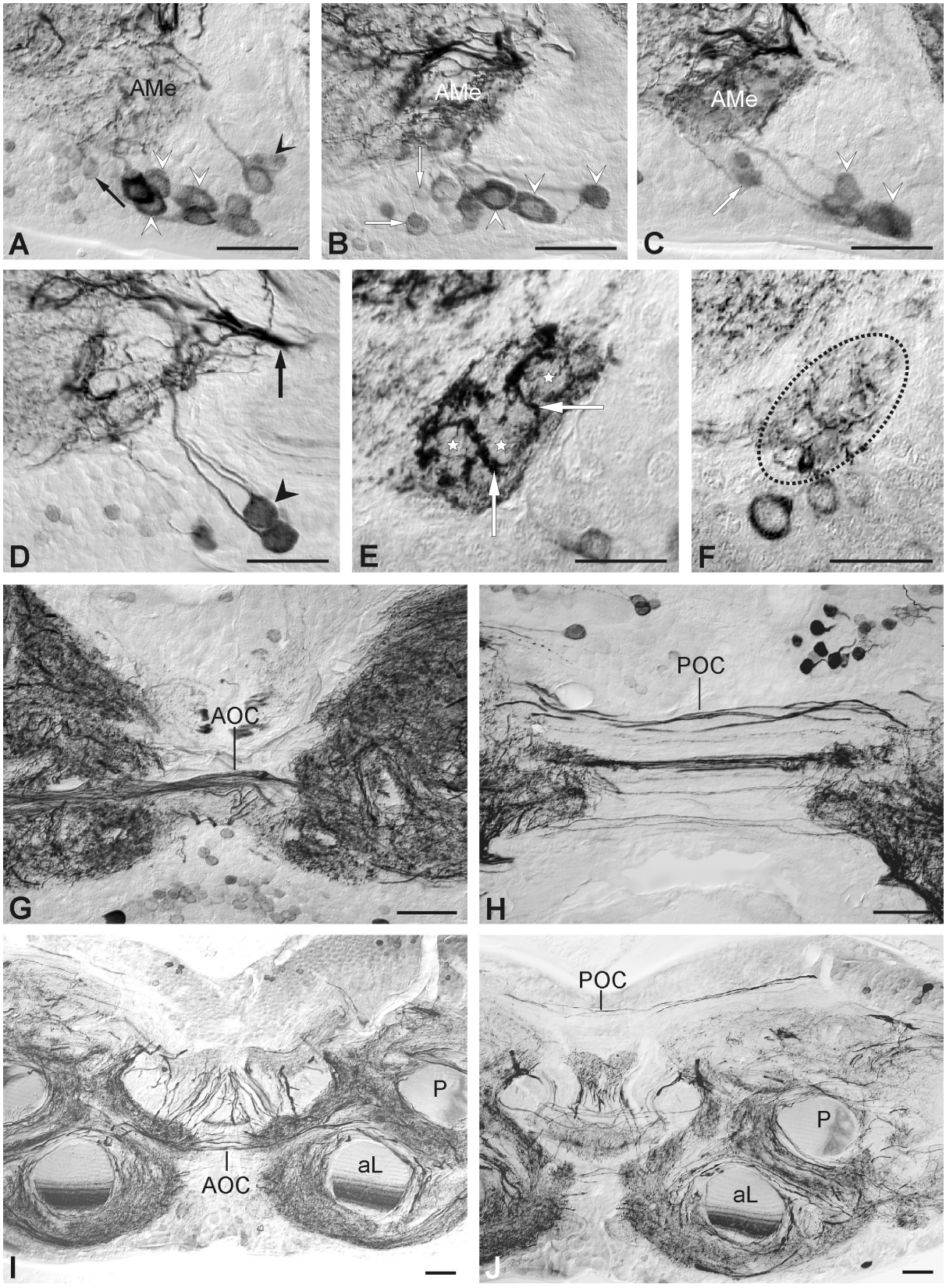
neurons (MNe; Fig. 1A, 7A, two neurons) and ventromedian neurons (VMNe; Fig. 1D, 7A, three neurons). The MNe are the medial-most, largest, and most heterogeneous soma group facing the lobula and are separated by a glia sheeth from the VNe. The VMNe send their primary neurites via the ventromedian bundle to the upper posterior edge of the AMe and join the lobula valley tract. Reischig et al. (2004) showed that many and perhaps all VMNe (about 35 neurons) connect both AMae. Accordingly, we found dense orcokinin immunoreactivity in the AOC (Fig. 1G, I) and in the POC (Fig. 1H, J). In the POC, six to eight orcokinin-ir fibers could be detected regularly (Fig. 1H).

The neuropil of the AMe is compartmentalized into a dense nodular core, which is interwoven by coarse internodular neuropil and surrounded by a shell of coarse neuropil. The latter continues into the anterior neuropil of the AMe (Reischig and Stengl 1996, 2003a). Orcokinin-ir neurons arborized most densely in the internodular and in the anterior neuropil (Fig. 1E, F). The nodular neuropil was invaded only sparsely by orcokinin-ir processes, but showed homogeneous immunostaining. Many of the prominent orcokinin-ir processes in the internodular neuropil could be traced to the VMNe neurons that project to, or originate from the contralateral AMe (Reischig et al. 2004). Owing to prominent and distinct immunolabeling, the projection patterns of the three VMNe neurons could be reconstructed through camera lucida drawings (Fig. 2). These neurons ramified in the internodular neuropil of the AMe and had dense arborizations in a median layer of the medulla. Axonal fibers, which were strongly immunostained, directly interconnected both AMae via the lobula valley tract and the POC (Fig. 2). The axonal projections of the neurons in the contralateral optic lobe were strongly immunostained, while cell bodies and ipsilateral processes were more weakly stained.

## Colocalization of immunostainings

Colocalization of orcokinin immunostaining with immunoreactivity for PDH, FMRFamide, GABA, Mas-allatotropin, and leucokinin was investigated by means of double immunofluorescence (Table 1). Three somata with colocalized orcokinin/PDH-immunoreactivity

**Fig. 1A–J** (next page) Orcokinin immunostaining in the accessory medulla (A–F) and in the anterior and posterior optic commissures (G–J) of *L. maderae*, detected with the PAP-technique. A–D Serial 30 $\mu$m vibratome sections through the accessory medulla (*AMe*), E, F 10 $\mu$m paraffin sections. I, J show horizontal sections, all other panels show frontal sections. **A–D** Orcokinin immunostained cell bodies are located in different soma groups of the anterior neuropil of the AMe. *Small black arrow* points to weakly orcokinin-ir distal frontoventral neurons (DFVNe). *White arrowheads* point to orcokinin-ir ventral neurons (VNe). Further, median neurons (MNe; *small black arrowhead*) and ventroposterior neurons (VPNe; *white arrows*) are stained. Three ventromedian neurons (VMNe) of the AMe (*black arrowhead*) show orcokinin immunoreactivity. They project via the lobula valley tract (*big arrow*) through the posterior optic commissure into the contralateral optic lobe. **E** Orcokinin-ir arborizations are concentrated in the internodular neuropil of the AMe (*arrows*), but the nodular neuropil (*asterisks*) is also stained above normal background. **F** Orcokinin immuostaining is less intense in the anterior neuropil of the AMe (*dotted line*). **G** Several fibers of the anterior optic commissure (*AOC*) show orcokinin immunostaining. **H** Six to eight fibers are immunostained in the posterior optic commissure (*POC*). **I** Horizontal section, showing orcokinin immunostaining in the AOC. *aL* $\alpha$-lobe of the mushroom body, *P* peduncle of the mushroom body. **J** Horizontal section showing orcokinin-ir fibers in the POC. *aL* $\alpha$-lobe of the mushroom body, *P* peduncle of the mushroom body. *Scale bars* 50 $\mu$m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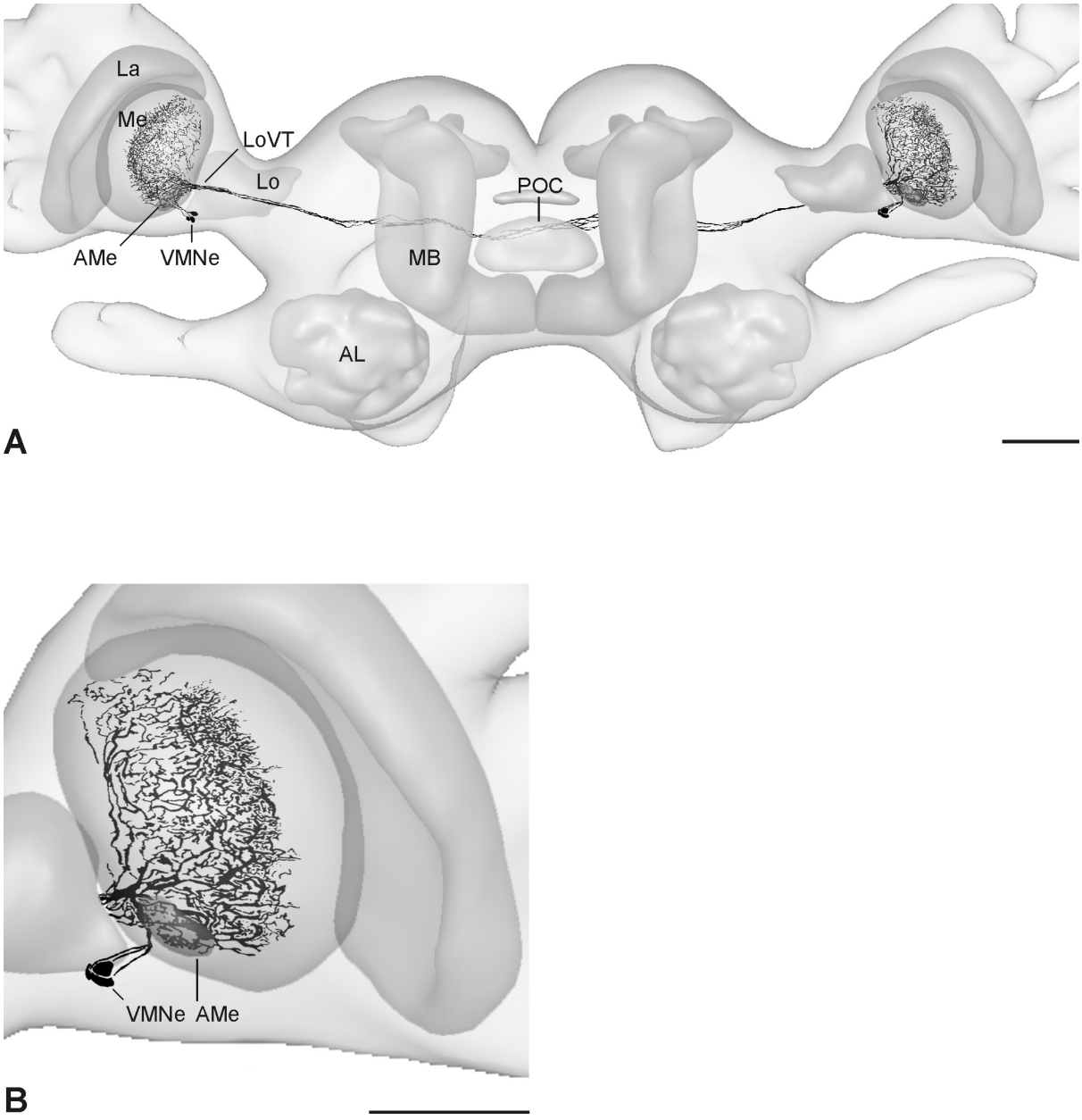


**Fig. 2A, B** Frontal reconstruction of three orcokinin-ir ventromedian neurons (*VMNe*) that connect the right and left accessory medulla (*AMe*) in the cockroach brain. The reconstruction is embedded into a three-dimensional scheme of the cockroach brain, provided by T. Reischig. **A** The neurons arborize in the AMe and in a median layer of the medulla (*Me*) and send axonal fibers through the lobula valley tract (*LoVT*) and the posterior optic commissure (*POC*) to the contralateral AMe. *AL* antennal lobe, *La* lamina, *Lo* lobula, *MB* mushroom body. **B** Higher magnification of arborizations in the optic lobe. The VMNe have characteristic tangential arborizations in a median layer of the medulla. *Scale bars* 200 $\mu$m

were detected in the group of the VNe (Figs. 3A, 7B). Although three PDH-ir VNe form a direct coupling pathway between the two pacemakers (Reischig et al. 2004), we did not find colocalized staining in the anterior (AOC) or in the posterior optic commissure (POC, Fig. 3B, C).

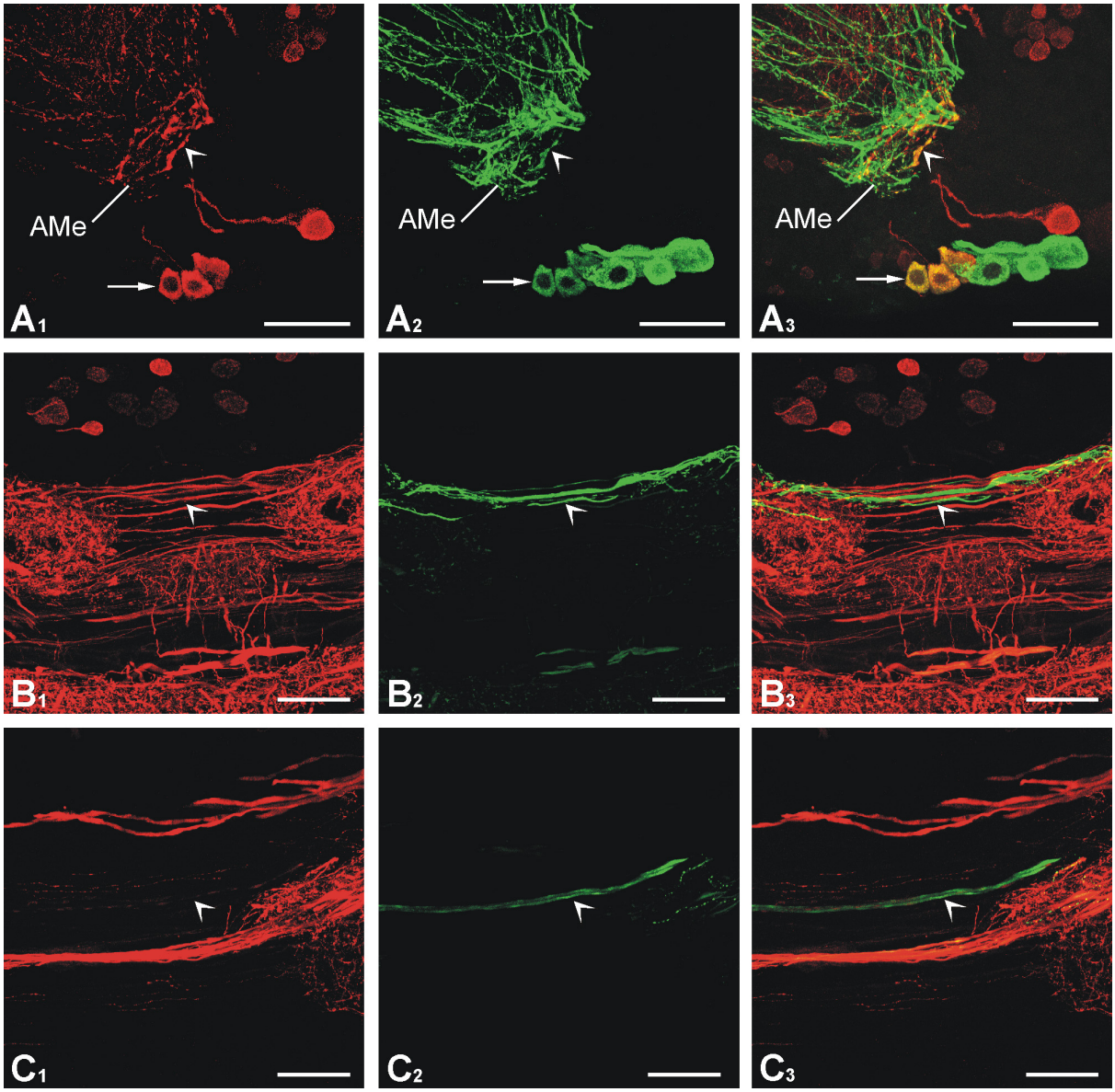


**Fig. 3A–C** Confocal laser images obtained from vibratome sections of the accessory medulla (*AMe*; A) and the anterior- (B) and posterior (C) optic commissure. Orcokinin immunoreactivity is shown in *red*, and PDH immunoreactivity in *green*. Panels on the right ($A_3$, $B_3$, $C_3$) show colocalization of orcokinin- and PDH immunostaining in *yellow*. $\mathbf{A}_{1-3}$ Stacks of 11 optical sections, z-distance between single sections = 2 $\mu$m. Three ventral neurons (VNe, *arrows*) show colocalized orcokinin/PDH immunostaining. Fibers with colocalized immunoreactivities project into the AMe (*arrowheads*). $\mathbf{B}_{1-3}$ Stacks of 17 optical sections, z-distance between single section = 1 $\mu$m. In the anterior optic commissure immunoreactivities are not colocalized (*arrowheads*). $\mathbf{C}_{1-3}$ Stacks of 17 optical sections, z-distance between single sections = 1 $\mu$m. Orcokinin- and PDH immunostaining is not colocalized in the posterior optic commissure (*arrowheads*). Instead, two fascicles of orcokinin-ir fibers cross the brain midline ventrally and dorsally to the PDH-ir fiber tract. *Scale bars* 50 $\mu$m

Similar results were obtained in the orcokinin/FMRFamide double labeling experiments. Three colocalized somata appeared in the group of the VNe (Figs. 4A, 7C). FMRFamide-ir fibers in the AOC and POC suggest that FMRFamide-ir neurons connect both AMae (Petri et al. 1995), but colocalization was not detected in either of the two

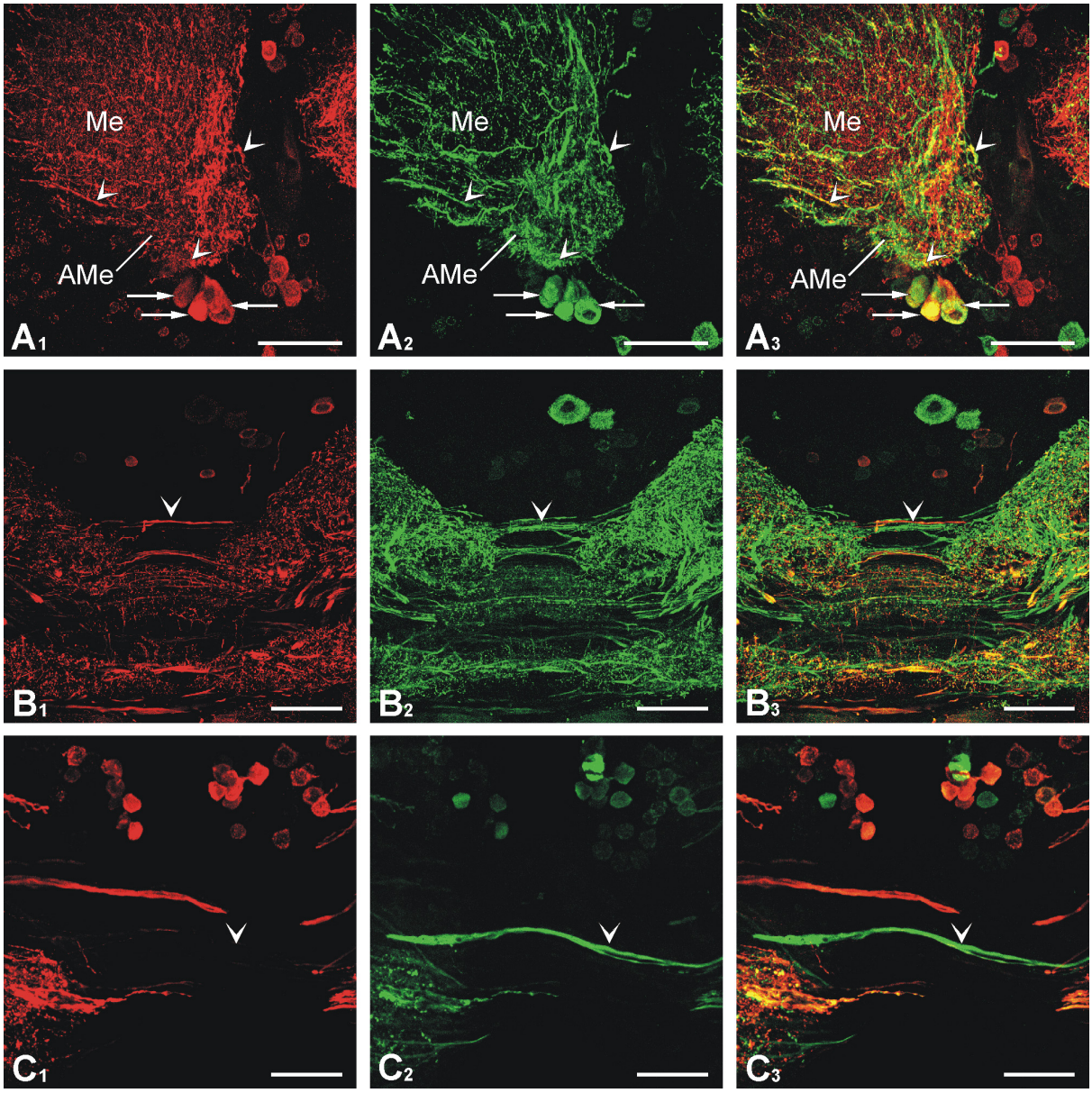


**Fig. 4A–C** Confocal laser images obtained from vibratome sections of the accessory medulla (*AMe*; A) and the anterior- (B) and posterior optic commissure (C). *Red* shows orcokinin immunoreactivity, *green* shows FMRFamide immunoreactivity. Panels on the right ($A_3$, $B_3$, $C_3$) show colocalization of orcokinin- and FMRFamide immunoreactivities in *yellow*. $\mathbf{A}_{1-3}$ Stacks of 7 optical sections, z-distance = 2 $\mu$m. Three ventral neurons (VNe) show colocalized orcokinin/FMRFamide immunoreactivities (*arrows*). Fine colabeled fibers (*arrowheads*) ramify in the AMe and medulla (*Me*). $\mathbf{B}_{1-3}$ Stacks of 13 optical sections, z-distance = 1 $\mu$m. No colabeling was detected in the anterior optic commissure (*arrowheads*). $\mathbf{C}_{1-3}$ Stacks of 13 optical sections, z-distance = 1 $\mu$m. Immunostainings are not colocalized in the posterior optic commissure (*arrowheads*). Orcokinin-ir fibers cross the posterior optic commissure dorsally to FMRFamide-ir fibers. *Scale bars* 50 $\mu$m

commissures. Instead, both in the AOC and POC orcokinin-ir fibers appeared dorsally to the FMRFamide-ir fibers (Fig. 4B, C).

