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Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*)

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Summary

The presence of an endogenous circadian clock in the brain of an animal was first demonstrated in the cockroach *Leucophaea maderae*. However, the clock's cellular basis remained elusive until pigment-dispersing hormone-immunoreactive neurons, which express the clock genes *period* and *timeless* in *Drosophila*, were proposed as pacemaker candidates. In several insect species, pigment-dispersing hormone-immunoreactive neurons are closely associated with the accessory medulla, a small neuropil in the optic lobe, which was suggested to be a circadian clock neuropil. Here, we demonstrate that ectopic transplantation of adult accessory medulla into optic lobe-less cockroaches restores circadian locomotor activity rhythms in *L. maderae*. All histologically examined cockroaches that regained circadian activity regenerated pigment-dispersing hormone-immunoreactive fibres from the grafts to original targets in the protocerebrum. The data show that the accessory medulla is the circadian pacemaker controlling locomotor activity rhythms in the cockroach. Whether pigment-dispersing hormone-immunoreactive neurons are the only circadian pacemaker cells controlling locomotor activity rhythms remains to be examined.

Key words: circadian rhythm, accessory medulla, locomotor activity rhythm, pigment-dispersing hormone neuron, pacemaker, cockroach, *Leucophaea maderae*.

Introduction

Lesion experiments demonstrated for the first time that the locomotor activity rhythm of the cockroach *Leucophaea maderae* is controlled by two bilaterally paired and mutually coupled endogenous circadian pacemakers, which reside in the brain's optic lobes (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Roberts, 1974; Sokolove, 1975; reviewed by Helfrich-Förster et al., 1998). It was convincingly shown in lesion experiments that cockroaches without optic lobes remained arrhythmic throughout their lifetime. But, because arrhythmic locomotion does not indicate the absence of an intact circadian clock, the most compelling evidence for the location of the circadian clock in the optic lobes of the cockroach was provided by transplantation experiments (Page, 1982). After exchange of whole optic lobes between animals with different circadian periods, the cockroaches regained circadian locomotor activity several weeks after the transplantation. Interestingly, the regained circadian period was controlled by the transplanted optic lobe (Page, 1982, 1983). The next step in localizing the oscillator in the cockroach was the discovery of the pigment-dispersing hormone-immunoreactive (PDH-ir) neurons, which share properties predicted for circadian pacemaker neurons (Homberg et al., 1991; Stengl and Homberg, 1994; Petri et al., 1995; reviewed by Helfrich-Förster et al., 1998). The PDH-ir neurons are associated with the accessory medulla (AMe), a small neuropil situated at the ventromedial edge of the medulla of the optic lobe (Homberg et al., 1991; Petri et al., 1995; Reischig and Stengl, 1996). Although lesion and transplantation experiments demonstrated that the suprachiasmatic nucleus (SCN) is necessary and sufficient for the control of circadian locomotor activity rhythms in mammals (Stephan and Zucker, 1972; Inouye and Kawamura, 1979; Sawaki et al., 1984; Ralph et al., 1990; Silver et al., 1996), conclusive evidence for the localization of the circadian clock in insects is still missing. In *Drosophila melanogaster*, PDH-ir lateral neurons contain the clock proteins PERIOD and TIMELESS, and these neurons have been suggested to be circadian pacemaker neurons in fruitflies and cockroaches (Zerr et al., 1990; Ewer et al., 1992; Helfrich-Förster and Homberg, 1993; Stengl and Homberg, 1994; Frisch et al., 1994; Helfrich-Förster, 1995, 1998, 2001; reviewed by Helfrich-Förster et al., 1998). In the absence of the lateral neurons, however, weak rhythmic activity still remains (Helfrich-Förster, 1998), and in the absence of the peptide pigment-dispersing factor fruitflies remain rhythmic with a shorter period for a few days in constant darkness before becoming arrhythmic (Renn et al., 1999). Thus, to clearly identify the insect circadian pacemaker it has to be shown that transplantation of the pacemaker candidate suffices for rhythm generation in arrhythmic animals. Here, we show that ectopic transplantation of fully differentiated AMe tissue grafts to an