GABA-ir neurons connect the nodular core of the AMe with the medulla and with the lamina via processes in the distal tract (Reischig and Stengl 1996; Petri et al. 2002). Up

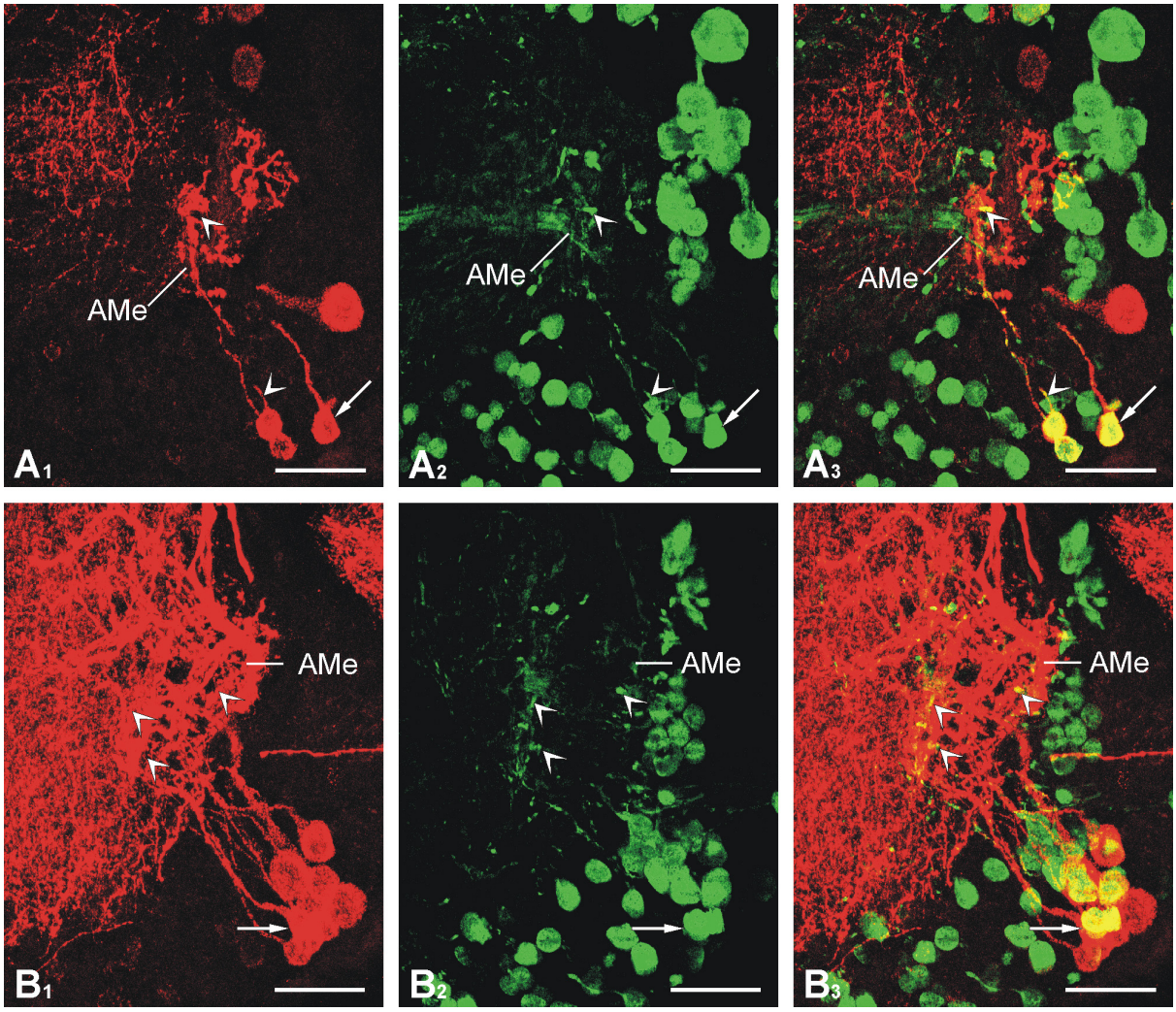


**Fig. 5A–B** Confocal laser images obtained from vibratome sections of the accessory medulla (*AMe*). *Red* shows orcokinin immunoreactivity, *green* shows GABA immunoreactivity. Panels on the right ($A_3$, $B_3$) show colocalization of orcokinin- and GABA immunoreactivities in *yellow*. **A** Stacks of 10, **B** of 11 optical sections (z-distance between single sections = 2 $\mu$m). $\mathbf{A}_{1-3}$ Three ventral neurons (VNe, *arrows*) of the AMe show colocalized orcokinin/GABA immunoreactivities. *Arrowheads* point to colabeled processes from these cells. $\mathbf{B}_{1-3}$ Section posterior to A. Three to four additional VNe show double immunofluorescence (*arrows*). Fine colabeled fibers (*arrowheads*) ramify in the AMe. *Scale bars* 50 $\mu$m

to six VNe showed colocalized orcokinin/GABA immunostaining (Figs. 5A, B, 7D). These neurons were more medial than the VNe described by Reischig and Stengl (2003a), but were here nevertheless included in the group of VNe. Double labeled orcokinin/GABA-ir arborizations were largely confined to the AMe (Fig. 5A, B), suggesting that these cells are local interneurons. However, the intensity of GABA immunostaining in the neuropil was very weak in these preparations. In contrast to GABA immunostaining, orcokinin immunoreactivity was not detected in the distal tract.

Most cell bodies of Mas-allatotropin-ir neurons were weakly immunostained, in contrast to other peptide immunostainings in the AMe (Reischig and Stengl 2003a). Their neuronal processes are concentrated in the nodular neuropil of the AMe (Petri and Stengl 1995; Fig. 6A). Only one soma in the group of the MNe and three somata of the VNe

showed prominent colocalized orcokinin/Mas-allatotropin immunoreactivity (Figs. 6A, B, 7E). Double labeling experiments with antisera against orcokinin and leucokinin showed dense leucokinin immunoreactivity in a subgroup of the DFVNe, but none of these cells showed colabeled orcokinin/leucokinin immunoreactivities (Fig. 6C).

## Discussion

In this study, we analyzed orcokinin immunostaining in the accessory medulla (AMe) of the cockroach *L. maderae*, a neuropil that controls circadian changes in locomotor activity (Reischig and Stengl 2003a; Homberg et al. 2003). In the cell cortex around the AMe, about 30 somata were orcokinin-ir, distributed in five identified neuron groups of the AMe. Double labeling experiments showed that orcokinin immunostaining is colocalized with immunostaining for PDH-, FMRFamide-, GABA-, and Mas-allatotropin in particular subgroups of the AMe neurons.

### Orcokinin-ir neurons in the accessory medulla

The AMe is a distinct, non-retinotopic neuropil in the optic lobe of the cockroach *L. maderae*. The processes of local and projection neurons appear to be differentially distributed in the AMe: local neurons ramify especially in the noduli, and projection neurons, in the surrounding coarse and internodular neuropil, possibly indicating functional subcompartments in the AMe (reviewed by Homberg et al. 2003).

The pattern of orcokinin-ir neurons in the AMe differs in several respects from the distribution of hitherto studied neuropeptides (Petri et al. 1995; Nässel et al. 2000). Most of the 30 orcokinin-immunostained neurons belong to the ventral neurons (VNe). Furthermore, three ventromedian neurons (VMNe) showed orcokinin immunostaining. This is the first evidence for the presence of a neuropeptide in this conspicuous group of neurons, which connect both AMae via the POC (Reischig et al. 2004). Loesel and Homberg (2001) characterized neurons of the VMNe group, termed PC2 neurons, through intracellular recordings combined with dye injection. Their reconstruction of a PC2 neuron shows striking similarities with the reconstruction of Fig. 2, particularly in the contralateral branching pattern of the neurons. Single cell dye fills revealed that the neurons have bilateral processes in the AMe, and ramifications concentrated dorsally in the ipsilateral medulla and in a medial layer in the contralateral medulla (Loesel and Homberg 2001). The neurons showed strong responses to light and, most interestingly, to polarized light. We, therefore, suggest that the three orcokinin-ir VMNe are light- and perhaps polarization sensitive. Reischig and Stengl (2003a) assumed that ipsilateral light input into the AMe is processed in the noduli, whereas contralateral light input enters the AMe preferentially via the anterior, shell, and internodular neuropil. Hence, orcokinin-related peptides possibly transmit light and/or coupling information to the contralateral pacemaker. Preliminary studies on the effect of $Asn^{13}$-orcokinin injections on circadian locomotor activity in the cockroach (Hofer et al. 2003) indeed suggest that orcokinin has a role in light entrainment.

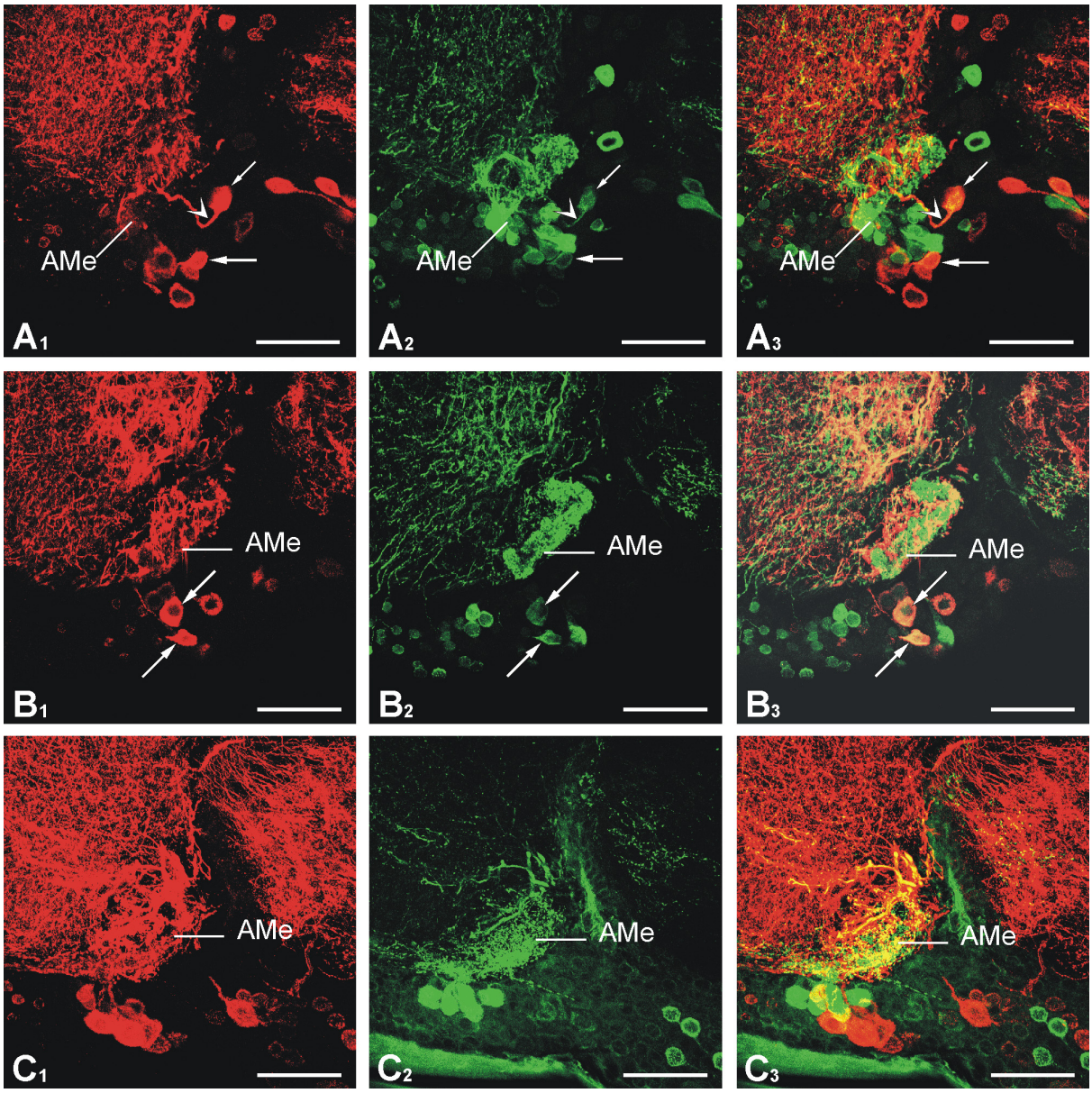


**Fig. 6A–C** Confocal laser images from vibratome sections of the accessory medulla (*AMe*). Orcokinin immunoreactivity is shown in *red*, and Mas-allatotropin (A, B) and leucokinin (C) immunoreactivities, in *green*. $A_3$–$C_3$ show colocalization of orcokinin- with Mas-allatotropin- ($A_3$, $B_3$) and leucokinin immunoreactivity ($C_3$) in *yellow*. Each image shows a stack of 8 optical sections (z-distance between single sections = 2 $\mu$m). $\mathbf{A}_{1-3}$ One prominent neuron in the group of the medial neurons (MNe, *small arrows*) and one ventral neuron (VNe, *large arrows*) show colocalized orcokinin/Mas-allatotropin immunoreactivity. The colabeled neurite (*arrowheads*) projects into the AMe. Most of the apparent colocalization is caused by overlap, but not colocalization of orcokinin-ir and Mas-allatotropin-ir processes. $\mathbf{B}_{1-3}$ Section posterior to (A). The *arrows* point to two orcokinin/Mas-allatotropin-ir ventral neurons (VNe). $\mathbf{C}_{1-3}$ Double labeling of orcokinin- and leucokinin-immunostaining at the level of the distal frontoventral neurons (DFVNe, *green* in $C_2$). No colocalization of orcokinin- and leucokinin immunoreactivities occurs. *Scale bars* 50 $\mu$m

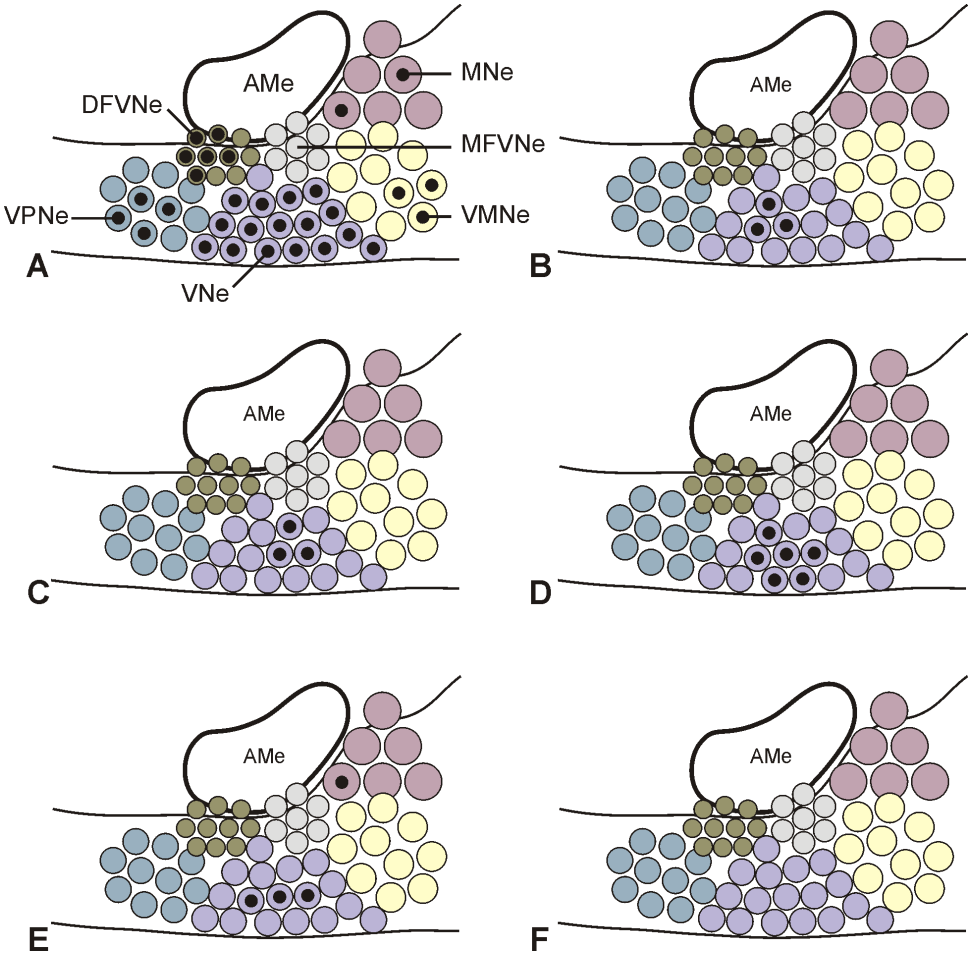


**Fig. 7A–F** Schematic diagram of the accessory medulla (*AMe*) with adjacent neuronal cell groups. The number of circles representing single somata do not correspond to the actual numbers of cells in the respective groups. **A** Distribution of orcokinin-ir neurons in the cell groups of the AMe (*filled circles*). Four ventroposterior neurons (*VPNe*), four to six distal frontoventral neurons (*DFVNe*), sixteen ventral neurons (*VNe*), two median neurons (*MNe*) and three ventromedian neurons (*VMNe*) show orcokinin immunostaining. **B** Distribution of colabeled orcokinin/PDH-ir neurons in the AMe. Three colabeled VNe were detected. **C** Colabeled orcokinin/FMRFamide-ir neurons. Three VNe were colabeled. **D** Colabeled orcokinin/GABA-ir neurons. Up to six VNe were colabeled. **E** Colabeled orcokinin/Masallatotropin-ir neurons in the AMe. One MNe and three VNe were colabeled. **F** None of the leucokinin-ir DFVNe were colabeled.

In addition, four to six distal frontoventral neurons (DFVNe), up to four ventroposterior neurons (VPNe), and two median neurons (MNe) showed orcokinin immunostaining. Reischig and Stengl (2003a) suggest that DFVNe are local neurons, which exclusively arborize in the noduli of the AMe. The weak uniform orcokinin immunostaining in the noduli might originate from these neurons. Up to now no immunoreactive substance was detected in the VPNe, whose branching patterns remain to be demonstrated. A subset of neurons that form the distal tract is thought to be among the MNe (Homberg et al. 2003), but although we found two orcokinin-ir neurons in this group, the distal tract was devoid of immunoreactivity.

### Colocalizations

In double labeling experiments we demonstrated colocalization of orcokinin with immunostaining for PDH, FMRFamide, GABA, and Mas-allatotropin. Among the VNe, three neurons showed colocalized orcokinin/PDH-, and orcokinin/FMRFamide-immunoreactivities, respectively. The PDH-ir neurons contain the circadian clock proteins PERIOD, TIMELESS, CLOCK and CYCLE in *D. melanogaster* and are thought to be pacemaker cells and clock outputs in the fruitfly and the cockroach (Homberg et al. 1991; Stengl and Homberg 1994; Helfrich-Förster 1995; Renn et al. 1999; Taghert 2001; Reischig and Stengl 2003a, 2003b; Stanewsky 2002; Homberg et al. 2003). Anatomical studies and microinjections of PDH into the AMe of *L. maderae* suggest that PDH transmits coupling information to the contralateral AMe (Petri and Stengl 1997; Reischig et al. 2004). Of four VNe connecting the AMe with its contralateral counterpart, three neurons are PDH-ir, with two of them projecting through the anterior optic commissure (AOC), and one projecting via the posterior optic commissure (POC; Reischig et al. 2004). Up to now only PDH- and FMRFamide-ir VNe were candidates to connect both AMae of the cockroach (Reischig et al. 2004), but here we additionally found orcokinin immunoreactivity in the AOC and POC. However, fibers exhibiting colocalized PDH- (or FMRFamide-)/orcokinin immunostaining were not detected in the AOC or in the POC. The occurrence of orcokinin-immunoreactivity in the AOC as well as in the POC fibers together with the lack of colocalized orcokinin/PDH immunostaining in these commissures thus implies the possibility that one orcokinin-ir VNe, namely the fourth, not PDH-ir VNe, connects both AMae.

Up to six VNe showed colocalized immunostaining for orcokinin and GABA. In the AMe, one neuron of the population of GABA-ir neurons was similar to projection neurons of the AMe that respond to light (Loesel and Homberg 2001; Petri et al. 2002). Other GABA-ir neurons terminating in the nodular neuropil of the AMe appear to form the main light entrainment pathway from the ipsilateral compound eye via the distal tract. Light-like phase response curves following GABA-injections into the AMe support this hypothesis (Petri et al. 2002). However, the somata of the neurons that form the distal tract are thought to be situated in the group of the MNe and not in the VNe (Homberg et al. 2003), as the orcokinin/GABA-ir neurons do, and orcokinin immunoreactivity was not observed in the distal tract. Therefore, the orcokinin/GABA-ir neurons are probably not involved in the direct light input pathway to the AMe, but may nevertheless regulate the processing of light information within the AMe. In addition to GABA, the neuropeptide Mas-allatotropin is also involved in light entrainment (Petri et al. 2002). In contrast to the orcokinin/GABA colocalizations, we found only one colabeled orcokinin/Mas-allatotropin neuron that belongs to the MNe, and additional three colocalized VNe. This suggests that orcokinin may act as copeptide or comodulator with Mas-allatotropin in the circadian system.

In summary, the colocalization of peptides related to PDH, FMRFamide, or Mas-allatotropin with orcokinin-like substances in the VNe and MNe, suggests that different peptides serve a cooperative role in these neurons. In addition, an orcokinin-related peptide might act as a neuromodulator in a subset of GABA-ir neurons of the AMe.

### The role of orcokinin in the circadian pacemaker of the cockroach *Leucophaea maderae*

The widespread occurrence of orcokinin-related peptides in different cell types of the AMe suggests that orcokinin serves a multitude of functions in the circadian system, possibly including a role in light entrainment, output pathways, and internal synchronization. Of particular interest is the presence of orcokinin immunostaining in the three pairs of heterolateral VMNe. Neurons of this group are highly sensitive to light and polarized light (Loesel and Homberg 2001). Page (1978) has demonstrated more than 30 years ago that the circadian pacemaker of *L. maderae* can be entrained by light to the contralateral eye. The VMNe fit exactly his proposed neural connection between both pacemakers for contralateral light entrainment, and orcokinin-related peptides might be the neuroactive compounds released from these neurons.

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## References

Bungart D, Dircksen H, Keller R (1994) Quantitative determination and distribution of the myotropic neuropeptide orcokinin in the nervous system of astacidean crustaceans. Peptides 15:393–400

Bungart D, Hilbich C, Dircksen H, Keller R (1995) Occurrence of analogues of the myotropic neuropeptide orcokinin in the shore crab, *Carcinus maenas*: evidence for a novel neuropeptide family. Peptides 16:67–72

Dircksen H, Burdzik S, Sauter A, Keller R (2000) Two orcokinins and the novel octapeptide orcomyotropin in the hindgut of the crayfish *Orconectes limosus*: identified myostimulatory neuropeptides originating together in neurones of the terminal abdominal ganglion. J Exp Biol 18:2807–2818

Helfrich-Förster C (1995) The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. Proc Natl Acad Sci USA 92:612–616

Hofer S, Dircksen H, Homberg U (2003) Involvement of a neuropeptide related to orcokinin in light entrainment of the circadian clock of the cockroach. In: Elsner N, Zimmermann H (eds) The neurosciences from basic research to therapy. Thieme, Stuttgart, pp 808–809

Hofer S, Dircksen H, Homberg U (2004) Comparative distribution of orcokinin immunostaining in the brain of selected dicondylian insects. J Comp Neurol (in revision)

Homberg U (1991) Neuroarchitecture of the central complex in the brain of the locust *Schistocerca gregaria* and *S. americana* as revealed by serotonin immunocytochemistry. J Comp Neurol 303:245–254

Homberg U, Würden S, Dircksen H, Rao KR (1991) Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. Cell Tissue Res 266:343–357

Homberg U, Reischig T, Stengl M (2003) Neural organization of the circadian system of the cockroach *Leucophaea maderae*. Chronobiol Int 20:577–591

Huybrechts J, Nusbaum MP, Bosch LV, Baggerman G, De Loof A, Schoofs L (2003) Neuropeptidomic analysis of the brain and thoracic ganglion from the Jonah crab, *Cancer borealis*. Biochem Biophys Res Commun 308:535–544

Li L, Pulver SR, Kelley WP, Thirumalai V, Sweedler JV, Marder E (2002) Orcokinin peptides in developing and adult crustacean stomatogastric nervous systems and pericardial organs. J Comp Neurol 444:227–244

Loesel R, Homberg U (2001) Anatomy and physiology of neurons with processes in the accessory medulla of the cockroach *Leucophaea maderae*. J Comp Neurol 439:193–207

Nässel DR, Persson MG, Muren JE (2000) Baratin, a nonamidated neurostimulating neuropeptide, isolated from the cockroach brain: distribution and actions in the cockroach and locust nervous systems. J Comp Neurol 422:267–286

Negoescu A, Labat-Moleur F, Lorimier P, Lamarcq L, Guillermet C, Chambaz E, Brambilla E (1994) F(ab) secondary antibodies: a general method for double immunolabeling with primary antisera from the same species. Efficiency control by chemiluminescence. J Histochem Cytochem 42:433–437

Page TL (1978) Interactions between bilaterally paired components of the cockroach circadian system. J Comp Physiol A 124:225–236

Page TL (1983) Regeneration of the optic tracts and circadian pacemaker activity in the cockroach *Leucophaea maderae*. J Comp Physiol A 152:231–240

Petri B, Stengl M (1997) Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. J Neurosci 17:4087–4093

Petri B, Stengl M, Würden S, Homberg U (1995) Immunocytochemical characterization of the accessory medulla in the cockroach *Leucophaea maderae*. Cell Tissue Res 282:3–19

Petri B, Homberg U, Loesel R, Stengl M (2002) Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. J Exp Biol 205:1459–1469

Reischig T, Stengl M (1996) Morphology and pigment-dispersing hormone immunocytochemistry of the accessory medulla, the presumptive circadian pacemaker of the cockroach *Leucophaea maderae*: a light- and electron-microscopic study. Cell Tissue Res 285:305–319

Reischig T, Stengl M (2003a) Ultrastructure of pigment-dispersing hormone-immunoreactive neurons in a three-dimensional model of the accessory medulla of the cockroach (*Leucophaea maderae*). Cell Tissue Res 314:421–435

Reischig T, Stengl M (2003b) Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). J Exp Biol 206:1877–1886

Reischig T, Petri B, Stengl M (2004) Pigment-dispersing hormone (PDH)-immunoreactive neurons form a direct coupling pathway between the bilaterally symmetric circadian pacemakers of the cockroach *Leucophaea maderae*. Cell Tissue Res (in press)

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802

Skiebe P, Dreger M, Meseke M, Evers JF, Hucho F (2002) Identification of orcokinins in single neurons in the stomatogastric nervous system of the crayfish, *Cherax destructor*. J Comp Neurol 444:245–259

Stanewsky R (2002) Clock mechanisms in *Drosophila*. Cell Tissue Res 309:11–26

Stangier J, Hilbich C, Burdzik S, Keller R (1992) Orcokinin: a novel myotropic peptide from the nervous system of the crayfish, *Orconectes limosus*. Peptides 13:859–864

Stengl M, Homberg U (1994) Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. J Comp Physiol A 175:203–213

Sternberger LA (1979) Immunocytochemistry. Wiley, New York

Taghert PH (2001) How does the circadian clock send timing information to the brain? Semin Cell Dev Biol 12:329–341

Yasuda-Kamatani Y, Yasuda A (2000) Identification of orcokinin gene-related peptides in the brain of the crayfish *Procambarus clarkii* by the combination of MALDI-TOF and on-line capillary HPLC/Q-TOF mass spectrometries and molecular cloning. Gen Comp Endocrinol 118:161–172

Chapter III. Evidence for a role of orcokinin-related peptides in the circadian clock of the cockroach *Leucophaea maderae*

Hofer S and Homberg U (2004) J Exp Biol (in preparation)

# Chapter III

# Evidence for a Role of Orcokinin-related Peptides in the Circadian Clock of the Cockroach *Leucophaea maderae*

Sabine Hofer and Uwe Homberg

## Abstract

Orcokinins are a family of myotropic neuropeptides that were identified in various decapod crustaceans, where they act both as hormones and as neuromediators within the nervous system. Evidence for the presence of orcokinins in the brain of insects was provided recently by immunocytochemistry. Particularly prominent orcokinin immunoreactivity was found in the accessory medulla (AMe), a small neuropil in the optic lobe associated with circadian pacemaker functions. In the cockroach *Leucophaea maderae*, about 30 somata in five of the six cell groups of the AMe show orcokinin immunostaining. By means of tracer injections into one AMe and immunostaining with anti-orcokinin antiserum, we show that one orcokinin-immunoreactive (-ir) ventral neuron and three ventromedian neurons directly connect both AMae. To determine a possible circadian function of orcokinin in the cockroach, we injected 150 fmol $Asn^{13}$-orcokinin into the vicinity of the AMe at different circadian times. These experiments resulted in stable phase-dependent resetting of the phase of circadian locomotor activity of the cockroach. The shape of the resulting phase response curve closely matched the phase shifting effects of light-pulses, and its amplitude was dependent on the amount of the injected peptide. Together with the anatomical data the results suggest that orcokinin-related peptides play an important role in light entrainment pathways to the circadian clock via the contralateral compound eye. This study, furthermore, provides the first evidence for a physiological role of an orcokinin-related peptide in insects.

**Keywords:** Orcokinin, Insect brain, Circadian rhythms, Accessory medulla, Phase response curve

## Introduction

Orcokinins are a family of neuropeptides that were originally purified from the nervous system of the crayfish *Orconectes limosus* (Stangier et al. 1992). All investigated or-

cokinins are highly conserved between different species but show some variation in isoforms (Bungart et al. 1994; Yasuda-Kamatani and Yasuda 2000; Skiebe et al. 2002; Huybrechts et al. 2003). Recently, we demonstrated that orcokinin-related peptides are also present in the brain of polyneopteran insects (such as cockroaches and locusts), but not in the brain of endopterygote species (Hofer et al. 2004). Furthermore, recent biochemical characterization suggests that orcokinins are present in the nervous systems of the locust *Schistocerca gregaria* and the cockroach *Leucophaea maderae* (H. Dircksen, personal communication). Immunostaining in the brain and retrocerebral complex suggests that orcokinin-related peptides function both as hormones and neuromodulators in these insects as described for crustaceans.

In this study, we analyzed the role of orcokinin-related peptides in the circadian clock of the cockroach *L. maderae*. A small neuropil at the anterior base of the medulla, the accessory medulla (AMe), has been associated with circadian pacemaker activity in several insects including the fruitfly *Drosophila melanogaster* and the cockroach *L. maderae* (Homberg et al. 2003; Helfrich-Förster 2003; Reischig and Stengl 2003a). The anatomical and neurochemical organization of the cockroach AMe has been studied in considerable detail (reviewed by Homberg et al. 2003). The AMe consists of a core of dense nodular neuropil, which is embedded in and surrounded by coarse neuropil (Petri et al. 1995; Reischig and Stengl 1996, 2003b). Six groups of somata near the AMe send neurites into the AMe neuropil (Reischig and Stengl 2003b). Neurons of the distal tract connect the medulla to the AMe (Reischig and Stengl 1996, 2003b; Petri et al. 2002); these neurons are likely to play a major role in light entrainment of the clock through photoreceptors of the compound eye (Roberts 1965; Nishiitsutsuji-Uwo and Pittendrigh 1968). Page (1978, 1981, 1983a) demonstrated that neural connections between both brain hemispheres in *L. maderae* serve a role in bilateral coupling of the clocks and, in addition, in light entrainment by the contralateral compound eye. Commissural neurons between both AMae, which might mediate these functions, have been identified recently (Loesel and Homberg 2001; Reischig and Stengl 2002, 2004). Immunocytochemical studies and injections of neuroactive substances suggest that a variety of neuropeptides and $\gamma$-aminobutyric acid (GABA) play roles as neuromediators in the circadian system of the cockroach (Petri et al. 1995, 2002; Petri and Stengl 1997). GABA-ergic neurons connect the medulla to the AMe via the so-called distal tract, which is discussed to constitute an important part of the ipsilateral light entrainment pathway to the clock (Reischig and Stengl 1996, 2003b; Petri et al. 2002). Neuropeptides related to Mas-allatotropin also play a role in the light entrainment pathway of the clock; they were detected immunocytochemically in local AMe neurons (Petri et al. 2002). Neurons immunoreactive to $\beta$-pigment-dispersing hormone (PDH) are output neurons of the clock, which connect the AMe to the lamina and selected parts of the midbrain (Reischig et al. 2004). Peptide and tracer injections revealed that three PDH-ir neurons connect both AMe via the anterior and posterior optic commissure and probably transmit coupling information into the contralateral clock of the cockroach (Reischig et al. 2004; Petri and Stengl 1997). Several other peptides including FMRFamide and leucokinin were detected immunocytochemically in the cockroach AMe but their functional roles remain to be investigated (Petri et al. 1995).

Most recently, peptides related to crustacean orcokinins were detected immunocytochemically in the AMe of the cockroach (Hofer et al 2004; Hofer and Homberg 2004).

Detailed mapping showed that staining was present in about 30 neurons of the AMe and included cell bodies in five of the six established cell groups of the AMe (Hofer and Homberg 2004). Staining in AMe neurons with axonal fibers in the posterior optic commissure was particularly prominent and suggested that orcokinin-immunoreactive neurons participate in coupling of the bilateral clocks. The present study shows that four pairs of orcokinin-ir neurons connect both AMe via the posterior and possibly the anterior optic commissure. Microinjections of $Asn^{13}$-orcokinin into the vicinity of the AMe, furthermore, resulted in phase dependent phase shifts in circadian wheel-running activity resembling the phase-shifting effects of light. These experiments revealed the first evidence for a physiological role of orcokinin in insects and suggest that the peptide plays a role in light entrainment of the clock via the contralateral compound eye.

## Materials and methods

### Animals

Male adult cockroaches (*Leucophaea maderae*) were taken from crowded colonies at the University of Marburg. Animals were reared under 12:12 hours light-dark (LD) photoperiod, at about 60 % relative humidity, and a temperature of 28 °C.

### Dextran injections

Animals ($n = 22$) were anesthetized with $CO_2$ and fixed in a mounting device. A small window was cut into the head capsule above the left optic lobe to expose the brain. An amount of 1–3 nl Texas Red dextran-solution (dextran conjugated with Texas Red, 3000 MW, lysine fixable, Molecular Probes Inc., USA; 0.1 mg/$\mu$l in water) was pressure-injected with a microinjector (Microinjector 5242, Eppendorf, Germany) under stereomicroscopic control into one AMe with a glass capillary (Clark, Pangbourne Reading, England). The capillary was pulled to a pipette as used for patch clamp experiments, with a tip diameter of 1–3 $\mu$m. After the injection, the head capsule was closed with wax to allow intracellular transport of the dye while the animal was surviving overnight.

### Immunocytochemistry

The next day the injected brains of the cockroaches were removed and fixed for 4 hours or overnight in 4 % paraformaldehyde/7.5 % saturated picric acid in sodium phosphate buffer (0.1 M, pH 7.4) at room temperature. The brains were embedded in gelatine/albumin (4.8 % gelatine and 12 % ovalbumin in demineralized water) and postfixed in 8 % formalin in sodium phosphate buffer (0.1 M, pH 7.4). The brains were sectioned using a vibrating blade microtome (Leica, Nussloch, Germany) in frontal plane at 40 $\mu$m thickness. The brain sections were washed in Tris-buffered saline (TBS; 0.1 M Tris-HCl/0.3 M NaCl, pH 7.4) containing 0.1 % Triton X-100 (TrX). They were preincubated in TBS with 0.5 % TrX and 5 % normal goat serum (NGS; DAKO, Hamburg, Germany). Primary antiserum,

anti-Asn$^{13}$-orcokinin (provided by Dr. H. Dircksen, Department of Zoology, Stockholm), was diluted at 1:4000 in TBS containing 0.5 % TrX and 1 % NGS and was applied to the sections for 18–20 h at room temperature. The sections were washed in TBS containing 0.1 % TrX and incubated with Cy2-conjugated goat anti rabbit antiserum (GAR; Dianova Hamburg, Germany, diluted 1:300) in TBS containing 0.5 % TrX and 1 % NGS for 1 h. Afterwards, the sections were thoroughly washed, and mounted on chromalum/gelatine coated microscope slides.

### Specificity controls

The anti-Asn$^{13}$-orcokinin antiserum has been characterized by Bungart et al. (1994), who tested HPLC-fractions of different astacidean crustaceans with an enzyme linked immunosorbent assay (ELISA). On cockroach brain sections, specificity of the antiserum was determined by liquid-phase preadsorption of the diluted primary antiserum with various concentrations ($10^{-4}$ M up to $10^{-11}$ M) of Asn$^{13}$-orcokinin (NFDEIDRSGFGFN-OH; Stangier et al. 1992; Bachem, Heidelberg, Germany), before adding the combined solution to the preparation. Immunostaining was abolished after preadsorption with 1 nM Asn$^{13}$-orcokinin for 18–20 h at room temperature.

### Evaluation and visualization

Microscopic images were captured with a Zeiss microscope equipped with a 2 megapixel digital camera (Polaroid, Cambridge, MA, USA). Positional information is given with respect to the body axes of the animals.

A Leica TCS SP2 confocal laser scan microscope equipped with a spectrophotometric emmission light detection system was used to evaluate the doublestaining experiments. All scans were performed using a Leica HPX PL apochromate 40x/1.25 oil immersion objective. To exclude crosstalk artifacts, the specimens were scanned sequentially, and the detection ranges were separated as far as possible. Cy2 fluorescence was excited with the 488 nm line of an argon laser and detected between 505 and 525 nm. Texas Red fluorescence was excited with the 543 nm line of a helium/neon laser and detected between 585 and 625 nm.

### Operation and injection in behavioral experiments

All manipulations were performed in dim red light with a microinjector (see above). The experimental animals were removed at different circadian times from their running wheels and mounted in a metal tube. The animals were anesthetized with $CO_2$. A small window was cut in the head capsule, and one optic lobe was injected with 2 nl of orcokinin solution or saline in the vicinity of the AMe, following the procedure described above for dextran injections. After the injection, the excised piece of cuticle was waxed back and the animal was returned to the running wheel. The time of injections did not

take more than 15 minutes. The injection volume (150 fmol in 2 nl saline with blue food dye [McCormick, Baltimore, MD]) was controlled before and after injection with test injections in mineral oil. The concentration of $10^{-4}$ M was chosen because similar doses were effective in previous peptide injection experiments (Petri and Stengl 1997; Petri et al. 2002). Concentrations of $10^{-8}$ M and $10^{-12}$ M orcokinin were tested at CT 13-15 h of the circadian cycle, corresponding to the peak of the phase response curve, to investigate dose dependency of the response. Control injections consisted of 10 % blue food dye in saline without orcokinin.

### Behavioral assays and data analysis

Circadian behavior was analyzed from cockroaches kept in constant darkness (DD) and constant temperature (28 °C) and humidity (60 %). Locomotor activity was recorded with running wheels (Wiedenmann 1977) equipped with a magnetic reed switch. One revolution of the running wheel resulted in one impulse. Impulses were continuously counted by a computer over 1 min intervals and condensed and processed by a custom-designed PC-compatible software (developed by H. Fink, University of Konstanz). The data were plotted in double plot activity histograms. The free-running period $\tau$ and the induced phase shifts were estimated by converting the raw data into ASCII format. They were then merged into 30 min intervals and analyzed with Chrono II software (provided by Till Roenneberg; Roenneberg and Morse 1993) on a Macintosh computer. The $\chi^2$-periodograms were calculated with Tempus 1.6 (Reischig 2003), an add-in for Microsoft Excel, on an IBM-compatible PC. Data were evaluated from 110 of the 157 animals used. The remaining 47 cockroaches died after the operation. The free-running periods before and after injection were calculated by linear regression through daily activity onset. Changes in $\tau$ ($\Delta\tau = \tau_{after} - \tau_{before}$) were calculated, with periods estimated by regression through activity onsets and by $\chi^2$-periodogram analyses (Enright 1965; Sokolove and Bushell 1978). Phase shifts were determined as time differences between the regression lines before and after injection extrapolated to the day after treatment. Phase delays were plotted as negative values and phase advances as positive values. Time on the $x$-axis of the resulting phase response curve is shown as circadian time (CT), with CT 12:00 h = activity onset = beginning of the subjective night. Daily activity onsets were determined by using Chrono II (Roenneberg and Morse 1993).

The behavioral data were merged into 2 h time intervals and the means and standard deviations (S.D.) were calculated for each bin. Changes of phases and periods in a given time interval were considered to be significantly different from zero, if the calculated 95 % confidence interval of the respective time interval did not contain the value zero. The phase and period changes were statistically analyzed by a two-tailed Student's t-test. Additionally, the peak values of the delay and advance portion of the resulting orcokinin-phase response curve (at CT 14 h and CT 18 h) were tested against all other values. Significance in all cases was taken as $p < 0.05$. Statistical analyses were performed with SPSS 11.0 (Superior Performing Software Systems; SPSS Inc.) and Excel XP (Microsoft). Smoothed phase response curves were produced with Excel.

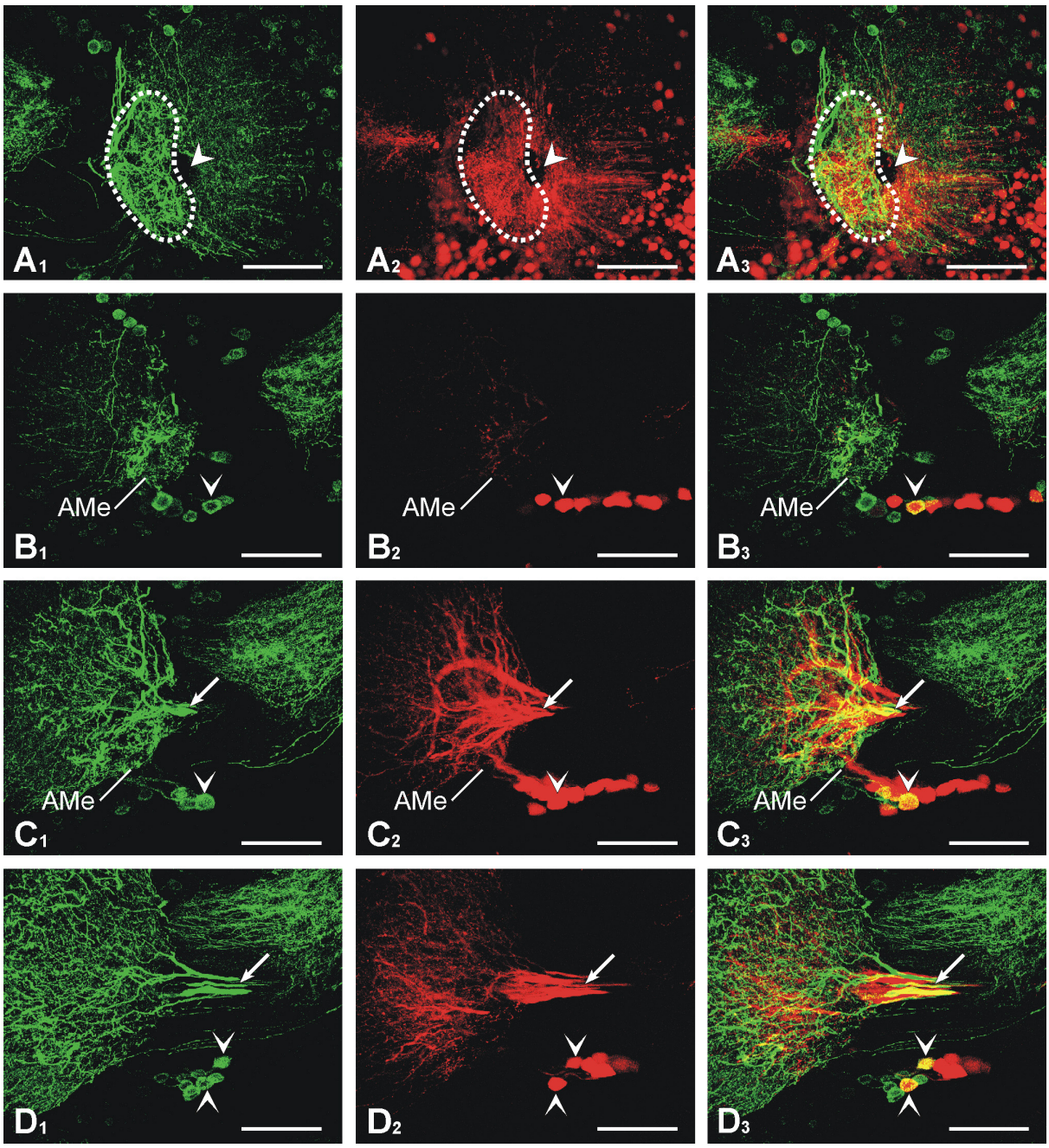


**Fig. 1A–D** Confocal laser images obtained from vibratome sections of the accessory medulla. Orcokinin immunoreactivity is shown in *green*, and Texas Red dextran (TRed-D) fluorescence in *red*. The right columns ($A_3$, $B_3$, $C_3$) show colocalization of orcokinin immunoreactivity and TRed-D fluorescence in *yellow*. Stacks of 7-11 optical sections (z-distance between single sections = 2 $\mu$m). $\mathbf{A}_{1-3}$ The TRed-D-injected accessory medulla (outlined by the *dotted line*). *Arrowheads* point to the injection site. $\mathbf{B}_{1-3}$ The contralateral accessory medulla (*AMe*) after injection of TRed-D into the opposite AMe revealed one colabeled orcokinin/TRed-D fluorescent ventral neuron (VNe; *arrowheads*). $\mathbf{C}_{1-3}$ Section posterior to B. Near the accessory medulla (*AMe*), one ventromedian neuron (VMNe, *arrowheads*) shows anti-orcokinin/TRed-D colabeling. Colabeled fibers from the injected AMe projected via the lobula valley tract (*arrows*) into the anterior and internodular neuropil of the contralateral AMe. $\mathbf{D}_{1-3}$ Section posterior to C. Two additional VMNe (*arrowheads*) and fibers in the lobula valley tract (*arrows*) show colabeled anti-orcokinin/TRed-D fluorescence. *Scale bars* 50 $\mu$m

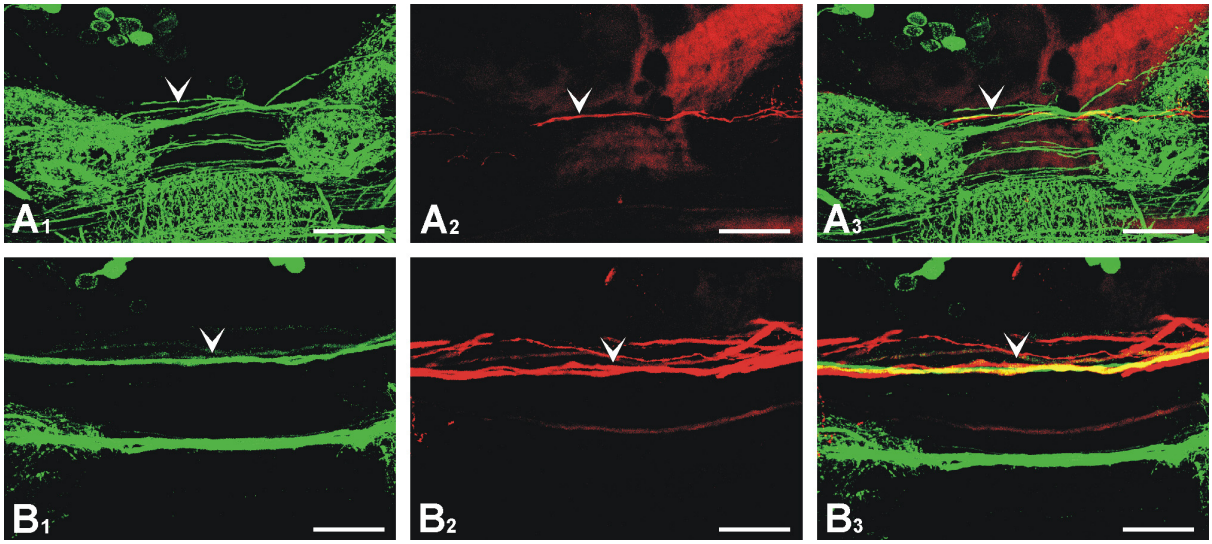


**Fig. 2A–B** Confocal laser images obtained from vibratome sections of the anterior- (A) and posterior optic commissure (B). *Green* shows orcokinin immunoreactivity, *red* shows Texas Red dextran (TRed-D) fluorescence, after injection of TRed-D into one AMe (see Fig. 1A). The right column ($A_3$, $B_3$,) shows colocalization of orcokinin immunoreactivity and TRed-D fluorescence in *yellow*. Stacks of 10 optical sections (z-distance between single sections = 1 $\mu$m). $\mathbf{A}_{1-3}$ Orcokinin- and TRed-D immunostained fibers project in parallel via the anterior optic commissure (*arrowheads*), but do not show colabeling. $\mathbf{B}_{1-3}$ Colabeled orcokinin/TRed-D immunostained fibers in the posterior optic commissure (*arrowheads*). *Scale bars* 50 $\mu$m

## Results

To determine whether subsets of orcokinin-ir accessory medulla (AMe) neurons qualify for direct, monosynaptic coupling of both AMae, we injected Texas Red-conjugated dextran (TRed-D) as neuronal tracer into one AMe. Brains were immunostained with anti-orcokinin antiserum ($n$ = 21), and the AMe contralateral to the injection site and commissures in the midbrain were examined for double fluorescence (Figs. 1, 2). These experiments showed that one bilateral pair of orcokinin-ir ventral neurons (VNe) and three pairs of ventro-median neurons (VMNe) provide direct connections between both AMae. To examine whether orcokinin influences circadian locomotor activity of the cockroach, we injected Asn$^{13}$-orcokinin into the vicinity of one AMe of the cockroach. These experiments resulted in phase-dependent phase shifts in circadian locomotor activity resembling the phase-shifting effects of light.