optic lobe-less cockroach restores circadian locomotor activity. Furthermore, we demonstrate histologically that transplanted PDH-ir neurons regenerate to their original targets in the midbrain. The data show that the AMe is the circadian pacemaker controlling locomotor activity rhythms in the cockroach. Because only a small number of animals with regained rhythmicity could be examined histologically, it cannot be excluded that, adjacent to PDH-ir neurons, there are also other circadian pacemaker candidates.

Materials and methods

Animals

To obtain cockroaches (*Leucophaea maderae* Fabr.) with different endogenous circadian periods, two populations were reared in different light regimens (11 h:11 h L:D and 13 h:13 h L:D; 25°C and 30% relative humidity) according to Page and Block (1980). The animals were fed with dried dog food, potatoes and water *ad libitum*.

Surgery

All operations were performed on male cockroaches under steady CO_2 anaesthesia. Cell culture medium (L 15; GIBCO, Eggenstein, Germany) containing penicillin and streptomycin was used to rinse the wounds. For the initial left optic lobe (OL) section, a triangular cuticular flap was cut into the head capsule to expose the OL. With an iridectomy scissor, the optic nerves and the optic stalks were cut, and the OL was removed; the cuticle was flapped back in place and sealed with wax.

For transplantation of AMe tissue into the right antennal lobe (AL) of a host cockroach (Fig. 1B), its right brain hemisphere was exposed. With a razorblade fragment, a pocket was cut into the right AL. Then, the donor animal was decapitated, its brain exposed, and the perineurium of one OL was removed. According to external markers, tissue containing the AMe with its adjacent PDH-ir cells (in controls: tissue out of the adjacent medulla) was excised from the donors brain with a fine glass pipette (tip-∅, 150–250 μm). The tip of the pipette was stuck into the AL of the host animal, the graft tissue was carefully blown out and occasionally its position was corrected with an eyebrow hair. Then, the right OL was removed; the cuticle was flapped back in place and sealed with wax. In the OL-to-OL transplantations, animals were donors and host at the same time (Fig. 1A) and the AMe graft was implanted into the location of the host's removed AMe.

Activity analysis

Locomotor activity was monitored in running-wheels in constant darkness at 26°C as described previously (Stengl and Homberg, 1994). Activity was visualized with double-plot activity histograms; the heights of the bars represent the number of revolutions per 5 min, truncated at 30 revs min^{-1} (Stengl and Homberg, 1994). To distinguish rhythmic from arrhythmic locomotor activity (Figs 2, 3) we used χ^2-periodograms and mass entropy spectral analysis (MESA; Dowse and Ringo, 1989) and averaged locomotor activity plots per circadian day to scrutinize rhythmic data obtained. For a more objective, automated judgement of rhythmicity, we developed a new software in Visual Basic for Applications (VBA); the 'scan periodogram analysis with Rhythm-Detector' allows distinction between rhythmic and arrhythmic episodes in long data sets. Raw data were merged into 30-min intervals and converted into Excel 97 format. χ^2-periodograms were calculated with VBA according to the algorithms of Sokolove and Bushell (1978). The scan periodogram analysis was performed as follows. Over a defined single periodogram length (s.p.l.) of at least 8 days, the program calculated a periodogram from day 1 to day X, then from day 2 to day X+1, until the last day of the recording. For every periodogram, the software determined the maximum (Q_p = peak height), the period τ, the χ^2 for P=0.01 at τ, and the width of the peak [at the intersections with the Sokolove significance line (SSL; χ^2 for P=0.01)]. To normalize Q_p against χ^2, the quotient Q_p/χ^2 was calculated and plotted against the number of the starting day of the respective periodogram on the data record (Q_p/χ^2 curve in Fig. 3C). The according normalised χ^2 value is 1 and was plotted as well (χ^2 for P=0.01 in Fig. 3C).