### Injection of TRed-D into the AMe

After injection of approximately 2 nl TRed-D into one AMe (Fig. 1A), we found up to four colabeled orcokinin/TRed-D somata in the contralateral AMe (Figs. 1B–D, 3). These somata could be identified as one orcokinin/TRed-D colabeled VNe and three colabeled VMNe. Prominent orcokinin/TRed-D colabeled fibers projected via the lobula valley tract into the AMe and arborized preferentially in the internodular neuropil (Fig. 1C, D). Colabeled orcokinin/TRed-D fluorescent fibers were visible in the posterior optic commissure (Fig. 2B, 3). These fibers connected both AMae without extending sidebranches to

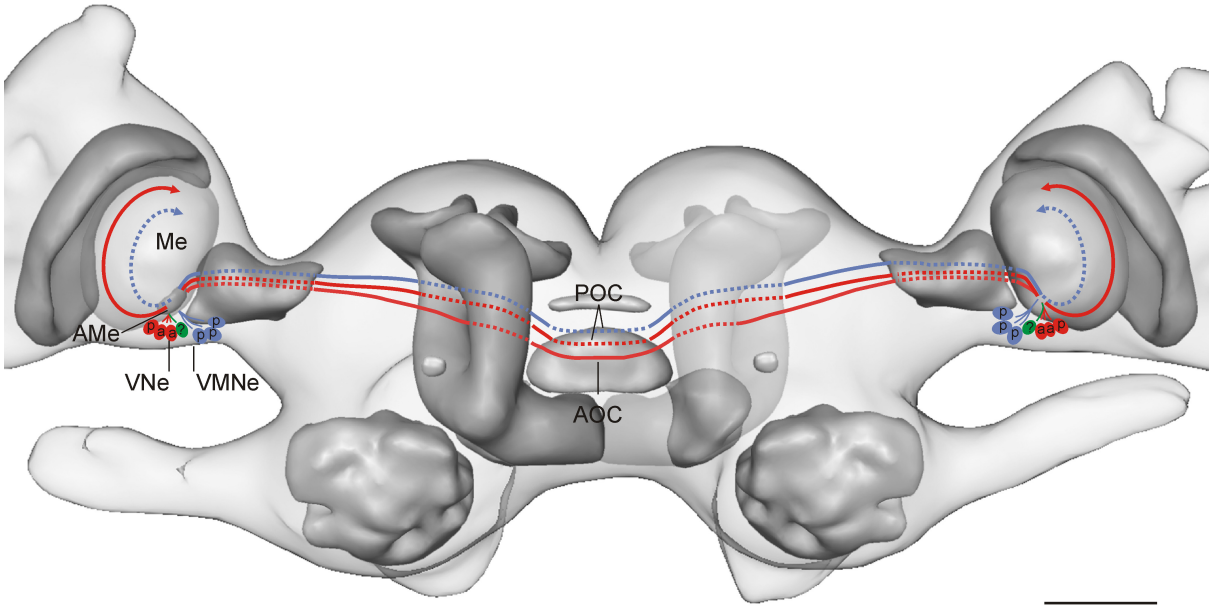


**Fig. 3** Three-dimensional model of the cockroach brain showing orcokinin-ir (*blue, green*) and PDH-ir (*red*) connections between both AMae. Three ventromedian neurons (*VMNe, blue, p*) project via the posterior optic commissure (*POC*) into the contralateral AMe and arborize in several median layers of the medulla. One ventral neuron (*VNe, green*) projects via the anterior or posterior commissure. One PDH-ir VNe (*red, p*) projects via the POC and two PDH-ir VNe (*a*) via the anterior optic commissure to the contralateral AMe and in the distalmost layer of the medulla. The 3D model was provided by T. Reischig and modified. *Scale bar* 200 $\mu$m

the midbrain, but the number of colabeled fibers could not be clearly determined. In contrast, no colocalization of orcokinin/TRed-D was found in the anterior optic commissure (Fig. 2A).

### Effects of Asn$^{13}$-orcokinin injections on circadian locomotor activity rhythms

To investigate whether orcokinin plays a role as an input signal to the circadian clock, we examined whether the peptide influences circadian locomotor activity of the cockroach. Asn$^{13}$-orcokinin was injected into the vicinity of one AMe at different circadian times, and locomotor activities of the free-running cockroaches were recorded before and after the injections (Fig. 4). Control injections with carrier solution alone (blue food dye in saline) did not cause significant phase shifts in circadian locomotor rhythm, except for a small but significant phase delay from CT 21–23 h (Table 1). Injections of orcokinin resulted in large and significant phase-dependent phase shifts in circadian activity at several circadian times. Maximal phase delays (−3.8 h) occurred when orcokinin was injected at CT 13 h, and maximal phase advances (2.2 h) were observed at CT 18 h (Fig. 5). As judged from the 95 % confidence intervals, significant orcokinin-dependent phase delays occurred from CT 7–15 h, and significant phase advances from CT 17–19 h (Table 1, Fig. 5, 6). Phase shifts during the rest of the cycle were not significantly different from zero. Orcokinin-induced phase shifts from CT 7–15 h and from CT 17–19 h were also significantly different from phase shifts induced by control injections. Finally, orcokinin-

**Table 1** Phase shifts (in circadian hours) resulting from injections of 150 fmol orcokinin and from control (saline) injections at different times of the circadian cycle (circadian time, CT)

| | Phase shifts (mean ± S.D.) | | 95 % CI (lower to upper limit) | | n | |
|---|---|---|---|---|---|---|
| CT (h) | Orcokinin | Saline | Orcokinin | Saline | Orcokinin | Saline |
| 01:00–03:00 | $0.39 \pm 1.12^{d}$ | $-0.12 \pm 0.33$ | 3.17 *to* −2.39 | −0.92 *to* 0.69 | 3 | 3 |
| 03:00–05:00 | $-0.07 \pm 1.27^{d,f}$ | $-0.02 \pm 0.11$ | 1.11 *to* −1.25 | −1.03 *to* 0.10 | 7 | 3 |
| 05:00–07:00 | $-0.45 \pm 1.44^{d,f}$ | $-0.30 \pm 0.59$ | 3.13 *to* −4.03 | −1.76 *to* 1.16 | 3 | 2 |
| 07:00–09:00 | $-1.50 \pm 0.54^{a,d,f}$ | $-0.09 \pm 0.35$ | $-0.83$ *to* $-2.17^{b}$ | −0.97 *to* 0.79 | 5 | 3 |
| 09:00–11:00 | $-2.16 \pm 0.49^{a,f}$ | $-0.10 \pm 0.85$ | $-1.38$ *to* $-2.94^{b}$ | −1.15 *to* 0.96 | 4 | 3 |
| 11:00–13:00 | $-2.13 \pm 1.11^{a,f}$ | $0.11 \pm 0.17$ | $-1.08$ *to* $-3.18^{b}$ | −0.16 *to* 0.38 | 3 | 5 |
| 13:00–15:00 | $-2.92 \pm 0.87^{a,c,f}$ | $-0.12 \pm 0.55$ | $-1.84$ *to* $-4.00^{b}$ | −0.70 *to* 0.45 | 5 | 4 |
| 15:00–17:00 | $-0.21 \pm 1.19^{d,f}$ | $0.12 \pm 0.45$ | 1.04 *to* −1.46 | −0.50 *to* 0.84 | 6 | 6 |
| 17:00–19:00 | $1.39 \pm 0.50^{a,d,e}$ | $-0.15 \pm 0.44$ | 2.01 *to* $0.77^{b}$ | −1.01 *to* 1.06 | 5 | 4 |
| 19:00–21:00 | $0.58 \pm 0.88^{d,f}$ | $0.15 \pm 0.53$ | 1.98 *to* −0.82 | −0.72 *to* 1.27 | 4 | 4 |
| 21:00–23:00 | $0.65 \pm 0.88^{a,d}$ | $-0.58 \pm 0.21$ | 2.54 *to* −0.24 | $-0.85$ *to* $-0.19^{b}$ | 2 | 4 |
| 23:00–01:00 | $0.20 \pm 0.91^{d,f}$ | $-0.28 \pm 0.24$ | 2.46 *to* −2.06 | −1.17 *to* 0.30 | 3 | 4 |

[a] Phase shifts significantly different from control injections ($p < 0.05$, two-tailed t-test)
[b] Phase shifts significantly different from zero as judged by the 95 % confidence interval (CI; see Materials and methods)
[c] Phase delay significantly different from orcokinin-dependent phase shifts at other circadian times ([d]) ($p < 0.05$, two-tailed t-test)
[e] Phase advance significantly different from orcokinin-dependent phase shifts at other circadian times ([f]) ($p < 0.05$, two-tailed t-test)

induced phase shifts at CT 21–23 h were significantly different from control injections, but not from zero (Table 1, Fig. 6). Orcokinin-induced phase shifts at CT 13–15 h were not significantly different from orcokinin-induced phase shifts from CT 9–13 h, but from all other orcokinin-induced phase shifts. Similarly, orcokinin-induced phase shifts between CT 17 h and CT 19 h were not significantly different from orcokinin-induced phase shifts at CT 21–23 h and CT 1–3 h.

## Dose dependency of orcokinin-induced phase shifts

The orcokinin-dependent phase shifts at CT 13–15 h where positively correlated with the dose of orcokinin-injections (Fig. 7). The phase delays decreased with decreasing amounts of injected peptide. Significant phase delays were caused by injection of 150 fmol ($-2.92 \pm 0.81$ h, mean ± S.D., CI = [−1.84; −4.00], $n = 5$) and 1.5 x $10^{-2}$ fmol ($-1.70 \pm 0.99$ h, mean ± S.D., CI = [−2.73; −0.66], $n = 6$) orcokinin. Phase shifts induced by injections of 1.5 x $10^{-6}$ fmol orcokinin ($-0.32 \pm 0.38$ h, mean ± S.D., CI = [−0.64; 0.00], $n = 8$) were neither significantly different from zero nor from control injections (Fig. 7).

## Effects of orcokinin injections on the period of the circadian locomotor rhythm

We did not find significant changes in the free running periods at any CT before and after injection within the saline or peptide injected groups, nor between these groups. The observed effects were always small, included both lengthening (by maximally 0.48 h) and shortening (by maximally −0.63 h) of the period, and were independent of the time of

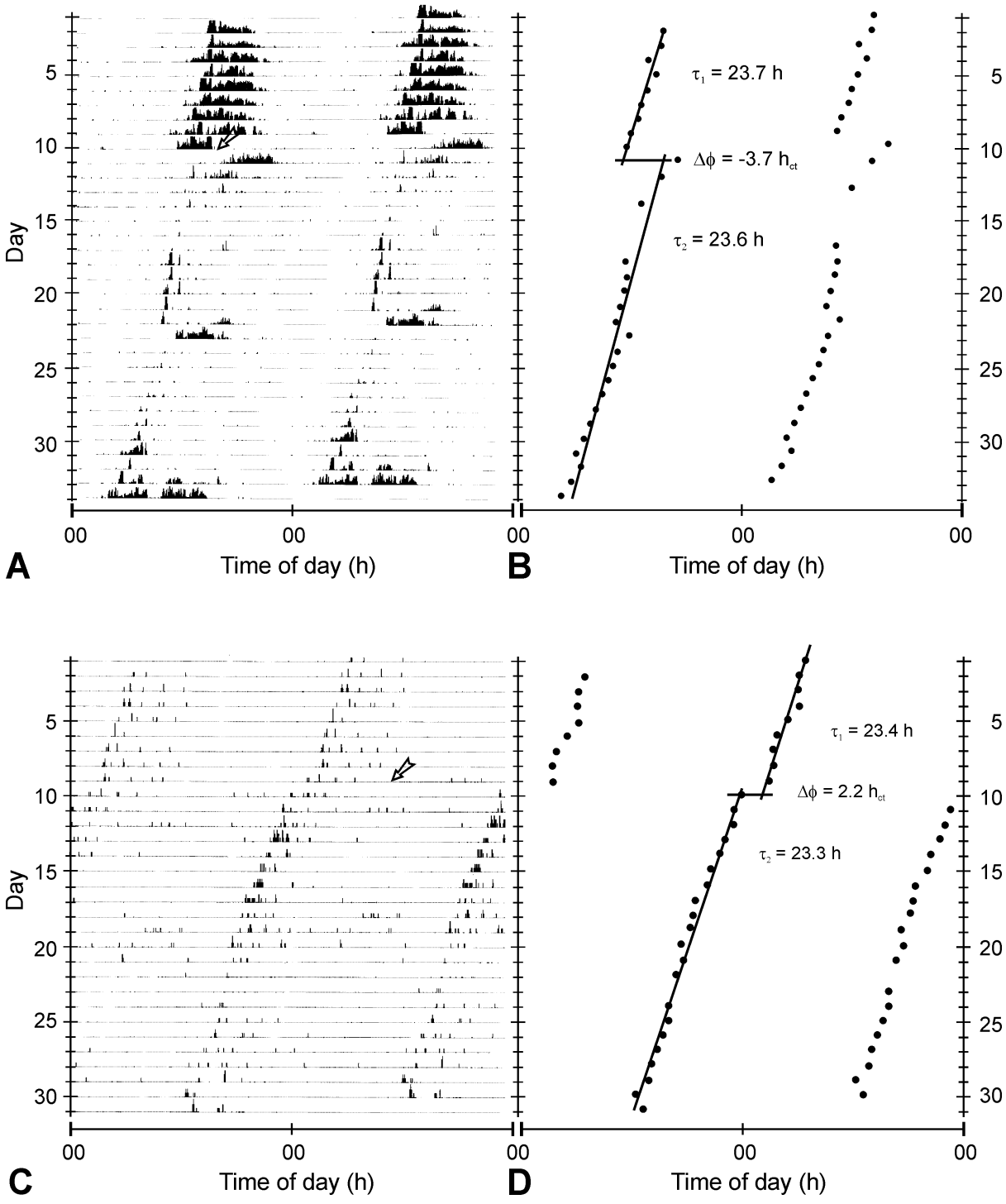


**Fig. 4A–D** Records of circadian wheel-running activity (A, C) and plots of activity onsets of cockroaches kept in constant darkness (B, D). **A, B** After injection of 150 fmol of $Asn^{13}$-orcokinin in 2 nl saline at CT 14 h of day 10 (*arrow* in A), regression analysis through consecutive activity onsets (B) revealed a phase delay $\Delta\phi$ of 3.7 circadian hours ($h_{ct}$) after the injection. **C, D** After injection at CT 18 h of day 10 (*arrow* in C), the regression analysis through consecutive activity onsets (D) revealed a phase advance $\Delta\phi$ of 2.2 $h_{ct}$.

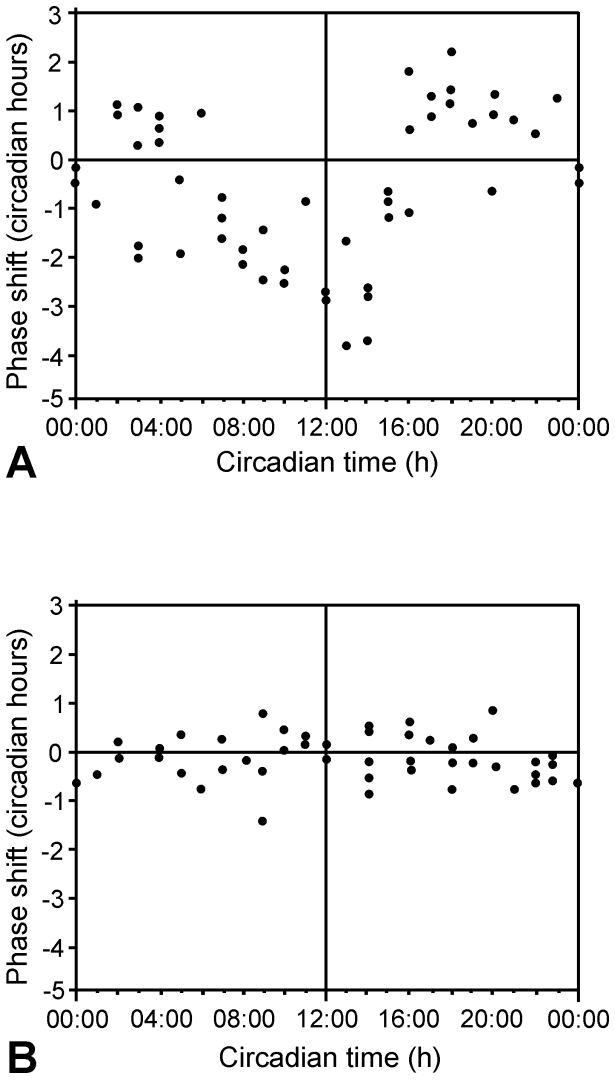


**Fig. 5A, B** Scatter plots of orcokinin- and saline-dependent phase shifts at different circadian times. **A** Orcokinin injections (150 fmol in 2 nl saline with blue food dye, $n = 50$) caused maximal phase delays during the early subjective night (up to −3.8 h at CT 13 h) and maximal phase advances during the middle of the subjective night (up to 2.2 h at CT 18 h). **B** Control injections (2 nl saline with blue food dye, $n = 46$) caused small and not significant phase shifts in both directions except a small significant phase delay at CT 22 h.

injection during the circadian cycle. On average, the mean period ($23.58 \pm 0.23$ h, mean ± S.D., $n = 96$) was altered neither by orcokinin (difference in period lengths before and after injection $0.00 \pm 0.19$ h, mean ± S.D., CI = [−0.05; 0.06], $n = 50$) nor by control injections ($-0.04 \pm 0.22$ h, mean ± S.D., CI = [−0.11; 0.02], $n = 46$).

## Discussion

The present study provides strong evidence for a physiological role of orcokinin-related peptides in the circadian system of the cockroach *L. maderae*. Injections of $Asn^{13}$-orcokinin into the vicinity of the AMe, the circadian pacemaker in the cockroach brain, resulted in phase-dependent phase shifts in circadian locomotor activity that closely match

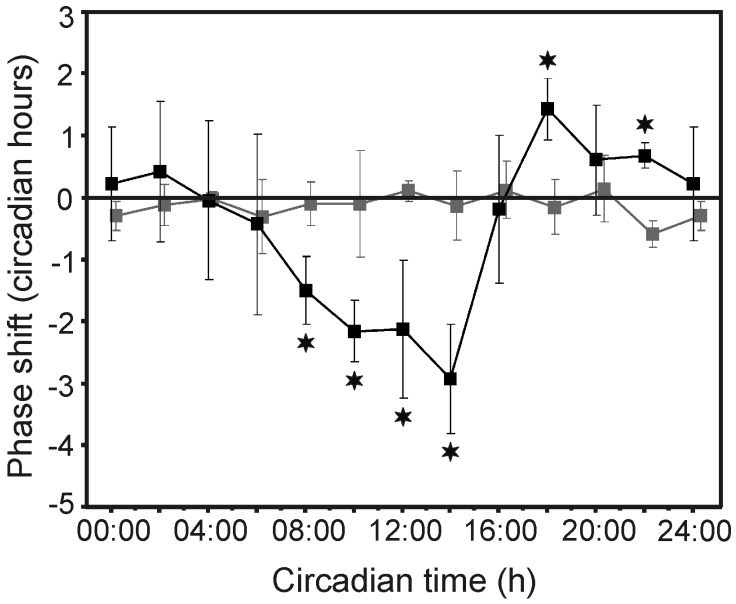


**Fig. 6** Phase response curves obtained in response to 150 fmol $Asn^{13}$-orcokinin and control injections. Data were merged into 2 h bins. Orcokinin-dependent phase shifts (*black*) and phase shifts following control injections (*gray*) are plotted. *Asterisks* indicate orcokinin-dependent phase shifts that were significantly different ($p < 0.05$) from control injections at the same circadian time.

the phase response curve obtained for light-pulses (Page and Barrett 1989). These data suggest an involvement of orcokinin-related peptides in circuits relaying photic information to the circadian pacemaker. In a previous study, we showed that up to 30 neurons distributed in five cell groups of the AMe of *L. maderae* are immunoreactive to an antiserum against $Asn^{13}$-orcokinin (Hofer and Homberg 2004). Dextran injections into one AMe combined with orcokinin immunostaining revealed that four pairs of orcokinin-ir AMe neurons provide direct connections between the bilateral pacemakers. The additional arborizations of some of these neurons in the medulla neuropil suggest that these neurons may be responsible for the light-like phase shifts seen in the peptide injection experiments.

### Orcokinin-ir neurons form a direct coupling pathway between both AMae

For a well synchronized circadian rhythm in behavior, the bilaterally distributed pacemakers in the insect brain have to be mutually coupled. In crickets, bilateral coupling of the clocks is relatively weak, but in the cockroach *L. maderae*, bilateral coupling is strong (Page et al. 1977; Wiedenmann and Loher 1984; Ushirogawa et al. 1997), and is assumed to be mediated by direct neuronal connections between the AMae in the right and left brain hemisphere (Page 1983a, b). Tracing studies showed that anterior neurons with cell bodies in two clusters termed MC I (4 cells) and MC II (35 cells) near the AMe connect both AMae directly (Reischig et al. 2004). The MC I neurons correspond to four ventral neurons (VNe), and the MC II cells are identical with the ventromedian neurons (VMNe) of Reischig and Stengl (2003b). Three of the four VNe that connect both AMae directly are PDH-ir (Reischig et al. 2004). The neurons project via the anterior (two neurons) and

posterior (one neuron) optic commissures, innervate the internodular and shell neuropils of the AMe, and send a fan of fibers along the distal surface of the medulla toward the first optic chiasm and lamina. Neurons of this morphological type were found to be unresponsive to light stimuli in intracellular recordings (Loesel and Homberg 2001). Together with the non-photic phase response curve obtained by PDH injection, Stengl and coworkers, therefore, concluded that the three PDH-ir VNe transmit phase information to the contralateral pacemaker (Petri and Stengl 2002; Reischig et al. 2004). We show here that one contralaterally projecting VNe is orcokinin-ir. Since colocalization between PDH and orcokinin occurs neither in the anterior nor in the posterior optic commissure (Hofer and Homberg 2004), this neuron has to be the forth non-PDH-ir VNe with projections to the contralateral AMe.

Page (1978, 1983a, 1983b) proposed that the bilateral optic lobe pacemakers of *L. maderae* do not only exchange phase information, but also receive entraining light signals from the contralateral eye. The pathway for contralateral light entrainment is most likely provided by the second group of commissural neurons, the VMNes. Neurons of this group have contralaterally projecting fibers in the posterior optic tract; they invade the internodular and shell neuropil of the AMe and, in contrast to VNes, have tangential arborizations in a median layer of the medulla (Reischig et al. 2004). Interestingly, neurons of this type are highly sensitive to light stimuli (Loesel and Homberg 2001). Ensemble reconstructions of orcokinin-ir VMNes (Hofer and Homberg 2004) and, in this study, tracer injections combined with immunocytochemistry clearly show that three of the 35 contralaterally projecting VMNes are orcokinin-ir. We, therefore, suggest that these neurons transmit light information to the contralateral AMe, and that release of an orcokinin-like substance is the output signal of these neurons. The neurotransmitter of the remaining 32 VMNes is not known.

The colabeling of dextran/orcokinin-immunostained fibers in the posterior but not in the anterior optic commissure suggests that the orcokinin-immunolabeled VNe, like the VMNes, traverse the brain midline via the posterior optic commissure. Since dextran transport through the anterior optic commissure was often only of low intensity (see also Reischig et al. 2004), we can, however not completely exclude the possibility, that the orcokinin-ir VNe projects via the anterior optic commissure but was not detected in the dextran injections.

### Orcokinin injections into the AMe

Microinjections of orcokinin into the vicinity of the AMe resulted in phase delays and phase advances of the circadian wheel running activity. The effects of peptide injections were dose dependent and were significantly different from control injections. The phase response curve observed after orcokinin injections was similar to the biphasic phase response curve after light-pulses (Page and Barret 1989; Fig. 8), as well as to phase response curves after GABA- and allatotropin injections (Petri et al. 2002).

Our recent immunocytochemical study revealed widespread occurrence of orcokinin-related peptides in different cell types, in addition to the commissural neurons, namely, the ventroposterior neurons (VPNe), distal frontoventral neurons (DFVNe), VNe, and

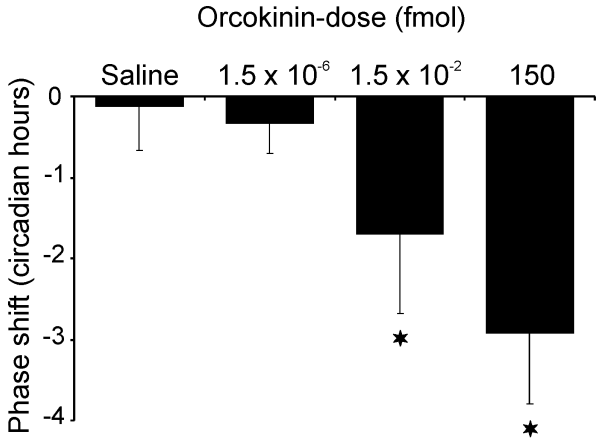


**Fig. 7** Dose dependency of $Asn^{13}$-orcokinin-induced phase shifts between CT 13 h and CT 15 h. *Bars* show phase shifts resulting from injections of saline ($n = 6$), $1.5 \times 10^{-6}$ orcokinin ($n = 6$), $1.5 \times 10^{-2}$ orcokinin ($n = 8$), and 150 fmol of orcokinin ($n = 5$) in 2 nl saline. *Stars* indicate orcokinin doses that induced phase shifts significantly different from control injections ($p < 0.05$).

median neurons (MNe) of the AMe (Hofer and Homberg 2004; for nomenclature see Reischig and Stengl 2003b). Therefore, we suggest that orcokinin serves a multitude of functions in the circadian system, possibly including a role in light entrainment, output pathways, and internal synchronization.