To distinguish 'rhythmic' from 'arrhythmic' periodogram peaks, we averaged peak values from optic lobe-less cockroaches with no recognizable periodicities (see next paragraph). This analysis revealed 26.6% of arrhythmic periodogram peaks exceeding the SSL. These peaks had low peak heights (9.2±7.8%, mean ± S.D., with 0%=χ^2 and 100%=2×χ^2) and narrow widths (0.3±0.1 h) as compared with periodogram peaks calculated over rhythmic activity phases. Therefore, only peaks with heights of ≥20% and widths of

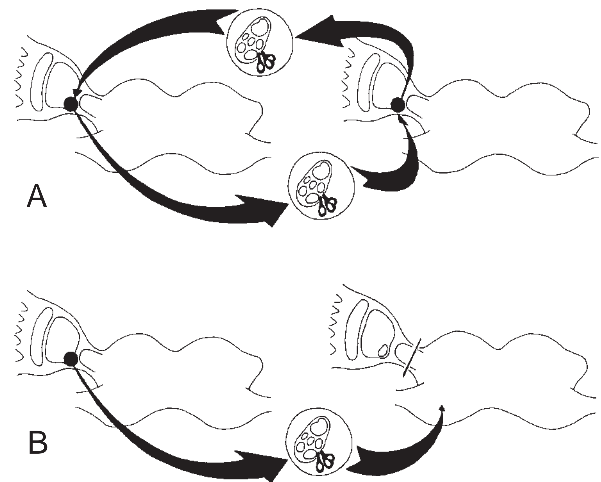

Fig. 1. Illustration of the two transplantation methods used in this study. (A) Accessory medulla (AMe)-grafts were exchanged between animals raised in 11 h:11 h L:D and 13 h:13 h L:D cycles. The left (in respect to the body axis) optic lobes were sectioned at least three weeks before the transplantations. (B) The AMe-grafts (in control experiments: medulla-grafts) were transplanted into the right antennal lobe of host animals. The remaining right optic lobes were subsequently removed. See Materials and methods for further details.

≥0.7 h, well above the values of arrhythmic periodogram peaks, were chosen to indicate rhythmicity. In this case, an arbitrary value of 0.5 was assigned to the 'rhythmicity' curve of the Rhythm-Detector plot; otherwise the value was set to zero (Fig. 3C). Hence, a locomotor record was judged as rhythmic if the postoperative scan periodogram analysis revealed at least one peak with a peak height of ≥20% together with a peak width of ≥0.7 h for at least two consecutive days (Fig. 3C). With MESA (Dowse and Ringo, 1989; with use of a demo version of El Temps 1.172, a chronobiological evaluation program written by Antoni Díez-Noguera, Barcelona, Spain) and averaged locomotor activity plots per circadian day, we confirmed rhythmicity in data sets obtained with our new evaluation software. Thus, we obtained a reliable, new, automated analysis method for long data records with objective measures of short episodes of rhythmicity, which avoided subjective selection of data sets and misjudgement of randomly generated activity peaks.