Interestingly, the maximal phase advance observed after orcokinin injection was not as high as that observed for GABA- and allatotropin injections. Furthermore, the peaks of the orcokinin-induced phase response curve were broader (with respect to the range of significant phase shifts) than the peak phase shifts after light-pulses, GABA-, allatotropin-, and PDH injections (Page and Barett 1989; Petri and Stengl 1997; Petri et al. 2002). These differences might be explained as follows: The AMae appear to be connected by two types of orcokinin-ir neurons, which are part of functionally different circadian coupling pathways: a pathway transmitting phase information (one orcokinin-ir VNe) and a pathway transmitting light information (three orcokinin-ir VMNe). Phase information appears to be carried by PDH-ir VNe, and if the orcokinin-ir VNe plays a similar role, a mixture of light-like (three neurons) and non-light-like (one neuron) phase responses should occur after orcokinin injections, which would then lead to flatter and broader peaks in the phase response curve compared to those observed after light-pulses, GABA or allatotropin injections (Page and Barett 1989; Petri et al. 2002). This assumption is underlined by the fact that orcokinin immunoreactivity occurs in five of the six morphologically distinguishable and obviously also functionally divergent neuron groups associated with the AMe. Nevertheless, after injection of orcokinin, the biphasic light-like phase response effects appear to dominate over an additionally expected overlapping non-photic phase response curve.

### The role of orcokinin in the circadian pacemaker of the cockroach *Leucophaea maderae*

Combining the results of this work with previous immunocytochemical studies (Hofer et al. 2004; Hofer and Homberg 2004), we propose that orcokinin has following functions in

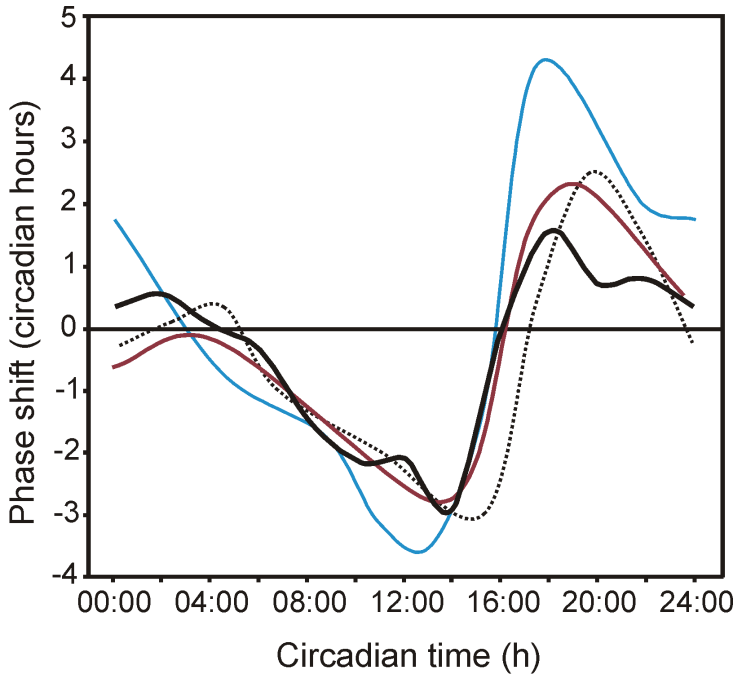


**Fig. 8** Smoothed phase response curves for injections of 150 fmol orcokinin (*black*) compared with the phase response curve for 6 h light-pulses (*blue*; Page and Barret 1989), GABA (*red*) and allatotropin (*dotted line*) injections (Petri et al. 2002).

individual AMe neurons: 1. Orcokinin-ir neurons (VMNe, VPNe and MNe) could play an important role in the light entrainment pathway; 2. Orcokinin-ir neurons (VNe) could form output pathways to different effectors of the clock; 3. Orcokinin-ir neurons (VNe) may transmit coupling information to the contralateral AMe. In summary, our work supports earlier studies suggesting that orcokinin plays multiple roles in the cockroach circadian system (Hofer et al. 2004; Hofer and Homberg 2004), and presents the first direct evidence of a physiological function of this peptide in insects.

## Acknowledgements

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## References

Bungart D, Dircksen H, Keller R (1994) Quantitative determination and distribution of the myotropic neuropeptide orcokinin in the nervous system of astacidean crustaceans. Peptides 15:393–400

Enright JT (1965) The search for rhythmicity in biological time series. J Theor Biol 8:426–468

Helfrich-Förster C (2003) The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster*. Microsc Res Tech 62:94–102

Hofer S, Homberg U (2004) Orcokinin immunoreactivity in the accessory medulla of the cockroach *Leucophaea maderae*. Cell Tissue Res (in preparation)

Hofer S, Dircksen H, Homberg U (2004) Comparative distribution of orcokinin immunostaining in the brain of selected dicondylian insects. J Comp Neurol (in revision)

Homberg U, Reischig T, Stengl M (2003) Neural organization of the circadian system of the cockroach *Leucophaea maderae*. Chronobiol Int 20:577–591

Huybrechts J, Nusbaum MP, Bosch LV, Baggerman G, De Loof A, Schoofs L (2003) Neuropeptidomic analysis of the brain and thoracic ganglion from the Jonah crab, *Cancer borealis*. Biochem Biophys Res Commun 308:535–544

Loesel R, Homberg U (2001) Anatomy and physiology of neurons with processes in the accessory medulla of the cockroach *Leucophaea maderae*. J Comp Neurol 439:193–207

Nishiitsutsuji-Uwo J, Pittendrigh CS (1968) Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? Z Vgl Physiol 58:14–46

Page TL (1978) Interactions between bilaterally paired components of the cockroach circadian system. J Comp Physiol A 24:225–236

Page TL (1981) Effects of localized low-temperature pulses on the cockroach circadian pacemaker. Am J Physiol 240:144–150

Page TL (1983a) Regeneration of the optic tracts and circadian pacemaker activity in the cockroach *Leucophaea maderae*. J Comp Physiol A 152:231–240

Page TL (1983b) Effects of optic-tract regeneration on internal coupling in the circadian system of the cockroach. J Comp Physiol 153:353–363

Page TL, Barrett RK (1989) Effects of light on circadian pacemaker development. II. Responses to light. J Comp Physiol A 165:41–49

Page TL, Caldarola PC, Pittendrigh CS (1977) Mutual entrainment of bilaterally distributed circadian pacemakers. Proc Natl Acad Sci USA 74:1277–1281

Petri B, Homberg U, Loesel R, Stengl M (2002) Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. J Exp Biol 205:1459–1469

Petri B, Stengl M (1997) Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. J Neurosci 17:4087–4093

Petri B, Stengl M, Würden S, Homberg U (1995) Immunocytochemical characterization of the accessory medulla in the cockroach *Leucophaea maderae*. Cell Tissue Res 282:3–19

Reischig T (2003) Identification and characterisation of the circadian pacemaker of the cockroach *Leucophaea maderae*. PhD-thesis, University of Marburg, Germany

Reischig T, Stengl M (1996) Morphology and pigment-dispersing hormone immunocytochemistry of the accessory medulla, the presumptive circadian pacemaker of the cockroach *Leucophaea maderae*: a light- and electron-microscopic study. Cell Tissue Res 285:305–319

Reischig T, Stengl M (2002) Optic lobe commissures in a three-dimensional brain model of the cockroach *Leucophaea maderae*: a search for the circadian coupling pathways. J Comp Neurol 443:388–400

Reischig T, Stengl M (2003a) Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). J Exp Biol 206:1877–1886

Reischig T, Stengl M (2003b) Ultrastructure of pigment-dispersing hormone-immunoreactive neurons in a three-dimensional model of the accessory medulla of the cockroach (*Leucophaea maderae*). Cell Tissue Res 314:421–435

Reischig T, Petri B, Stengl M (2004) Pigment-dispersing hormone (PDH)-immunoreactive neurons form a direct coupling pathway between the bilaterally symmetric circadian pacemakers of the cockroach *Leucophaea maderae*. Cell Tissue Res (in press)

Roberts SK (1965) Photoreception and entrainment of cockroach activity rhythms. Science 148:958–959

Roenneberg T, Morse D (1993) Two circadian oscillators in one cell. Nature 362:362–364

Skiebe P, Dreger M, Meseke M, Evers JF, Hucho F (2002) Identification of orcokinins in single neurons in the stomatogastric nervous system of the crayfish, *Cherax destructor*. J Comp Neurol 444:245–259

Sokolove PG, Bushell WN (1978) The chi square periodogram: its utility for analysis of circadian rhythms. J Theor Biol 72:131–160

Stangier J, Hilbich C, Burdzik S, Keller R (1992) Orcokinin: a novel myotropic peptide from the nervous system of the crayfish, *Orconectes limosus*. Peptides 13:859–864

Ushirogawa H, Abe Y, Tomioka K (1997) Circadian locomotor rhythms in the cricket, *Gryllodes sigillatus*. II. Interactions between bilaterally paired circadian pacemakers. Zool Sci 14:729–736

Wiedenmann G (1977) Two activity peaks in the circadian rhythm of the cockroach *Leucophaea maderae*. J Interdiscipl Cycle Res 8:378–383

Wiedenmann G, Loher W (1984) Circadian control of singing in crickets: two different pacemakers for early-evening and before-dawn activity. J Insect Physiol 30:145–151

Yasuda-Kamatani Y, Yasuda A (2000) Identification of orcokinin gene-related peptides in the brain of the crayfish *Procambarus clarkii* by the combination of MALDI-TOF and on-line capillary HPLC/Q-TOF mass spectrometries and molecular cloning. Gen Comp Endocrinol 118:161–172

Chapter IV. Extraretinal photoreceptors in the cockroach *Leucophaea maderae*: involvement of cryptochrome and UV-opsin in light entrainment?

# Chapter IV

# Extraretinal Photoreceptors in the Cockroach *Leucophaea maderae*: Involvement of Cryptochrome and UV-Opsin in Light Entrainment?

Sabine Hofer

## Abstract

Many vertebrates and invertebrates have supplementary retinal and extraretinal photoreceptors that are implicated in entrainment of circadian rhythms. In the cockroach *Leucophaea maderae*, two putative extraocular photoreceptor organs near the lamina (lamina organ) and near the lobula (lobula organ) were recently described. Cells of both organs are characterized by rhabdom-like microvilli, and both organs showed immunoreactivity to an antiserum against the blue-light photoreceptor molecule cryptochrome (CRY). CRY immunostaining in the lamina and lobula organs of the cockroach was confirmed in this study using two different antisera, but in contrast to former studies, CRY immunoreactivity was also detected in various other brain areas including the accessory medulla, the putative circadian pacemaker center in *L. maderae*. Western blot analyses demonstrated a daily cycle of CRY in total brain extracts of the cockroach as has been shown for *Drosophila*. In addition, I investigated whether opsin-based photoreceptors are also present in these putative photoreceptor organs. A variety of antisera against opsins and arrestins were tested immunocytochemically. An antiserum against *Manduca sexta* UV-opsin resulted in staining of the lamina and lobula organs, but the staining pattern differed from that of CRY immunostaining. The evidences for the presence of two photoreceptors known to be sensitive for short wavelength light underline the assumption that the lamina and lobula organs of the cockroach are extraretinal photoreceptor organs with a maximum sensitivity in the short wavelengths of the light spectrum.

## Introduction

It has long been known that insects have two types of photosensory organs. Photic entrainment of circadian rhythms can occur either through retinal/ocular (compound eyes and ocelli) or via extraretinal/extraocular photoreceptors (in the brain or elsewhere). Although the retinal phototransduction process is well understood in insects (Zuker 1996;

Hardie 2003), little is known about the molecular basis of extraretinal photoreceptors in these species. Extraocular photoreceptors lack the capacity for complex image analysis but often are implicated in the entrainment of circadian rhythms and the timing of photoperiodic response (Helfrich-Förster et al. 2001; Fleissner and Fleissner 2003). The existence of such extraocular photoreceptors has been described in many invertebrate and vertebrate species (Adler 1976; Truman 1976; Bennett 1979; Page 1982; Fleissner et al. 1993; Fleissner and Fleissner 2003). In the fruitfly, a putative extraocular photoreceptor, the so called Hofbauer-Buchner eyelet (H-B eyelet) lies close to the compound eyes (Hofbauer and Buchner 1989; Helfrich-Förster et al. 2002). It innervates a small neuropil at the edge of the medulla, the accessory medulla (AMe), which is suggested to constitute the pacemaker center controlling circadian behavior (Helfrich-Förster et al. 1998; Helfrich-Förster 2004). *Drosophila* uses several light input pathways for entrainment of the circadian clock. In addition to the compound eyes and the H-B eyelet, *Drosophila* engages neurons containing cryptochrome (CRY), a blue light photoreceptor and probably the ocelli for circadian entrainment (Rieger et al. 2003). Only after complete elimination of all known photoreceptors the fly is blind with respect to circadian light entrainment (Helfrich-Förster et al. 2001).

Extraocular photoreceptor organs in the optic lobe have been found in a variety of hemi- and holometabolic insect orders, such as beetles, hymenopterans, and orthopterans (Fleissner and Fleissner 2003). These organs are different from ocular photoreceptors known so far. They lack a dioptric apparatus and the photoreceptor cells are not regularly arranged. In the optic lobes of beetles and cockroaches, two putative extraocular photoreceptor organs have been discovered, the lamina and lobula organs (Fleissner and Fleissner 1992; Fleissner et al. 1993; Fleissner et al. 2001). The lamina organ is an elongated structure distal to the first optic chiasm, at the anterior edge of the lamina. The lobula organ is situated at the anterior distal surface of the lobula. Both organs lack shielding pigments. The diameter of the lamina organ is close to 50 $\mu$m with a length of about 400 $\mu$m, while the lobula organ measures 40 $\mu$m in diameter and is about 150 $\mu$m long (Fleissner et al. 2001). The arrangement of cell bodies around the organs, as well as the rhabdom-like membrane specializations suggest that the lamina and lobula organs serve a photoreceptive role. Frisch et al. (1996) showed in the beetle, that fiber fascicles connect both organs with the accessory medulla. In a more recent study, Fleissner et al. (2001) described in the cockroach cryptochrome immunostaining using an antiserum against *Arabidopsis* CRY II.

To further characterize the identity of cryptochrome in the optic lobe of the cockroach, antisera against *Arabidopsis*- and *Drosophila* CRY were used in immunohistochemical experiments and in Western blot analysis of cockroach brains. Western blots showed that CRY protein levels in the brain were more abundant during late night than at other Zeitgeber times. Because of structural similarities of the lamina and lobula organs to known photoreceptor organs in other insects, including the fruitfly (Yasujama and Meinertzhagen 1999), I additionally tested different antisera against opsins and arrestins and found immunoreactivity to *M. sexta* UV-opsin in the lamina organ.

## Materials and methods

### Animals

Adult cockroaches (*Leucophaea maderae*) and flies (*Drosophila melanogaster*) were taken from crowded colonies at the University of Marburg. Animals were reared under 12:12 hours light-dark (LD) photoperiod, at about 60 % relative humidity, and a temperature of 28 °C.

### Immunocytochemistry

Immunocytochemical staining for cryptochrome (CRY) was performed by use of the indirect peroxidase-antiperoxidase (PAP) technique of Sternberger (1979). A polyclonal antiserum against *Arabidopsis* CRY II, raised in rabbits (Hoffmann et al. 1996), was donated by Prof. A. Batschauer; a rabbit antiserum against *Drososphila* CRY was donated by Dr. R. Stanewsky. The cockroaches were anesthetized by cooling to 4 °C and decapitated. The brains were dissected and fixed for 4 hours or overnight in 4 % paraformaldehyde/7.5 % saturated picric acid in sodium phosphate buffer (0.1 M, pH 7.4) at room temperature. Then, the brains were dehydrated through a graded series of aqueous ethanol solutions and toluene, and embedded in Paraplast Plus (Monoject Scientific, St. Louis, MO, USA). Serial sections at 10 $\mu$m were cut with a rotary microtome. The following incubation steps were carried out on slide. The sections were preincubated in Tris-buffered saline (TBS; 0.1 M Tris-HCL/0.3 M NaCl, pH 7.4) containing 0.5 % Triton X-100 (TrX) and 5 % normal goat serum (NGS; DAKO, Hamburg, Germany). Primary antisera, anti-*Arabidopsis*-CRY II and anti-*Drosophila*-CRY, were diluted at 1:1000 in TBS containing 0.5 % TrX and 1 % NGS. Secondary antiserum (goat anti rabbit; Sigma, Deisenhofen, Germany) was used at a dilution of 1:40, and rabbit PAP (DAKO, Hamburg, Germany) at a dilution of 1:300 in TBS containing 0.5 % TrX and 1 % NGS. Afterwards, the sections were thoroughly washed in sodium phosphate buffer, stained with 0.03 % 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Deisenhofen, Germany) and 0.025 % $H_2O_2$ in sodium phosphate buffer (0.1 M, pH 7.4) for 20–30 minutes.

For immunocytochemical staining of UV-opsin (in cooperation with Dr. G. Fleissner; *Manduca* opsin 2 = UV-sensitive opsin) the brains were sectioned using a vibrating blade microtome (Leica, Nussloch, Germany) in frontal plane at 30 $\mu$m thickness. The free-floating sections were washed in TBS containing 0.1 % TrX and were preincubated in TBS with 0.5 % TrX and 5 % NGS. Primary antiserum, anti-UV-opsin, was diluted at 1:200 in TBS containing 0.5 % TrX and 1 % NGS for 18–20 h at room temperature. As secondary antibody, a Cy3-conjugated goat anti rabbit antiserum (GAR; Dianova Hamburg, Germany, diluted 1:300) in TBS containing 0.5 % TrX and 1 % NGS was applied for 1 h. After that, the sections were washed as described, and then mounted on chromalum/gelatine coated microscope slides. In addition, opsin-immunostaining was performed by use of the indirect PAP technique, as described above.

A variety of antisera against other opsins were tested: Anti-bovine-rod opsin (rho4D2), anti-bovine-blue opsin (JH 455) and anti-bovine-green opsin (JH 492) in cooperation with Dr. G. Fleissner (Goethe University Frankfurt; antisera were provided by Prof. Dr. Peichl,

MPI-Hirnforschung, Frankfurt; see Peichl et al. 2004; for specification of the antisera, see Hicks and Molday 1986 [rho4D2] and Wang et al. 1992 and Chiu and Nathans 1994 [JH 455 and JH 492]). The antisera were used at dilutions ranging from 1:500 to 1:5000. Moreover, antisera against several *Drosophila* opsins were examined: Anti-Rh1, anti-Rh3, anti-Rh4, anti-Rh5 and anti-Rh6 (courtesy of Steve Britt, University of Colorado; Chou et al. 1999). Further, an anti-PglRh4 (against a *Papilio glaucus* long wavelength rhodopsin, provided by Dr. A. Briscoe, Dept Ecology and Evolutionary Biology, Irvine; Briscoe 2000) was tested. The antisera were used at dilutions between 1:10–1:50.

In addition, different antisera raised against proteins involved in the phototransduction pathway were tested: An anti-$G_{\alpha q/11}$ (Santa Cruz Biotechnologie, Inc., Heidelberg), and different monoclonal antisera against bovine arrestin: anti-MabF4C1, anti-MabC10C10, anti-MabD9F2, provided by Dr. L. Donoso (Wills Eye Hospital, Philadelphia; Knopse et al. 1988; Lieb et al. 1991). The immunostaining protocols were performed as described above. The antisera were used at dilutions ranging from 1:200 to 1:2000.

### Preinkubation of *Drosophila* CRY antiserum with *Drosophila* embryos

To reduce background in immunostaining using the *Drosophila* CRY antiserum, we preadsorbed the antiserum with wildtype fly embryos (provided by Dr. Renkawitz-Pohl, University of Marburg). The decorionized embryos were incubated for 1 h with the antiserum. Following this treatment and after centrifugation, only the supernatant was used.

### Evaluation and visualization

Microscope images were captured with a Zeiss microscope equipped with a 2 megapixel digital camera (Polaroid, Cambridge, MA, USA). Contrast and brightness of the micrographs were optimized in Adobe Photoshop 6.0. Positional information is given with respect to the body axes of the animals.

### Western blot analysis

For immunoblot analysis, 3 $\mu$g of total protein extracts from whole brains, optic lobes, and protocerebra of the cockroach, and of *Drosophila* heads were separated by sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and incubated with the two anti CRY antisera 1:2000–1:4000 (*Drosophila* CRY and *Arabidopsis* CRY II).

For sample preparation, cockroach brains were dissected at specific Zeitgeber times and collected in liquid nitrogen. After completion of the time series with 2–4 brains per Zeitgeber time, all frozen samples were processed simultaneously. The frozen brains were homogenized in extraction buffer (SST 0.01 M, pH 7.4; $Na_3VO_4$, 200 mM; phenylmethylsulfonyl flouride (PMSF) 100 mM; SDS 1 %) using a rotating micro-blade (Ultra-Turrax). The extracts were incubated for 10 min at 95 °C, centrifuged, and only the supernatants

were used for further analyses. The protein concentration was determined photometrically after Bradford (1979). For Western blotting, each lane of the 7.5 % polyacrylamide/SDS mini gels was loaded with equal amounts of protein from the brain extracts (3 $\mu$g) in buffer (50 mM Tris-HCl, pH 6.8; glycerol 10 %; bromphenol blue 0.1 %; mercaptoethanol 5 %; 0.1M dithiothreitol [DTT]; SDS 2 %). After electrophoresis, gels were electroblotted onto nitrocellulose using a semidry blotting chamber. The blots were incubated for 1 h in blocking solution (phosphate buffered saline [PBS] pH 7.5 with 5 % milk powder and 0.1 % Tween 20). The antisera were applied at 1:2000–1:4000 over night at 4 ℃. Subsequently, the blots were washed in buffer (PBS, Tween 20, 0.05 %; Triton X-100, 1 %; SDS 0.1 %) and incubated for 1 h with peroxidase-conjugated goat anti-rabbit antiserum at a dilution of 1:5000. Visualization was performed with a chemoluminescence kit (Supersignal, Pierce, Rockford IL). All blot experiments were repeated at least three times.

## Results

### Cryptochrome- and opsin-like immunostaining on brain sections

The pattern of immunostaining obtained with the antiserum against *Arabidopsis* CRY II was identical to the staining pattern described by Fleissner et al. (2001). Immunostaining extended as a narrow band over nearly the entire dorsoventral extension on the proximal edge of the lamina organ, and the cell bodies were free of immunostaining. As described by Fleissner et al. (2001), staining was concentrated to the central core of the organ. In contrast, immunostaining of the lobula organ was less intense and in some cases not visible (not shown). The central brain was devoid of immunolabeled fibers (not shown). A very similar pattern of immunostaining was observed with the antiserum against *Drosophila* CRY, with the exception of additional dense immunostaining in the lamina (Fig. 1A, B). Interestingly, after preadsorption with *Drosophila* embryos, the *Drosophila* CRY antiserum revealed additional dense staining in the central brain that was not present when using the non-preadsorbed antiserum (Fig. 1C, D). The mushroom bodies, the antennal lobes, and the antennal nerves were prominently stained (Fig. 1D). In contrast to homogenous immunostaining in the mushroom body, fibrous staining appeared in the antennal lobe, which may originate from local interneurons of the antennal lobe connecting different glomeruli. Also in the antennal nerve, fine stained fibers appeared.

Many different opsin and arrestin antisera were tested, but only an antiserum against *M. sexta* UV-opsin revealed positive staining in the brain. Dense UV-opsin immunostaining appeared in the lamina as well as in the lobula organs (Fig. 2A, B). In contrast to the anti-CRY staining, the anti-UV-opsin staining had a more inhomogeneous appearance, and, in case of the lamina organ, the stained band was broader. Although the cryptochrome and UV-opsin immunostainings were apparently different, it was impossible to discriminate between the immunostained structures in the lamina and lobula organs. Other brain neuropils and brain areas were free of immunolabeling. No immunostaining appeared in the lamina and lobula organs with any other of the tested antisera.