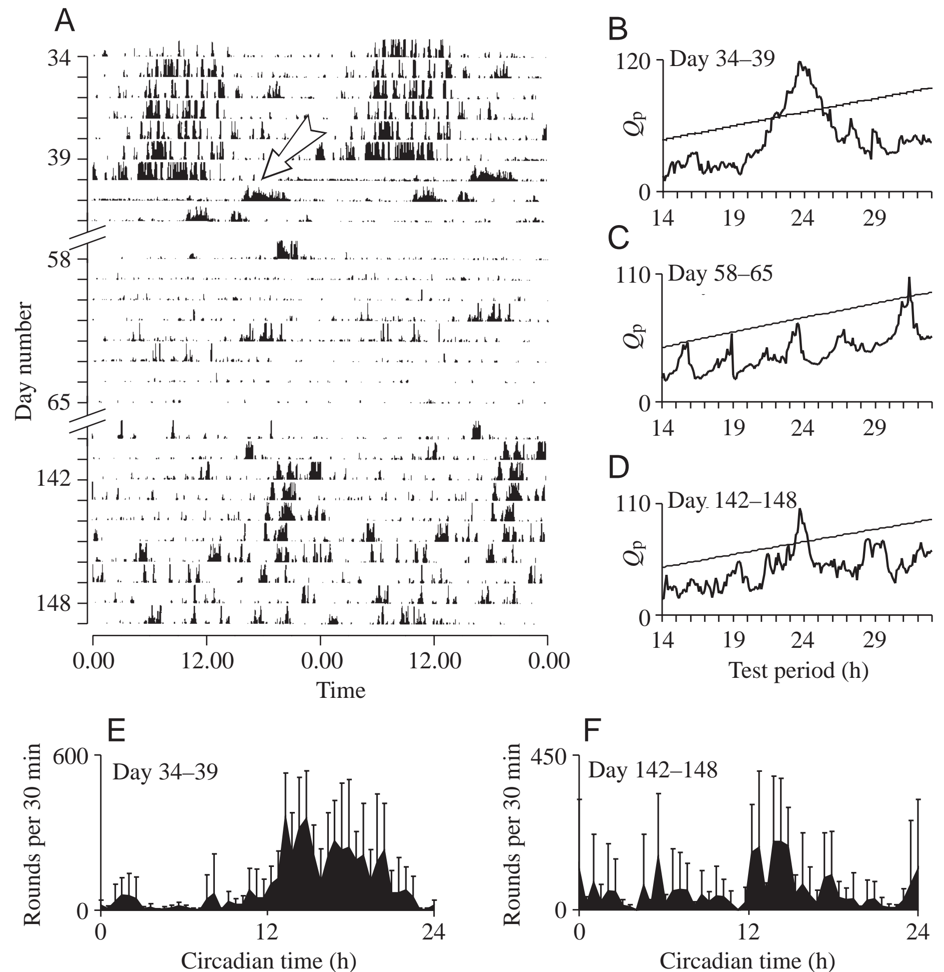

Fig. 2. Regained circadian rhythmic locomotor activity in optic lobe-less cockroaches after transplantation of one accessory medulla into the antennal lobe (animal ID 13/84; Table 2). (A) Double-plot activity histograms show circadian wheel-running activity (τ=24.2 h) in constant darkness before the operation (B; day 34–39, χ^2-periodogram). The operated cockroach is arrhythmic for 101 days after the operation, as shown in the χ^2-periodogram (C; day 58–65). Then, the cockroach regains rhythmic activity with τ=23.7 h, as shown in the χ^2-periodogram analysis (D; day 142–148; periodogram peak height=40.7%, width=1 h). Additionally, two activity plots show the mean locomotor activity ± S.D. of the animal during the course of a circadian day before (E; day 34–39) and after (F; day 142–148) the transplantation. Histological data of this animal are shown in Fig. 4C,D.

For the averaged activity plots, the circadian period lengths of the activity episodes in question were determined. The activity values were processed into a matrix using the same algorithms as for periodogram analysis (Sokolove and Bushell, 1978), with the determined period lengths as the test period. The means ± S.D. of activity amounts for every 30 min bin representing the same circadian time were calculated. The circadian day was normalized to 24 h. The activity onset of the first day of the examined activity episode was used as phase reference point and set to CT 12.

Analysis of 'arrhythmic' periodogram peaks

To analyse height and width of periodogram peaks in actual arrhythmic data records, we selected 30 bilobectomised, arrhythmic animals (as judged by eye on activity histograms and selective χ^2-periodogram analysis). Using the scan periodogram analysis, we calculated consecutive 10-day χ^2-periodograms (day 1 to 10, day 2 to 11, etc.; *N*=3445 for all animals) over the whole postoperative data record. The means ± S.D. of the heights and widths of all periodogram peaks that exceeded the SSL were calculated for every animal. Then, the total mean, as well as the mean of the standard deviations, for all 30 animals was calculated.