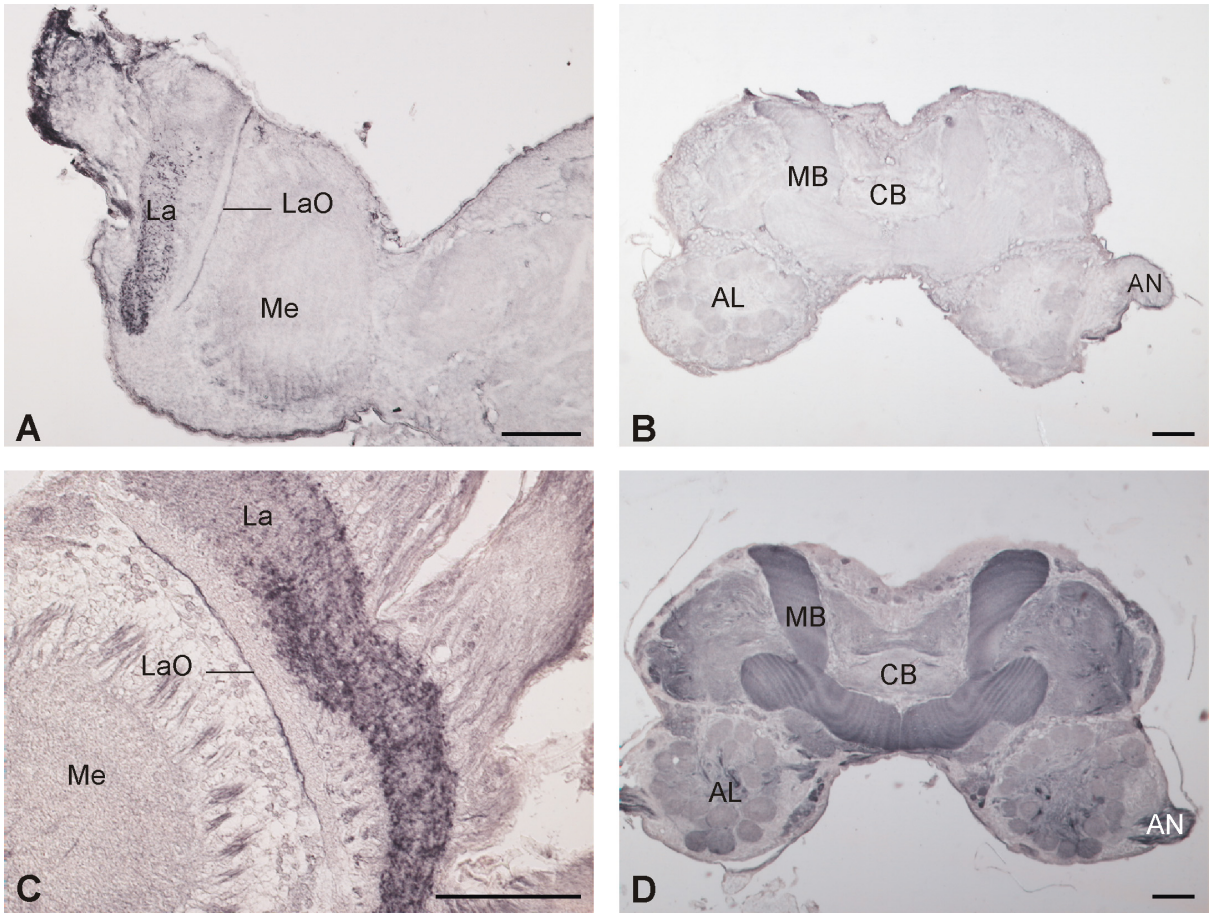


**Fig. 1A–D** Immunostaining with an antiserum against *Drosophila* CRY in the brain of the cockroach *L. maderae*. **A** Frontal paraffin section through the optic lobe of the cockroach after staining with non-preadsorbed anti-*Drosophila* CRY. Dense staining appeared in the lamina (*La*) and lamina organ (*LaO*). *Me* medulla. **B** The central brain is devoid of staining. C, D Immunostaining with the preadsorbed *Drosophila* anti-CRY antiserum. **C** The lamina (*La*) and the lamina organ (*LaO*) show dense anti-CRY immunoreactivity. *Me* medulla. **D** In the central brain the antennal lobe (*AL*), the antennal nerves (*AN*), the central body (*CB*), the mushroom bodies (*MB*) and surrounding neuropils were immunostained. *Scale bars* 100 $\mu$m

### Western blot analysis of cryptochrome-like immunoreactivity

For Western blot analysis, the *Arabidopsis* CRY II antiserum as well as the preadsorbed *Drosophila* CRY antiserum were used to analyse extracts from whole brains, separated optic lobes, and protocerebra of the cockroach. The results obtained with both antisera were very similar. To analyze daily fluctuations in the amount of protein, whole cockroach brains were collected at specific Zeitgeber times, and the protein extracts were separated by Western blot analysis. With both antisera, a single protein band around 65 kDa was detected, similar to the molecular weight reported by Fleissner et al. (2001). Some variation in CRY-related protein levels occurred, but in all experiments the protein was always more abundant during the late night than at other Zeitgeber times (Fig. 3A). As described by Emery et al. (1998) for *Drosophila*, an additional thin fuzzy band at about 70 kDa was sometimes visible in the cockroach extracts. In contrast to the immunostaining experiments of Fleissner et al. (2001), which did not show anti-CRY staining in

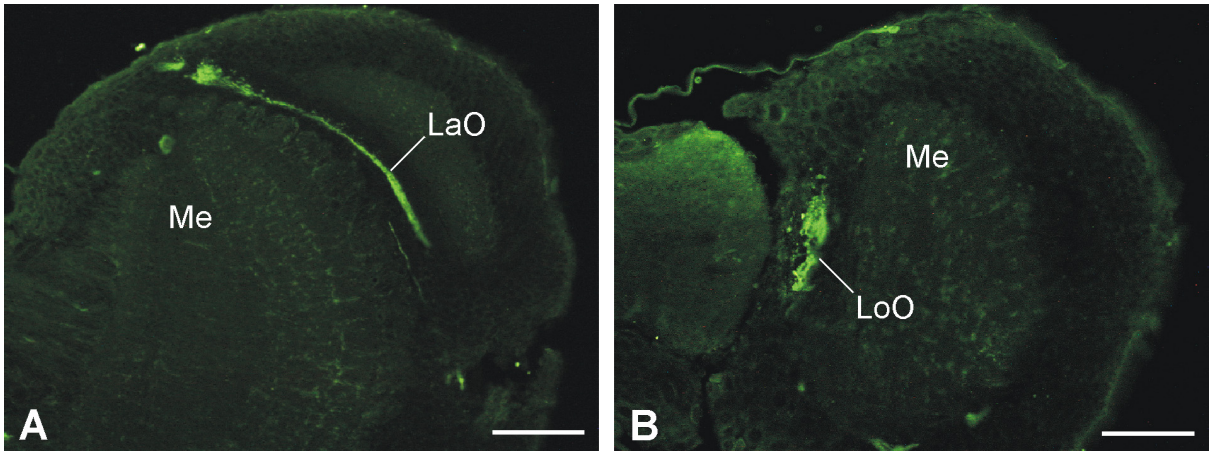


**Fig. 2A, B** Immunostaining in the cockroach brain with an antiserum against bovine UV-opsin, frontal paraffin sections. **A** The lamina organ (*LaO*) showed prominent immunofluorescence. The staining pattern had an inhomogeneous appearance. *Me* medulla. **B** Immunostaining of the lobula organ (*LoO*). *Me* medulla. *Scale bars* 100 $\mu$m

other brain areas than the lamina and lobula organs, I observed a putative CRY also in extracts of accessory medullae (AMe; Fig. 3A), of whole optic lobes, and of protocerebra without optic lobes (Fig. 3B, C). These results are consistent with immunostaining using the preadsorbed anti-*Drosophila* antiserum, which was also detected in various areas of the brain (see above). A comparison of cockroach brain extracts with *Drosophila* head extracts showed that the protein recognized by the antisera against *Arabidopsis* CRY II and *Drosophila* CRY was nearly of the same molecular weight (Fig. 3B, C).

## Discussion

Extraretinal photoreceptors are widespread among insects and play a role in the photoperiodic control of development and in entrainment of circadian rhythms (Truman 1976). The effects of light on the daily regulation of brain neuroendocrine activity are mediated by retinal- and extraretinal photoreceptors (Truman 1976). By means of light- and electron microscopic techniques, two putative extraretinal photoreceptor organs, the lamina and lobula organs, were described in the cockroach *Leucophaea maderae* (Fleissner et al. 2001). Both organs contain multiple receptor cells forming a common rhabdom-like microvillar complex in the center of the organs. In beetles, microvillar cells of corresponding structures are immunoreactive with antibodies against proteins of the phototransduction cascade (Fleissner et al. 1993; Fleissner and Fleissner 2003), thus indicating that these organs are functional photoreceptor organs. To date cockroaches are the only species, in which CRY immunoreactivity was detected in these organs. CRY immunoreactivity is restricted to a laminated structure in the lumen of the lamina and lobula organs. The aim of this study was to investigate possible daily oscillations of CRY and to provide further evidence for a photoreceptor function of the lamina and lobula organs of *L. maderae* through immunocytochemical detection of other molecules involved in phototransduction.

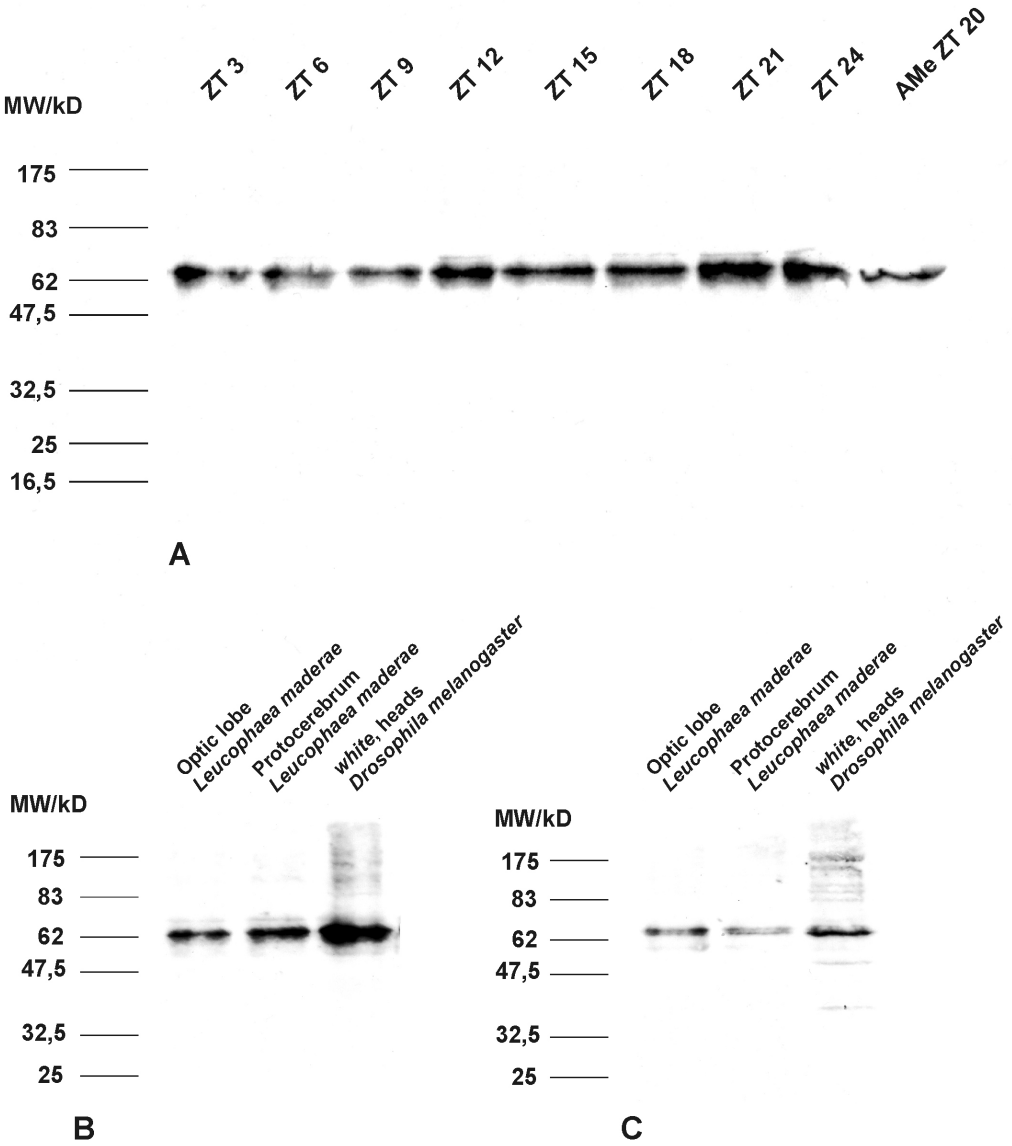


**Fig. 3A–C** CRY protein levels under LD conditions. Immunoblot detection of putative CRY in brain extracts of cockroaches and fruitflies. **A** Three micrograms of total protein extracts from heads and accessory medulla of *L. maderae* were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to membranes and probed with anti-*Arabidopsis* CRY II antiserum. A cycling of a CRY-like band (at approximately 65 kDa) in brain extracts is visible with maximum protein levels around ZT 21–24. Putative CRY protein is also present in the accessory medulla (AMe). **B, C** Three micrograms of total protein extracts from optic lobes and protocerebra of *L. maderae* and heads of *D. melanogaster* were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In all cases a putative CRY (band at approximately 65 kDa) protein was detected with anti-*Arabidopsis* CRY II antiserum (B) and anti-*Drosophila* CRY antiserum (preadsorbed; C).

## Cryptochrome immunostaining in the lamina and lobula organs

CRY is up to now the best candidate for an additional extraocular photoreceptor molecules in insects. In *D. melanogaster*, CRY is expressed in clock neurons of the brain, called lateral neurons (LN; Stanewsky 2002). These neurons play an important role in generating circadian rhythms in *Drosophila* by a transcriptional/translational feedback loop, in

which two negative factors PERIOD (PER) and TIMELESS (TIM) regulate their own transcription by interacting with two positive factors CLOCK and CYCLE (Allada et al. 1998; Rutila et al. 1998; Stanewsky 2002). CRY plays an important role in the entrainment of the molecular feedback loop to LD cycles in the fly. The important clock protein TIM is degraded by light, but is not directly light-sensitive. Thus, light signals have to be transferred via a photoreceptor to TIM, and there is growing evidence that CRY is involved in this process (Emery et al. 1998; Stanewsky et al. 1998; Foster and Helfrich-Förster 2001). In CRY mutants ($Cry^b$), TIM is not degraded in the presence of light, but stays at a constant high level in the photoreceptor cells of the compound eyes (Stanewsky et al. 1998). In *Drosophila*, the latest evidence places CRY firmly into the photoentrainment pathway and raises the possibility that it acts as photoreceptor, capable of both absorbing light and transducing that information directly to the oscillator (Klarsfeld et al. 2004).

In the cockroach *L. maderae* Fleissner et al. (2001) described CRY immunoreactivity in the lamina and lobula organs. The central brain and the accessory medulla were devoid of anti-CRY staining. In contrast, with the preadsorbed *Drosophila* CRY II antiserum I found CRY immunoreactivity in the central brain by both immunocytochemistry and Western blot analysis. In addition, CRY immunoreactivity was detected in the accessory medulla by protein blot analysis. The immunostaining patterns obtained with the preadsorbed and non-preadsorbed anti-*Drosophila* CRY II antiserum differed in that unexpected way that with the preadsorbed antiserum considerably more structures were stained than with the non-preadsorbed antiserum. The reason for this is unknown, but it may be speculated that non-specific binding sites of antibody molecules of the polyclonal antiserum mutually form complexes and therefore, cannot recognize lower concentrations of the specific antigen in the tissue. This could be a reason why the non preadsorbed antiserum solely labeled the lamina and lobula organs of the cockroach *L. maderae*, where the antigen might occur at higher concentrations.

Immunostaining experiments with the preadsorbed antiserum are compatible with the results revealed by means of protein blot analyses, because both the optic lobe and the median protocerebrum showed a prominent CRY protein band. Hence, the preadsorbed *Drosophila* CRY antiserum recognized more structures in the brain than shown previously (Fleissner et al. 2001). Krishnan et al. (2001) showed that CRY contributes to oscillator function and output rhythms in the antenna during and after entrainment to LD cycles and after photic inputs are eliminated by entraining flies to temperature cycles. The authors postulated a fundamental different role for CRY in central and peripheral oscillator mechanisms, because in the antennae of *Drosophila* CRY has a photoreceptor-independent role. In the cockroach, CRY immunoreactivity also appeared in the antennae. It is, therefore, possible that also in the cockroach CRY serves different functions in central and peripheral structures of the brain.

Interestingly, CRY protein levels in the cockroach brain showed a daily rhythm with highest abundance during the late night as shown for fruitflies by Emery et al. (1998). As in the fly, CRY might be unstable in the light, which might be the reason for an accumulation of CRY levels during the night. Light mediated changes in CRY levels may be important for signal transduction mediated by CRY. However, also a circadian oscillation of CRY concentration is possible.

Although Fleissner et al. (2001) postulated an involvement of putative CRY only in the lamina and lobula organs, I suggest that CRY is not only present in putative photoreceptor structures, which would again be similar to the situation in *Drosophila* where CRY is expressed in central and peripheral tissues (Krishnan et al. 2001; Stanewsky 2003). For further study on CRY functions in the cockroach, it will be important to identify and characterize *cry* genes in the cockroach, and compare this with known *cry* sequences and CRY proteins. In pilot studies I started to clone *L. maderae cry* by genomic DNA extraction and already obtained segments of gene sequences, but complete sequencing of the gene will require additional efforts.

An unexpected novel role for CRY in light-mediated magnetoreception in birds has recently been discussed by Ritz et al. 2000 (reviewed by Wiltschko and Wiltschko 2002). The authors provide a theoretical model for a radical-pair process located in photoreceptor cells of the retina that is involved in magnetoreception. In this model, changes in orientation within the geomagnetic field cause variation in the reaction yields of radical-pair processes that in turn affect the visual transduction pathway. The model fits well to behavioral and physiological characteristics of magnetoreception in birds. Birds orient to the direction and not to the polarization of the magnetic field. They obtained absolute directional information from the inclination of the magnetic field (Wiltschko and Wiltschko 1972), which would be explainable by a radical-pair based magnetic compass. The model further requires an appropriate photoreceptor molecule coupled to the radical-pair process. The search for this photoreceptor has focused on CRY, since (i) phototransduction of CRY may engage a radical-pair process due to its homology to photolyases (reviewed in Cashmore et al. 1999) and (ii) light-dependent magnetoreception of birds works properly only in light conditions covering the short wavelength range (Wiltschko and Wiltschko 1995, 2001). Light-dependent magnetoreception has also been shown in insects like fruitflies (Phillips and Sayeed 1993) and mealworm beetles (*Tenebrio molitor*, Vácha and Soukopová 2004). Also for the fruitfly, correct orientation in the magnetic field has only been shown for short wavelength or full spectrum light. An involvement of CRY in light-dependent magnetoreception of the fruitfly might be tested with CRY-mutant strains in appropriate behavioral assays. Interestingly, diffuse ambient light is sufficient to allow magnetic compass orientation in the mealworm beetle. Since the lamina and organs of the cockroach are placed inside the head capsule, they can only be reached by diffuse light lacking all directional information. In that context and due to their immunoreactivity to anti-CRY antisera, the possibility that these organs are involved in magnetic field perception should be considered.

### UV-opsin immunostaining in the lamina and lobula organs

Although CRY might act as a photoreceptor in entrainment of circadian rhythms in the cockroach, it is, as shown for *Drosophila*, certainly not the only photoreceptor involved in photoentrainment (Foster and Helfrich-Förster 2001). Opsin-based photopigments in the compound eyes and the eyelet of *Drosophila*, likewise, contribute importantly to entrainment of the circadian system to LD cycles (Helfrich-Förster et al. 2001).

By measuring the action spectrum for entrainment of the clock by imposed photic stimuli, Mote and Black (1981) showed that the cockroach circadian system is most

sensitive to green light and less sensitive to blue light. Mote and Black (1981) as well as Roberts (1965) suggested that light entrainment of the circadian clock is mediated via the compound eyes. These experiments might easily be interpreted as evidence against a role of CRY, which normally is maximally sensitive to blue light, in light entrainment of the cockroach. However, Roberts (1965) could not completely exclude, that sensory input to the circadian system is possible by direct photostimulation of the brain. Both the lamina and the lobula organs are situated in the brain, and the head capsule is slightly permeable to light. Hence it may be that CRY, which occurs in both organs, has an additional effect to light perception of the circadian system.

Although CRY appears to be present in the lamina and lobula organs and may play a photoreceptive role, additional photoreceptive proteins appear to occur in these organs. The most prominent cell type in the lamina and lobula organs has rhabdom-like microvilli. The extracellular space around the microvilli is occluded by desmosoms, which is typical for invertebrate photoreceptors (Shaw 1978). Fleissner and Fleissner (1992) observed in beetles opsin- and arrestin immunoreactivity in these microvillar structures. This underlines the assumption that both organs, indeed, serve a photoreceptor function in beetles. In *L. maderae*, I tested several antisera against different arrestins and opsins. Only an anti-*M. sexta* UV-opsin antiserum labeled a structure in the lamina and lobula organs, respectively. However, because of the dense anti-UV-opsin immunoreactivity in both organs, I could not discriminate in which cell types the immunoreactivity occurred. The immunostaining pattern of anti-UV-opsin is apparently different to the CRY immunostaining pattern but the immunostained structures can probably only be identified with electronmicroscopic techniques. However, the differential distribution of CRY and UV-opsin immunoreactivity might be a first indication that these organs are assemblages of photoreactive cell types employing different molecular photoreceptors. In summary, these results underline the assumption that both, the lamina and the lobula organs of the cockroach, are extraretinal photoreceptors.

In future experiments, it will be important to find fiber connections of the lamina and lobula organs to other neuropils in the brain of the cockroach, in order to reveal the brain regions that process the signals of these organs. In beetles, anti-histamine antisera and anti-PERIOD antisera marked the organ's cells and their fiber connections to neighboring neuropils, including the accessory medulla (Frisch et al. 1996; Fleissner and Fleissner 2003). In addition, PER-immunoreactive projections were found via the optic tract to the dorsal lateral brain. Additional experiments will be essential to reveal the function of the lamina and lobula organs. Electronmicroscopic studies of CRY and anti-UV-opsin immunostaining should characterize the cell types that express these substances. Characterization of CRY protein and the *cry* gene are necessary to investigate the spatial and temporal expression pattern of CRY in the cockroach brain. Finally, electrophysiological recordings combined with light stimuli and subsequent single cell dye injections seem to be the most appropriate way to demonstrate a photoreceptor function of these interesting and still mysterious organs.

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## References

Adler K (1976) Extraocular photoreception in amphibians. Photophysiol 23:275–298

Allada R, White NE, So WV, Hall JC, Rosbash M (1998) A mutant *Drosophila* homolog of mammalian CLOCK disrupts circadian rhythms and transcription of period and timeless. Cell 93:791–804

Bennett MF (1979) Extraocular light receptors and circadian rhythms. In: Autrum H, editor. Handbook of sensory physiology. Vol VII, 6A. Comparative physiology and evolution of vision in invertebrates. Invertebrate photoreceptors. Springer, Berlin, pp 641–663

Bradford MM (1997) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Briscoe AD (2000) Six opsins from the butterfly *Papilio glaucus*: Molecular phylogenetic evidence for paralogous origins of red-sensitive visual pigments in insects. J Mol Evol 51:110-121

Chou WH, Huber A, Bentrop J, Schulz S, Schwab K, Chadwell LV, Paulsen R, Britt SG (1999) Patterning of the R7 and R8 photoreceptor cells of *Drosophila*: evidence for induced and default cell-fate specification. Development 126:607-616

Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679

Cashmore A, Jarillo J, Wu YJ, Liu D (1999) Cryptochromes: blue light receptors for plants and animals. Science 284:760–765

Chiu MI, Nathans J (1992) A sequence upstream to the mouse blue visual pigment gene directs blue cone-specific transgene expression in mouse retinas. Vis Neurosci 11:733–780

Fleissner G, Fleissner G (2003) Nonvisual photoreceptors in arthropods with emphasis on their putative role as receptors of natural Zeitgeber stimuli. Chronobiol Int 20:593–616

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 beetles. J Comp Neurol 433:401–414

Fleissner G, Fleissner G, Frisch B (1993) A new type of putative non-visual photoreceptors in the optic lobe of beetles. Cell Tissue Res 273:435–445

Fleissner G, Fleissner G (1992) Neuronal organization of the circadian system in the optic lobe of the beetle *Pachymorpha sexguttata* and the scorpion *Androctonus australis*. Rhythmogenesis. Stuttgart, Thieme, p 295

Foster RG, Helfrich-Förster C (2001) The regulation of circadian clocks by light in fruitflies and mice. Philos Trans R Soc Lond B Biol Sci 356:1779–1789

Frisch B, Fleissner G, Brandes C, Hall JC (1996) Staining in the brain of *Pachymorpha sexguttata* mediated by an antibody against a *Drosophila* clock-gene product: labeling of cells with possible importance for the beetle's circadian rhythms. Cell Tissue Res 286:411–429

Hardie RC (2003) Regulation of TRP channels via lipid second messenger. Annu Rev Physiol 65:735–759

Helfrich-Förster C, Edwards T, Yasuyama K, Wisotzki B, Schneuwly S, Stanewsky R, Meinertzhagen IA, Hofbauer A (2002) The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function. J Neurosci 22:9255–9266

Helfrich-Förster C, Stengl M, Homberg U (1998) Organization of the circadian system in insects. Chronobiol Int 15:567–594

Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 30:249–261

Hofbauer A, Buchner E (1989) Does *Drosophila* have seven eyes? Naturwissenschaften 76:335–336

Hicks D, Molday RS (1986) Differential immunogold-dextran labeling of bovine and frog rod and cone cells using monoclonal antibodies against bovine rhodopsin. Exp Eye Res 42:55–71

Hoffmann PD, Batschauer A, Hays JB (1996) At-PHH1, a novel gene from *Arabidopsis thaliana* related to microbial photolyases and plant blue light receptors. Mol Gen Genet 253:259–265

Homberg U, Reischig T, Stengl M (2003) Neural organization of the circadian system of the cockroach *Leucophaea maderae*. Chronobiol Int 20:577–591

Klarsfeld A, Malpel S, Michard-Vanhée C, Picot M, Chélot E, Rouyer F (2004) Novel features of cryptochrome-mediated photoreception in the brain circadian clock of *Drosophila*. J Neurosci 24:1468–1477

Knopse VK, Donoso LA, Banga JP, Kasp K, Gregerson DS (1988) Epitope mapping of bovin retinal s-antigen with monoclonal antibodies. Curr Eye Res 7:1137–1147

Krishnan B, Levine JD, Lynch MK, Dowse HB, Funes P, Hall JC, Hardin PE, Dryer SE (2001) A new role for cryptochrome in a *Drosophila* circadian oscillator. Nature 411:313–317

Lieb WE, Smith-Lang L, Singh Dua H, Christensen AC, Donoso LA (1991) Identification of an s-antigen-like molecule in *Drosophila melanogaster*: An immunohistochemical study. Exp Eye Res 53:171–178

Mote MI, Black KR (1981) Action spectrum and threshold sensitivity of entrainment of circadian running activity in the cockroach *Periplaneta americana*. Photochem Photobiol 43:257–265

Page TL (1982) Transplantation of the cockroach circadian pacemaker. Science 216:73–75

Peichl L, Nemec P, Burda H (2004) Unusual cone and rod properties in subterranean African mole rats (Rodentia, Bathyergidae) Eur J Neurosci 19:1545–1558

Phillips J, Sayeed O (1993) Wavelength-dependent effects of light on magnetic compass orientation in *Drosophila melanogaster*. J Comp Physiol A 172:303–308

Rieger D, Stanewsky R, Helfrich-Förster C (2003) Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. J Biol Rhythms 18:377–391

Ritz T, Adem S, Schulten K (2000) A model for photoreceptor-based magnetoreception in birds. Biophys J 78:707–718

Roberts SK (1965) Photoreception and entrainment of cockroach activity rhythms. Science 148:958–959

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila* period and timeless. Cell 93:805–814

Shaw SR (1978) The extracellular space and blood-eye barrier in an insect retina: an ultrastructural study. Cell Tissue Res 188:35–61

Stanewsky R (2002) Clock mechanisms in *Drosophila*. Cell Tissue Res 309:11–26

Stanewsky R (2003) Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. J Neurobiol 54:111–147

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC (1998) The $cry^b$ mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95:681–692

Sternberger LA (1979) Immunocytochemistry, 2 edn. Wiley & Sons, New York

Truman JW (1976) Extraretinal photoreception in insects. Photochem Photobiol 23:215–225

Vácha M, Soukopová H (2004) Magnetic orientation in the mealworm beetle *Tenebrio* and the effect of light. J Exp Biol 207:1241–1248

Wang Y, Macke JP, Merbs SL, Zack DJ, Klaunberg P, Bennet J, Gearhart J, Nathans J (1992) A locus control region adjacent to the human red and green visual pigment genes. Neuron 39:429–440

Wiltschko W, Wiltschko R (1972) Magnetic compass of European robins. Science 176:62–64

Wiltschko W, Wiltschko R (1995) Migratory orientation of European robins is affected by the wavelength of light as well as by a magnetic pulse. J Comp Physiol A 177:363–369

Wiltschko W, Wiltschko R (2001) Light dependent magnetoreception in birds: the behavior of European robins, *Erithacus rubecula*, under monochromatic light of various wavelengths and intensities. J Exp Biol 204:3295–3302

Wiltschko W, Wiltschko R (2002) Magnetic compass orientation in birds and its physiological basis. Naturwissenschaften 89:445–452

Yasuyama K, Meinertzhagen IA (1999) Extraretinal photoreceptors at the compound eye's posterior margin in *Drosophila melanogaster*. J Comp Neurol 412:193–202

Zuker CS (1996) The biology of vision of *Drosophila*. Proc Natl Acad Sci USA 93:571–576

Chapter V. Involvement of the ocelli of the cockroach *Leucophaea maderae* in entrainment of the circadian clock

# Chapter V

# Involvement of the Ocelli of the Cockroach *Leucophaea maderae* in Entrainment of the Circadian Clock

Sabine Hofer

## Abstract

The circadian locomotor rhythm of the cockroach *Leucophaea maderae* is entrained by light to the daily solar cycle. While a contribution by ocelli and extraocular photoreceptors to entrainment was shown in flies, moths, and a cricket, light perception for entrainment of the circadian clock in the cockroach has been assumed to be exclusively via the compound eyes. To investigate whether the ocelli play a role in light entrainment of the cockroach *Leucophaea maderae* at low light intensities, the absolute threshold for entrainment to white light was determined in running wheel assays. Light stimuli were presented with a 12:12 h light-dark (LD) regime with twilight simulation in intensities ranging from $5.8\,\mathrm{x}\,10^{-6}$ to $3.6\,\mathrm{x}\,10^{-12}\,\mathrm{W/cm^2}$. Stepwise light reductions were combined with 6 h phase delays, and only animals that adjusted to the new light regime were judged to entrain. The threshold was defined by the light intensity to which 50 % of the animals still entrained, and lay between $1.5\,\mathrm{x}\,10^{-10}\,\mathrm{W/cm^2}$ (73 % of the animals entrained) and $3.6\,\mathrm{x}\,10^{-12}\,\mathrm{W/cm^2}$ (32 % of the animals entrained). The 50 %-threshold was therefore interpolated at $7.7\,\mathrm{x}\,10^{-11}\,\mathrm{W/cm^2}$. To test whether the ocelli (or extraretinal photoreceptors) alone play a role in light entrainment to the circadian clock, the optic nerves of cockroaches were surgically removed and the locomotor activities were monitored, but no operated animal entrained to the light regime. After surgical removal of the ocelli of intact animals, 77 % of the animals still entrained to $1.5\,\mathrm{x}\,10^{-10}\,\mathrm{W/cm^2}$, but no animal entrained to $3.6\,\mathrm{x}\,10^{-12}\,\mathrm{W/cm^2}$ anymore. These results suggest an involvement of the ocelli in light entrainment of the cockroach at least under low light conditions.

## Introduction

To provide information about the local time, internal circadian clocks of insects are entrained by environmental time cues. Among several parameters, the daily changes in light environment provide the most important and universal Zeitgeber signal for the circadian clock. Insects can be classified into two groups, according to the identity of different photoreceptors and their contribution in clock entrainment. In flies, mosquitoes, and moths,

photoreceptors of the compound eyes participate in light entrainment, but are not necessary for entrainment of the rhythm (Truman 1974; Helfrich and Engelmann 1983; Helfrich et al. 1985; Kasai and Chiba 1987; Cymborowski et al. 1994; Helfrich-Förster et al. 2001). In *Drosophila melanogaster*, entrainment is completely abolished only in mutants that lack all known extraretinal (the blue light photoreceptor cryptochrome [CRY] and the Hofbauer-Buchner eyelet [H-B eyelet]) and retinal (compound eyes, ocelli) photoreceptors (Helfrich-Förster et al. 2001). In other dipterans such as the fly *Musca domestica*, the blowfly *Calliphora vicina* and the mosquito *Culex pipiens pallens*, activity rhythms still entrain to LD cycles after removal of the optic lobes (Helfrich et al. 1985; Kasai and Chiba 1987; Cymborowsky et al. 1994). These results support the idea that in these insects the compound eyes are not the only photoreceptors to provide LD information to the circadian clock. In orthopteroid insects (with the exception of two species, see later) such as crickets and cockroaches, on the other hand, the compound eyes are indispensable for entrainment of circadian activity rhythm (Nishiitsutsuji-Uwo and Pittendrigh 1968; Loher 1972; Tomioka and Chiba 1984; Abe et al. 1997). To date, only for two orthopteroid species extraretinal photoreceptors for photic entrainment have been reported. In the New Zealand weta, *Hemideina thoracica*, synchronization to the LD cycles persists even after optic nerve severance and complete occlusion of the compound eyes and ocelli (Waddell et al. 1990). The bandlegged cricket, *Dianemobius nigrofasciatus*, also showed weak entrainment to LD cycles after bilateral severance of the optic nerves (Shiga et al. 1999).

Most adult insects have two or three simple eyes, the ocelli, in addition to compound eyes, and in some cases additional extraocular photoreceptors. These eyes are not well suited for image detection, but several studies showed that ocelli can detect changes in light intensity and play various roles in the behavior of insects (Goodman 1981; Mizunami 1994a). However, it is not yet clear at all why insects need these photoreceptor structures, in addition to their compound eyes. Anatomical, physiological, and behavioral studies revealed that ocelli are superior to compound eyes in terms of absolute sensitivity and speed of signal transmission (Chappell and Dowling 1972; Wilson 1978; Goodmann 1981; Mizunami et al. 1986; Mizunami 1994b, 1995). Thus in locusts, the ocellar response induced by motion of an artificial horizon appeared earlier than the compound eye response (Taylor 1981). One reason might be that the interneurons of the ocellar system are among the largest in the insect nervous system, which allows for the faster transmission of signals (Wilson 1978; Guy et al. 1979). Cockroaches have two ocelli with an extremely large visual field. The ocellar lens is the largest known among insects. The focal length of the ocellar lens does not allow for image formation in the retinal layer, but does serve as a large window for photon entry (Weber and Renner 1979). All structural specializations are designed to enhance light-gathering power, apparently as an adaptation to a low light habitat. The ocellar pathway is quite complex in the cockroach brain. Ocellar interneurons project to different areas in the brain, e.g., to thoracic motor centers mediating locomotor behavior, to higher association centers, and to primary sensory centers, including the lobula and medulla of the optic lobe, the antennal lobe, and the deuto- and tritocerebrum (Mizunami et al. 1982; Strausfeld and Bassemir 1985; Mizunami and Tateda 1986, 1988; Ohyama and Toh 1990; Mizunami 1990, 1994a, 1995).

Loesel and Homberg (2001) described an ocellar interneuron in the cockroach *L. maderae* connecting the accessory medulla, the circadian pacemaker center in the optic lobe, to the ocellar nerve. This was the first evidence for a neuronal connection of the ocelli with the circadian system of an insect, suggesting an involvement of ocelli in light entrainment of the cockroach *L. maderae*. Earlier experiments by Roberts (1965), however, had ruled out an involvement of ocelli in entrainment of the circadian system of *L. maderae,* but Roberts' experimental setup only used lights on/off stimulation with relatively high light intensities. I, therefore, decided to reinvestigate the possible contribution of ocelli to light entrainment of the clock by introducing a twilight simulation instead of a lights on/off regime. This more natural condition resulted in more precise entrainment to light-dark changes in scorpions (Fleissner G. et al. 1996; Fleissner and Fleissner 2003) and in other species (hamster: Boulos et al. 1996; humans: Danilenko et al. 2000). Given the high absolute sensitivity of the circadian system, particular attention was paid to the possible role of the ocelli in entrainment of *L. maderae* under low light intensities. After surgical ablation of the ocelli, the absolute threshold for light entrainment was increased significantly, suggesting an involvement of the ocelli in light pathway into the circadian clock of the cockroach at least under low light conditions.

## Materials and methods

### Animals

Male adult cockroaches (*Leucophaea maderae*) were taken from crowded colonies at the University of Marburg. Animals were reared under 12:12 h light-dark (LD) photoperiod, at about 60 % relative humidity, and a temperature of 28 °C.

### Behavioral assays and data analysis

Behavior analyses were performed in 12:12 h LD and constant temperature and humidity. The cockroaches were kept individually in light tight chambers (25 x 25 x 25 cm) and were provided with food and water for up to 18 months. Each chamber was equipped with three cool white light emitting diodes (WU-7-750 SWC, UHB-LED, Conrad, Germany) mounted behind a diffusor, to provide diffuse and uniform lighting within the box. The LD cycles were controlled by an electronic timer. This device simulated a dawn and dusk situation with regard to light intensity but without changes in wavelength. At the beginning and end of each light phase, light intensity was continuously increased and decreased for 1 h, respectively.

Light intensity in the photophase ranged from $5.8 \times 10^{-6}$ to $1.5 \times 10^{-12}$ W/cm$^2$. Starting from the highest intensity, light intensity was reduced stepwise (by $2.0 \times 10^{-1}$, $1.0 \times 10^{-1}$, $5.0 \times 10^{-2}$, or $2.5 \times 10^{-2}$ log units, respectively) through insertion of neutral density filters into the light path, if more than half of the animals used in mostly 10 parallel experimental trials still showed stable entrainment ($\tau = 24$ h, or $\tau > 24$ h if the light cycle was shifted by a 6 h delay) to the former light regime. Several days later or simultaneously, the phase of the LD cycle was shifted by a 6 h delay. Only animals that properly synchronized to

this new light phase (i.e., activity onset had to occur at the beginning of the dark phase) were considered to successfully entrain. This procedure was continued until more than half of the animals showed activity patterns that were no longer entrained by the imposed LD cycle. The intensity threshold for entrainment was defined as the light intensity to which half of the animals were still able to entrain, and therefore called 50 %-threshold.

Locomotor activity was recorded with running wheels (described by Wiedenmann 1977) equipped with a magnetic switch. One revolution of the running wheel resulted in one impulse. The impulses were continuously counted by a computer over 1 min intervals and condensed and processed by custom-designed PC-compatible software (developed by H. Fink, University of Konstanz). The data were plotted in double plot activity histograms. The free-running periods $\tau$ and the induced phase shifts were estimated by converting the raw data into ASCII format. Rhythmicity was evaluated by $\chi^2$-periodograms (Sokolove and Bushell 1978; calculated with Tempus 1.6 [Reischig 2003], an add-in for Microsoft Excel) as well as by visual inspection. Cockroaches with periods of 24 h and appropriate phase relationships to the LD cycle were judged to be synchronized to the light cycle. Also, cockroaches with periods of $\tau > 24$ h (after changing the light phase) were judged to correctly synchronize to the light cycle, since *L. maderae* usually has a freerunning period of about 23.7 h (Barrett and Page 1989). Cockroaches with periods $\tau < 24$ h were judged to freerun (the photophase was always shifted in delay). Failure in entrainment was also assumed, if animals showed a period of 24 h that is not in phase with the actual light regime.

Every single "experimental trial" (later referred only as "trial") denotes one run of stepwise light reduction (until the 50 % threshold was exceeded) with one animal. Since some animals were used more than once for experimental trials (but only within a certain experimental group), the number of trials in two experimental groups is larger than the number of animals used. In total, 19 animals were used in the intact (control) experimental group, 10 animals in the ocelli-less group, and 10 animals in the optic nerve-less group. Statistical analyses were performed with SPSS 11.0 (Superior Performing Software Systems; SPSS Inc.)

Irradiance of the LEDs (maximum intensity) was measured with a radiometer (Photodyne 18XTA, silicium detector). Light levels after inserting filters were extrapolated using the filter's transmission values (measured and verified in the laboratory of Prof. Dr. Paul Galland, Marburg).

### Operation

The experimental animals were removed from the running wheels and mounted in metal tubes. The animals were anesthetized with $CO_2$, and a small window was cut in the head capsule. In one group of animals, the optic nerves were cut on both sides of the brain. In another group of animals, the ocelli were removed following severance of the ocellar nerves. The success of the operations was controlled by eye. After the operations, the cuticle was waxed back and the animals were returned to the running wheels.

## Results

This study investigated the possible involvement of the ocelli in light entrainment of the circadian system of the cockroach *Leucophaea maderae*. At first, the threshold sensitivity was determined at which 50 % of the animals still entrained to the LD cycle (50 %-threshold). After optic nerve severance, none of the operated animals entrained to the imposed light regime (even at the highest light intensity), as shown in a previous study (Roberts 1965). After surgical removal of the ocelli in cockroaches that were entrained to low light conditions, the light sensitivity of the circadian system was significantly reduced.

### Behavior of intact animals under dim LD conditions

Cockroaches whose locomotor activity was continuously recorded in a running wheel assay, were entrained to a 12:12 h LD cycle of white light with twilight simulation and a light intensity of $5.8 \times 10^{-6}$ W/cm$^2$. The light intensity was stepwise reduced in intervals of at least one weak, and simultaneously or few days later, the LD cycle was shifted by a 6 h delay. Only animals that correctly re-synchronized to this new light phase were considered to be entrained. At a light intensity down to $7.3 \times 10^{-10}$ W/cm$^2$ all animals were still entrained. After reducing the light to $1.5 \times 10^{-10}$ W/cm$^2$, in 7 of 30 trials (23 %) the animals freeran with a period of less than 24 h (Table 1). In one trial (3 % of 30) the animal continued to run with a 24 h period without following the new phase. In the other 22 trials (73 % of 30) the cockroaches still entrained correctly to this light intensity (Table 1; Fig. 1). After further reducing the light to $3.6 \times 10^{-12}$ W/cm$^2$, in 7 of 19 trials (37 %) the animals freeran, and in 6 trials (32 % of 19) the animals continued to run with a 24 h period, but did not follow the new 6 h delayed light cycle (Fig. 1). Since this effect occurred considerably more often than animals with a period of 24 h occur in a normal population of freerunning cockroaches, it was assumed to be an after-effect from the previous LD cycle. In total, therefore, in 69 % of the 19 trials the animals did not re-entrain to this light condition, and in 6 trials (32 % of 19) the cockroaches successfully entrained according to the determined criteria (Table 1; Fig. 2A–D). Thus, by linear interpolation of these results, the 50 % intensity threshold for white light for circadian clock entrainment of *L. maderae* was determined at $7.7 \times 10^{-11}$ W/cm$^2$.

**Table 1** Locomotor activity of intact cockroaches in low light LD cycles

| Light intensity | $1.5 \times 10^{-10}$ W/cm$^2$ | | | $3.6 \times 10^{-12}$ W/cm$^2$ | | |
|---|---|---|---|---|---|---|
| $n$ total | 30 | | | 19 | | |
| | Entrainment | Freerun | After-effect | Entrainment | Freerun | After-effect |
| $n$ | 22<br>73 % | 7<br>23 % | 1<br>3 % | 6<br>32 % | 7<br>37 % | 6<br>32 % |

$n$ number of experimental trials

**Table 2** Locomotor activity of ocelli-less cockroaches in low light LD cycles

| Light intensity | $1.5\,\mathrm{x}\,10^{-10}\,\mathrm{W/cm^2}$ | | | $3.6\,\mathrm{x}\,10^{-12}\,\mathrm{W/cm^2}$ | | |
|---|---|---|---|---|---|---|
| $n$ total | 18 | | | 16 | | |
| | Entrainment | Freerun | After-effect | Entrainment | Freerun | After-effect |
| $n$ | 14<br>77 % | 2<br>11 % | 2<br>11 % | 0<br>0 % | 11<br>69 % | 5<br>31 % |

$n$ number of experimental trials

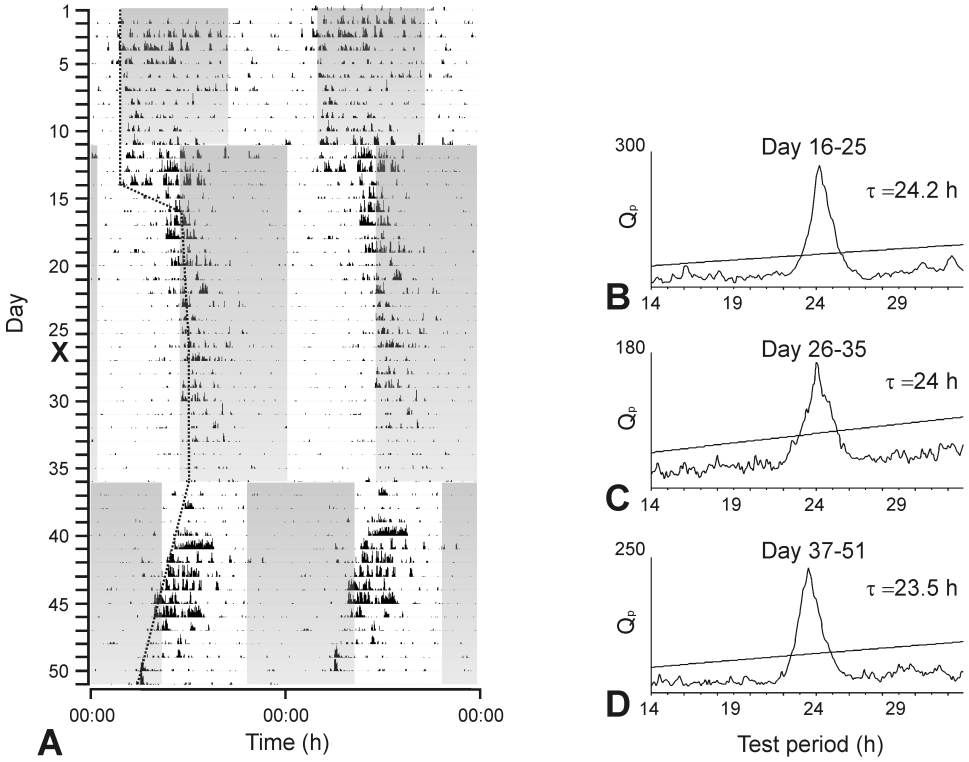
## Behavior of animals with severed optic nerves

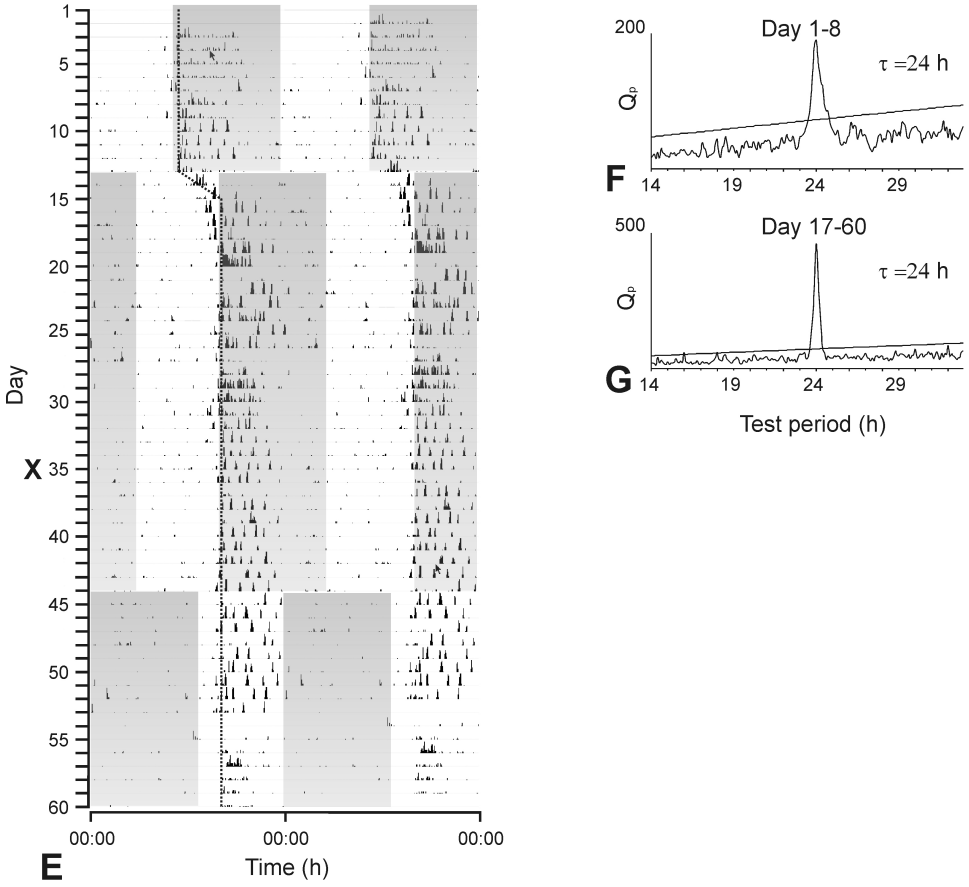
In 1965, Roberts surgically removed the optic nerves to test whether extraocular photoreceptive structures are involved in light entrainment of the circadian clock of the cockroach. To repeat this experiment in a simulated dawn and dusk situation, both optic nerves were cut in 10 cockroaches, and the animals were returned to the running wheels. None of these animals were re-entrained to LD at $5.8\,\mathrm{x}\,10^{-6}\,\mathrm{W/cm^2}$ or at lower light intensities. All cockroaches freeran after surgical removal of the optic nerves.

## Behavior of animals without ocelli

To test whether the ocelli play a role in the light entrainment of circadian locomotor activity, I submitted animals without ocelli in a 12:12 h LD cycle at low light intensities around the previously determined threshold to six hours phase delays. In two of the 18 trials (11 %), the animals freeran or showed an after-effect at an irradiance of $1.5\,\mathrm{x}\,10^{-10}\,\mathrm{W/cm^2}$ (Table 2). In fourteen trials, the cockroaches (77 % of 18) successfully entrained to the new photophase in this light condition (Table 2; Fig. 2E, F). After dimming the light to $3.6\,\mathrm{x}\,10^{-12}\,\mathrm{W/cm^2}$, none of the ocelli-less animals entrained, but in 11 trials (67 % of 18) the animals freeran (Table 2; Fig. 2E, G), and in 5 trials (31 % of 18) the animals showed an after-effect (Table 2). As tested with a two-tailed two-by-two frequency table (also called crosstab, a statistical method that involves a $\chi^2$-test to compare frequencies of experimental effects with respective control experiments) this difference was significant ($p < 0.01, \chi^2 = 6.098, df = 1$). In contrast, at the intensity of $1.5\,\mathrm{x}\,10^{-10}\,\mathrm{W/cm^2}$, there was no significant difference between the intact group and the ocelli-less group.