Moreover, we performed the periodogram peak analysis described above on randomly permutated activity data records (with Monte Carlo simulations) of 10 untreated, free-running cockroaches with prominent circadian rhythmic locomotor activity. The data records were about 10 weeks long, and every record was randomised and subsequently analysed 10 times. This resulted in a total of 7020 single periodograms, of which 88.1% showed peaks exceeding the SSL. The median height of these peaks was 10.2±9.8%, and the median width was 0.2±0.1 h; these values are within the range obtained by the respective evaluation of the generically arrhythmic animals and, therefore, further support the rhythmicity threshold

selected for the Rhythm-Detector. Interestingly, the number of periodogram peaks exceeding the SCL obtained with the randomly permutated data was much higher than in the arrhythmic animals. This is apparently due to a more even distribution of activity over the whole data record after the randomisation compared with generically arrhythmic animals. This results in a more even distribution of the Q_p values just below the SCLs in the periodograms and, therefore, leads to a higher probability of single Q_p values slightly exceeding the SCL.

We further analysed permutated activity records of the mentioned rhythmic animals with our Rhythm-Detector analysis. Automated evaluation allowed us to perform 1000 permutations and subsequent analyses for every data record, with single periodogram lengths of 10 days. This resulted in a total number of 10 000 Rhythm-Detector analyses, of which nine (0.09%) indicated rhythmicity applying to the rhythmicity criteria stated above. Thus, the Rhythm-Detector judges 99.91% of randomly permutated data records as arrhythmic and, thus, has a negligible error rate.

Immunocytochemistry

Following activity recordings, brains of operated animals (together with those of untreated animals to act as a control for staining) were dissected, fixed in a formaldehyde solution and either embedded in gelatine/albumin or in paraffin. Serial sections (gelatine, 30 µm; paraffin, 10 µm) were cut and stained using anti-β-PDH antiserum (Dircksen et al., 1987) with the three-step peroxidase–anti-peroxidase method according to Sternberger (Sternberger, 1979; see also Reischig and Stengl, 1996); detection of peroxidase was carried out with 3,3′-diaminobenzidine/H_2O_2. The paraffin sections were counterstained in 1% methylene blue.

To determine whether control or test animals regain rhythmicity in the locomotor assays, cockroaches were left in the running-wheels for as long as possible. Once the cockroach appeared to approach its natural death (when it became weak and showed either decreased or strongly increased activity), it was sacrificed and its brain was removed for immunocytochemistry. This focus on the long-term analysis of locomotor activity records of transplanted and control animals necessarily takes into account that several of the operated animals will die unexpectedly before they can be examined immunocytochemically.