**Fig. 1A–G** (next page) Actograms of individual intact *Leucophaea maderae* over a period of 51 (A) and 60 (E) days, respectively, and the corresponding $\chi^2-$periodograms (B–D; F, G). *Shaded areas* indicate scotophase. **A** Double-plot activity histogram showing wheel-running activity at different light regimes. When the light phase was changed (day 11), the animal entrained to $1.5\,\mathrm{x}\,10^{-10}\,\mathrm{W/cm^2}$, as shown in the corresponding $\chi^2$-periodogram (**B** $\tau = 24.2\,\mathrm{h}$; **C** $\tau = 24\,\mathrm{h}$). At day 26 ($X$), the light was dimmed ($3.6\,\mathrm{x}\,10^{-12}\,\mathrm{W/cm^2}$), and after the change of photoperiod (day 36) the animal freeran (**D** $\tau = 23.5\,\mathrm{h}$). **E** Activity histogram showing wheel-running activity with after-effect. At days 1–8, the animal entrained to the light regime of $1.5\,\mathrm{x}\,10^{-10}\,\mathrm{W/cm^2}$ as shown in the corresponding $\chi^2$-periodograms (**F** $\tau = 24\,\mathrm{h}$). After change of the photophase at day 13, the activity rhythm followed the phase delay and remained entrained (**G** $\tau = 24\,\mathrm{h}$). At day 35 ($X$), the light was dimmed to $3.6\,\mathrm{x}\,10^{-12}\,\mathrm{W/cm^2}$ and after change of the photoperiod the animal did not entrain to the new phase, but maintained a 24 h periodicity in phase with the previous LD rhythm (after-effect).


Day
X
00:00
00:00
00:00
Time (h)
A
Day 16-25
τ =24.2 h
B
Day 26-35
τ =24 h
C
Day 37-51
τ =23.5 h
D
Qp
Test period (h)


Day
X
00:00
00:00
00:00
Time (h)
E
Day 1-8
τ =24 h
F
Day 17-60
τ =24 h
G
Qp
Test period (h)

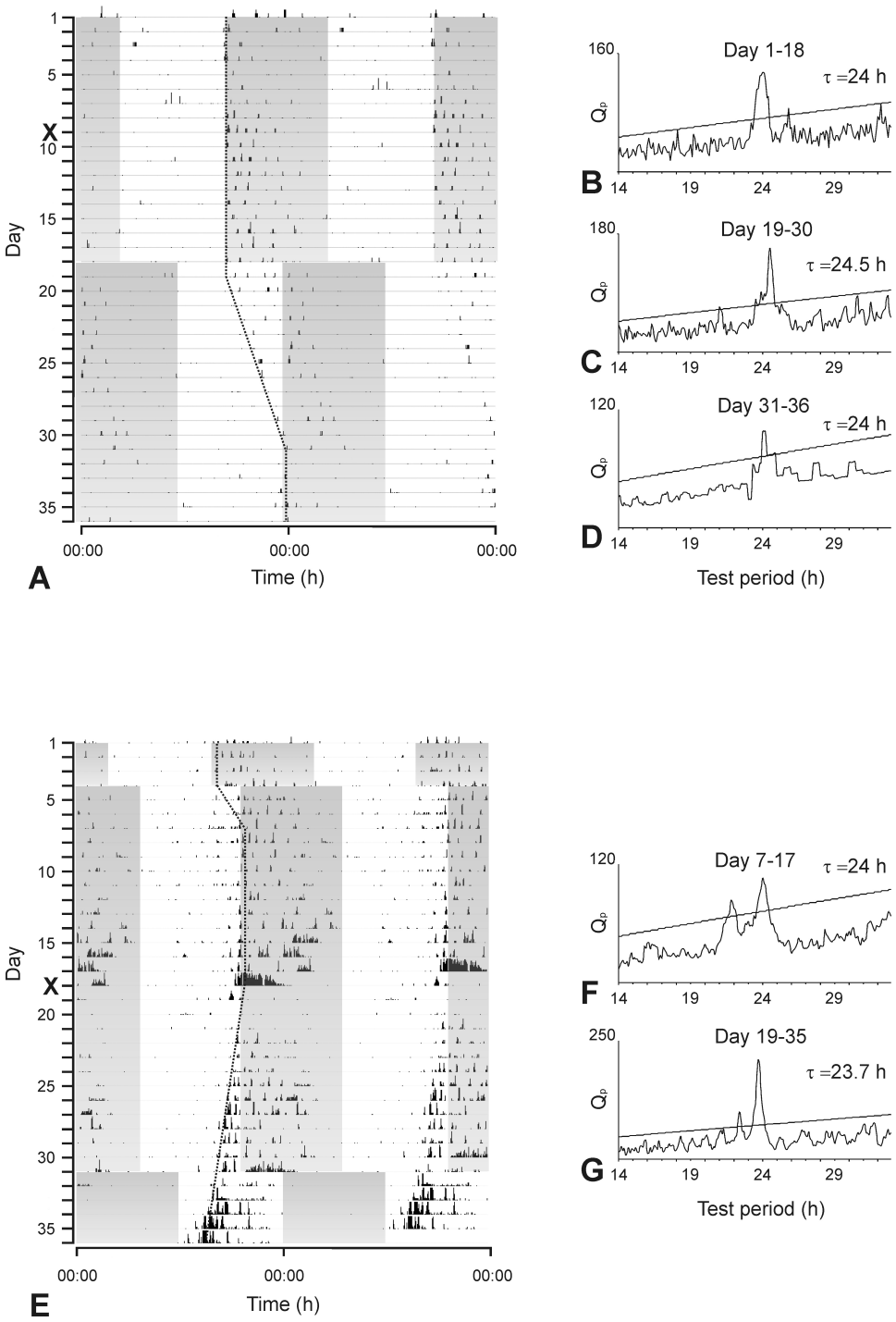



**Fig. 2A–G** Actograms of an intact (control) cockroach with ocelli (A) and of a cockroach without ocelli (E) over a period of 36 days, and the corresponding $\chi^2$-periodograms (B–D, F, G). Shaded areas indicate scotophase. **A** Double-plot activity histogram showing wheel-running activity in different light regimes of an intact individual. From day 1–18, the animal entrained to LD at $1.5 \times 10^{-10}$ W/cm$^2$, as shown in the corresponding $\chi^2$-periodograms (**B** $\tau = 24$ h). At day 9 ($X$), the light was dimmed ($3.6 \times 10^{-12}$ W/cm$^2$) and after phase delay of the LD cycle the animal entrained (**C** $\tau = 24.5$ h; **D** $\tau = 24$ h). **E** Double plot activity histogram of an ocelli-less individual. From day 7–17, the animal entrained to the new photophase ($1.5 \times 10^{-10}$ W/cm$^2$) as shown in the corresponding $\chi^2$periodograms (**F** $\tau = 24$ h). At day 18 ($X$), the light was dimmed ($3.6 \times 10^{-12}$ W/cm$^2$) and the animal showed a freerunning rhythm (**G** $\tau = 23.7$ h).

## Discussion

In the present work, the 50 %-threshold intensity for entrainment to white light for the cockroach *Leucophaea maderae* was determined in running wheel assays. The light stimuli were presented in a 12:12 h LD regime from $5.8 \times 10^{-6}$ to $3.6 \times 10^{-12}$ W/cm$^2$ with dawn and dusk simulation. After the 50 %-threshold was determined, I studied the involvement of the ocelli in entrainment to white light by surgical removal of this photoreceptor structure. To date, only high light intensities in an on/off light regime without twilight simulation were used for entrainment experiments in *L. maderae*. The present results suggest that the ocelli play a role in the direct or indirect light entrainment of the circadian clock.

### Light sensitivity of the circadian system of the cockroach

Entrainment of circadian rhythmic behavior by light has been investigated in a variety of insect species (Roberts 1965; Mote and Black 1981; Abe et al. 1997; Helfrich-Förster et al. 2001). Studies in dipterans, especially *Drosophila melanogaster*, have identified retinal and extraretinal structures beneath the compound eyes that participate in entrainment of the circadian clock (Helfrich-Förster et al. 2001). In contrast, in the cockroach, the compound eyes have been regarded as the sole photoreceptor organ involved in photic entrainment (Roberts 1965; Nishiitsutsuji-Uwo and Pittendrigh 1968; Sokolove 1975; Page 1978).

In an earlier study, Mote and Black (1981) examined the light sensitivity of the circadian system controlling locomotor activity of the cockroach *Periplaneta americana* by measuring the action spectrum for light entrainment. They found that light entrainment is dominated by green-sensitive photoreceptors, and that as few as 5 photons/eye/sec (corresponding to $6 \times 10^{-11}$ W/cm$^2$) of green light are sufficient to entrain locomotor behavior in 50 % of the cockroaches. In my experiments it turned out after linear interpolation between the light intensities to which about 70 % and 30 %, respectively, of the experimental trials the animals entrained, that the 50 %-threshold for *L. maderae* for entrainment to white light should be situated around $7.7 \times 10^{-11}$ W/cm$^2$. Because white light was used in my experiments instead of single wavelengths it is not possible to specify the light intensity in photons/eye/sec. Further, the linear interpolation between two light intensities, by which the 50 %-threshold for light entrainment was approximated, may not reflect the exact course of decrease of entrained animals while dimming off the light. However, both threshold intensities in the experiments of Mote and Black (1981) and the data presented here are nearly identical ($6 \times 10^{-11}$ W/cm$^2$ versus $7.7 \times 10^{-11}$ W/cm$^2$), although Mote and Black applied monochromatic green light (495 nm). This is remarkable since green light comprises only a part of the white light, which, as drawn from the spectrum supplied by the manufacturer of the LEDs, approximately may carry about one third of the total light energy of a given intensity. If this is not only a result in differences of the experimental setups, the circadian system of *L. maderae* appears to be even more light sensitive than that of *P. americana*. Another reason for the low threshold intensity in my experiments may be that the additional stimulation of short wavelength photoreceptors by use of white light (which provides a more natural condition than the use of monochromatic light) has a synergistic effect on the light sensitivity of the circadian system, even if entrainment

via the short wavelength photoreceptors alone requires three log units more light in *P. americana* (Mote and Black 1981).

Further, it may also be possible that the twilight simulation of my experimental setup influenced the light sensitivity of the circadian system. This condition may have a stronger synchronizing effect on the circadian system than the normally used light on/off stimuli. This has to be revealed in further studies. A positive influence of twilight simulation on the precision of light entrainment has been shown in scorpions (Fleissner et al. 1996; Fleissner and Fleissner 2003).

In conclusion, cockroaches display a remarkably high sensitivity for light entrainment of their circadian systems, which very likely is an adaptation to allow these night-active animals to stay synchronized to day-night cycles even in habitats that never receive direct daylight.

### The role of the ocelli in circadian rhythm entrainment of the cockroach

All structural specializations of the cockroach's ocelli are designed to improve light-gathering power, apparently an adaptation to low light habitats (Weber and Renner 1979). Ocellar neurons project to different areas in the brain, e.g., to primary sensory centers including the lobula and medulla of the optic lobe (Mizunami et al. 1982; Strausfeld and Bassemir 1985; Mizunami and Tateda 1986, 1988; Ohyama and Toh 1990; Mizunami 1990, 1995). Because of the innervation of visual neuropils, including the medulla, it seems not unlikely that the ocelli play a role in the entrainment of the circadian clock, which is located in the accessory medulla that is closely attached to the medulla (Reischig and Stengl 2003). As shown in this study, in no case an ocelli-less animal entrained to a light photophase with an intensity of $3.6 \,\mathrm{x}\, 10^{-12}\,\mathrm{W/cm^2}$, which is more than one log-unit below the interpolated 50 %-threshold, but in one third of the trials with intact animals entrainment occured. In contrast, at $1.5 \,\mathrm{x}\, 10^{-10}\,\mathrm{W/cm^2}$, which is above the 50 %-threshold, there was no significant difference in the number of entrained trials between the intact and the ocelli-less group. Therefore, I suggest that the ocelli play a role for entrainment at low light conditions near the sensitivity limit of the circadian system to entrain successfully.

In a previous study, Rence et al. (1988) showed an indirect role of the ocelli on circadian rhythmicity of the cricket *T. commodus*. The freerunning periods of crickets stridulating in constant light depended on the light intensity, with the lengths of the periods decreasing if the light intensity was decreased within a certain range of intensities. Ocelli-less crickets always exhibited a shorter freerunning period than intact animals. Further, the authors found an ocellar neuron which projected from the lateral ocellar nerve to the ipsilateral optic lobe. It was suggested that the ocelli increased the sensitivity of the compound eye photoreceptors for photic entrainment of the pacemaker.

More recently, Rieger et al. (2003) showed that the ocelli participate in circadian photoreception in *Drosophila melanogaster*. The authors demonstrated that in $so^1$ mutants, which lack ocelli and compound eyes, significantly fewer flies entrain to short and long days than in $cli^{eya}$ mutants (without eyes, but still with ocelli). In *Drosophila*, the ocelli have no direct connection to the pacemaker neurons, but indirect contacts be-

tween fibers from the pacemaker cells and from secondary ocellar neurons might exist in the dorsal protocerebrum, where circadian information appears to be transferred to downstream neurons (Helfrich-Förster 2003; Rieger et al. 2003). In the cockroach the situation appears similar. Ten years ago Mizunami (1994b) postulated that third order ocellar neurons transmit ocellar signals to different neuropil areas including the optic lobes and the protocerebrum. Intracellular recordings in *L. maderae*, indeed, revealed a light-sensitive neuron that connects the accessory medulla to the ocellar nerve (Loesel and Homberg 2001). This was the first direct neuronal connection found between the circadian pacemaker and the ocellar tract neuropil. In addition, several presumptive pacemaker neurons, for example the pigment-dispersing hormone immunoreactive medulla neurons, project into the medulla and into the protocerebrum (Chapters I–III, Reischig et al. 2004). However, whether the pacemaker output and/or input neurons and the ocellar neurons described by Mizunami (1994b) are indeed linked with each other must be resolved in further studies. As was confirmed in this study, the ocelli alone are not capable to entrain circadian activity in cockroaches. Nevertheless, I suggest that the ocelli play an indirect role in light entrainment of the circadian clock of the cockroach, by enhancing light sensitivity of the circadian clock to synchronizing compound eye input, similar as shown for the cricket and the fly (Rence et al. 1988; Rieger et al. 2003). It is not yet clear whether the ocelli are directly acting onto the pacemaker, or whether they act onto the light information pathway upstream to the pacemaker. A probable function of the ocelli could be to help adjusting the compound eyes of the night-active cockroach to very low light conditions.

### After-effects in low light conditions?

A few years ago, Page (2001, 2000) described a history dependency of circadian pacemaker period in an isolated eye of the mollusk *Bulla gouldiana* and the cockroach *L. maderae*. The freerunning period of the locomotor activity rhythm in constant darkness of animals raised in LD 12:12 h was shorter after entrainment to LD 11:11 h than after entrainment to LD 13:13 h; this effect was visible for up to 40 days. Interestingly, inhibition of transcription and translation, which resets the phase of the circadian oscillation, had no effect on the after-effects expressed in the isolated eye (Page 2000). Further, in the cockroach, the after-effects were unaffected by resetting the oscillator phase by low-temperature pulses (Page 2001). After-effects appear to be a consequence of a stable, long lasting change of a parameter of the circadian system. The author suggests that light entrainment of the pacemaker period and entrainment of the pacemaker phase may be mediated by two different target molecules. This may be a novel mechanism to control the period by light input into the circadian pacemaker system (Page 2000). In the present study, up to 32 % of the cockroaches in a low light regime exhibited a freerunning period of $\tau = 24$ h in phase with the prior light regime, but out of phase with the actual light-phase (shifted by 6 h delay), independent of the existence of the ocelli. After several days or weeks the periods shortened. These instances were to frequent to be interpreted as a random occurrence of a $\tau$ of 24 h in animals that normally have an average freerunning period of 23.7 h (Barret and Page 1989). The nature and implication of this behavior is not yet understood, but it is suggested that this is an after-effect from the previous

LD cycle, which in the context of entrainment to very low light conditions has not been observed so far. The details of this phenomenon remain to be elucidated.

In future experiments it would be important to additionally test sham operated animals and to compare three different groups, the intact, sham operated, and ocelli-less cockroaches to further support the role of the ocelli in light entrainment. However, this study provides the first evidence for an involvement of the ocelli in the circadian system of the cockroach *Leucophaea maderae.*

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## References

Abe Y, Ushirogawa H, Tomioka K (1997) Circadian locomotor rhythms in the cricket, *Gryllodes sigillatus*. I. Localization of the pacemaker and the photoreceptor. Zool Sci 14:719–727

Barrett RK, Page TL (1989) Effects of lights on circadian pacemaker development. I. The freerunning period. J Comp Physiol A 165:41–49

Boulos Z, Terman JS, Terman M (1996) Circadian phase response curves for simulated dawn and dusk twilights in hamsters. Physiol Behav 60:1269–1275

Chappell RL, Dowling JE (1972) Neural organisation of the median ocellus of the dragonfly. Intracellular electrical activity. J Gen Physiol 60:121–147

Cymborowski B, Lewis RD, Hong SF, Saunders DS (1994) Circadian locomotor activity rhythms and their entrainment to light-dark cycles continue in flies (*Calliphora vicina*) surgically deprived of their optic lobes. J Insect Physiol 40:501–510

Danilenko KV, Wirz-Justice A, Krauchi K, Weber JM, Terman M (2000) The human circadian pacemaker can see by the dawn's early light. J Biol Rhythms 15:437–446

Fleissner G, Fleissner G (2003) Nonvisual photoreceptors in arthropods with emphasis on their putative role as receptor of natural Zeitgeber stimuli. Chronobiol Int 20:593–616

Fleissner G, Riewe P, Lüttgen M, Fleissner G (1996) Dusk and dawn Zeitgebers synchronise circadian rhythms better than light on/off. In: Elsner E, Schnitzler HU (eds) Brain and evolution. Proceedings of the $24^{th}$ Göttingen Neurobiology Conference, Vol. I. Thieme, Stuttgart, p 221

Goodmann LJ (1981) Organization and physiology of the insect dorsal ocellar system. In Autrum H (ed) Invertebrate visual centers and behavior II, Vol VII, 6c. Springer, Heidelberg, pp 201–286

Guy RG, Goodman LJ, Mobbs PG (1979) Visual interneurons in the bee brain: Synaptic organisation and transmission by graded potentials. J Comp Physiol 134:253–264

Helfrich-Förster C (2003) The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster*. Microsc Res Tech 62:94–102

Helfrich C, Engelmann W (1983) Circadian rhythm of the locomotor activity in *Drosophila melanogaster* and its mutants 'sine oculis' and 'small optic lobes'. Physiol Entomol 8:257–273

Helfrich C, Cymborowski B, Engelmann W (1985) Circadian activity rhythm in the house fly continues after optic tract severance and lobectomy. Chronobiol Int 2:19–32

Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 30:249–261

Kasai M, Chiba Y (1987) Effects of optic lobe ablation on circadian activity in mosquito, *Culex pipiens pallens*. Physiol Entomol 12:59–65

Loesel R, Homberg U (2001) Anatomy and physiology of neurons with processes in the accessory medulla of the cockroach *Leucophaea maderae*. J Comp Neurol 439:193–207

Loher W (1972) Circadian control of stridulation in the cricket *Teleogryllus commodus* WALKER. J Comp Physiol 79:173–190

Mizunami M (1990) Nonliniar signal transmission between second- and third-order neurons of the cockroach ocelli. J Gen Physiol 95:297–317

Mizunami M (1994a) Processing of contrast signals in the insect ocellar system. Zool Sci 11:175–190

Mizunami M (1994b) Information processing in the insect ocellar system: Comparative approaches to the evolution of visual processing and neural circuits. Adv Insect Physiol 25:151–265

Mizunami M (1995) Neural organization of ocellar pathways in the cockroach brain. J Comp Neurol 352:458–468

Mizunami M, Tateda H (1986) Classification of ocellar interneurons in the cockroach brain. J Exp Biol 125:57–70

Mizunami M, Tateda H (1988) Synaptic transmission between second- and third-order neurons of cockroach ocelli. J Exp Biol 140:557–561

Mizuami M, Yamashita S, Tateda H (1982) Intracellular stainings of the large ocellar second order neurons in the cockroach. J Comp Physiol 149:215–219

Mizunami M, Tateda H, Naka KI (1986) Dynamics of cockroach ocellar neurons. J Gen Physiol 88:275–292

Mote MI, Black KR (1981) Action spectrum and threshold sensitivity of entrainment of circadian running activity in the cockroach *Periplaneta americana*. Photochem Photobiol 43:257–265

Nishiitsutsuji-Uwo J, Pittendrigh CS (1968) Central nervous system control of circadian rhythmicity in the cockroach. III. The optic lobes, locus of the driving oscillation? Z Vgl Physiol 58:14–46

Ohyama T, Toh Y (1990) Morphological and physiological characterization of small multimodal ocellar interneurons in the American cockroach. J Comp Neurol 301:501–510

Page TL (1978) Interactions between bilaterally paired components of the cockroach circadian system. J Comp Physiol A 124:225–236

Page TL (2000) A novel mechanism for the control of circadian clock period by light. J Biol Rhythms 15:155–162

Page TL, Mans C, Griffeth G (2001) History dependence of circadian pacemaker period in the cockroach. J Insect Physiol 47:1085–1093

Reischig T (2003) Identification and characterisation of the circadian pacemaker of the cockroach *Leucophaea maderae*. PhD-thesis, University of Marburg, Germany

Reischig T, Stengl M (2003) Ultrastructure of pigment-dispersing hormone-immunoreactive neurons in a three-dimensional model of the accessory medulla of the cockroach *Leucophaea maderae*. Cell Tissue Res 314:421–435

Reischig T, Petri B, Stengl M (2004) Pigment-dispersing hormone (PDH)-immunoreactive neurons form a direct coupling pathway between the bilaterally symmetric circadian pacemakers of the cockroach *Leucophaea maderae*. Cell Tissue Res (in press)

Rence BG, Lisy MT, Garves BR, Quinlan BJ (1988) The role of ocelli in circadian singing rhythms of crickets. Physiol Entomol 13:201–212

Rieger D, Stanewsky R, Helfrich-Förster C (2003) Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. J Biol Rhythms 18:377–391

Roberts SK (1965) Photoreception and entrainment of cockroach activity rhythms. Science 148:958–959

Shiga S, Numata H, Yoshioka E (1999) Localization of the photoreceptor and pacemaker for the circadian activity rhythm in the band legged ground cricket, *Dianemobius nigrofasciatus*. Zool Sci 16:193–201

Sokolove PG (1975) Localization of the cockroach optic lobe circadian pacemaker with microlesions. Brain Res 87:13–21

Sokolove PG, Bushell WN (1978) The chi square periodogram: its utility for analysis of circadian rhythms. J Theor Biol 72:131–160

Strausfeld N, Bassemir UK (1985) Lobula plate and ocellar interneurons converge onto a cluster of descending neurons leading to neck and leg motor neuropil in *Calliphora erythrocephala*. Cell Tissue Res 240:617–640

Taylor CP (1981) Contribution of compound eyes and ocelli to steering of locust in flight. I. Behavioral analysis. J Exp Biol 93:1–18

Tomioka K, Chiba Y (1984) Effects of nymphal stage optic nerve severance or optic lobe removal on the locomotor rhythm of the cricket, *Gryllus bimaculatus*. Zool Sci 1:385–394

Truman JW (1974) Physiology of insect rhythms. IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoth. J Comp Physiol 95:281–296

Waddell B, Lewis RD, Engelmann W (1990) Localization of the circadian pacemakers of *Hemideina thoracica* (Orthoptera; Stenopelmatidae). J Biol Rhythms 5:131–139

Weber G, Renner M (1976) The ocellus of the cockroach *Periplaneta americana* (Blattariae). Receptory area. Cell Tissue Res 168:209–222

Wiedenmann G (1977) Two activity peaks in the circadian rhythm of the cockroach *Leucophaea maderae*. Interdiscip J Cycle Res 8:378–383

Wilson M (1978) The functional organisation of locust ocelli. J Comp Physiol 124:297–316

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Ich versichere, daß ich meine Dissertation

The circadian system of the cockroach *Leucophaea maderae*: role of the neuropeptide orcokinin and light entrainment

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den

(Sabine Hofer)