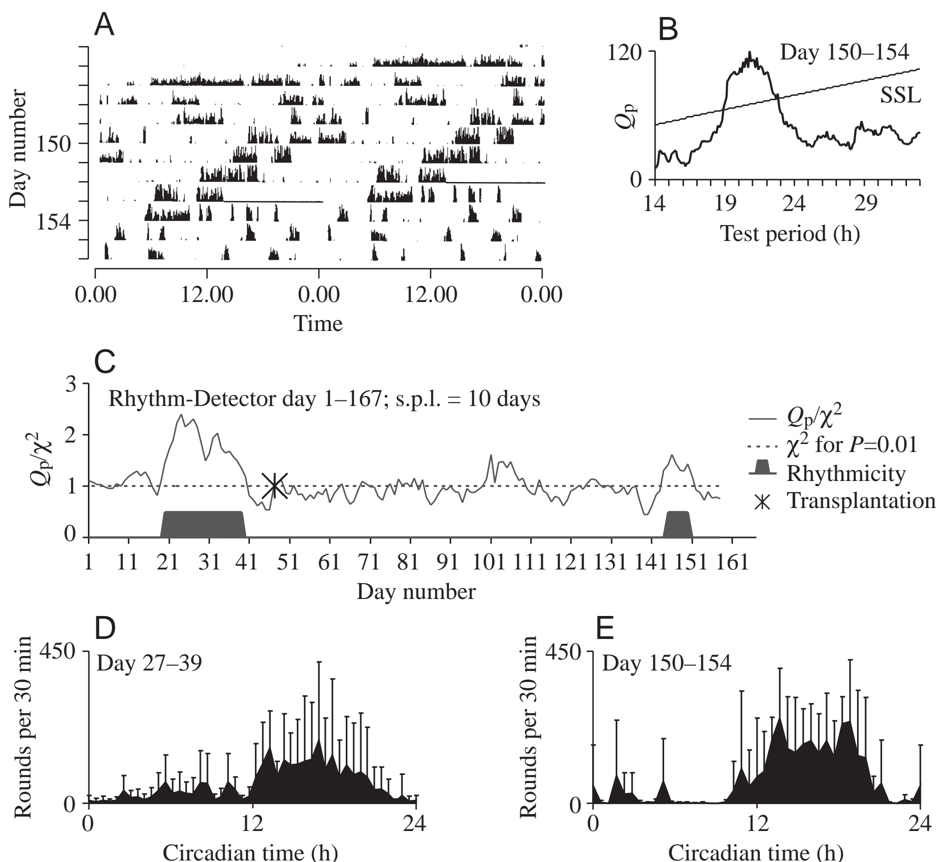

Fig. 3. (A–E) Double-plot activity histogram (A; day 145–156) and χ^2-periodogram analysis (B; day 150–154; Sokolove significance line=SSL=χ^2 for P=0.01) of another optic lobe-less cockroach (animal ID 13/21; Table 2) shows circadian wheel-running activity (τ=20.8 h) in constant darkness 148 days after the transplantation. The solid line at day 153 indicates computer failure. (C) The rhythm scan periodogram plot (Q_p/χ^2) over the complete length of the wheel-running recording (day 1–167) detects rhythmic peaks in consecutive 10-day-χ^2-periodograms (rhythmicity) before removal of the remaining optic lobe (day 19–39) and after the transplantation (day 143–151). s.p.l. = single periodogram length. Additionally, rhythmic activity can be seen in the two activity plots, which show the averaged locomotor activity ± S.D. of the animal during the course of a circadian day before (D; day 27–39) and after (E; day 150–154) the transplantation.

Results

To determine whether the AMe with PDH-ir output neurons is sufficient for controlling locomotor activity rhythms in insects, we performed transplantation experiments in a large insect, the cockroach *L. maderae* (Figs 1–4). Grafts containing the AMe with PDH-ir cells (Fig. 4A) were selectively excised from optic lobes of adult cockroaches using glass micropipettes and transplanted into the brains of adult host cockroaches. Then, the host animals were set back into running-wheels to search for regained circadian locomotor activity. Because many wild-type cockroaches with intact circadian pacemakers do not show circadian locomotor activity in running-wheel

assays, the absence of rhythmicity does not necessarily indicate the absence of an intact clock. Only positive behavioural evidence indicates the presence of an intact circadian clock (Stengl and Homberg, 1994). Thus, we wanted to know whether transplantation of the AMe alone, but not transplantation of adjacent medulla tissue, allows recovery of circadian locomotor activity. In addition, we wanted to examine whether all cockroaches with regained circadian activity also show regeneration of ectopic PDH-ir neurons into original target areas in the protocerebrum. This would suggest that PDH-ir neuronal connections to the central brain are necessary for expression of circadian locomotor rhythms, especially since the PDH-ir somata only comprise 1% of all grafted somata.

AMe-grafts measured approximately 150–250 μm in diameter and included the neuropil of the AMe with approximately 1000 associated cells (970±258, *N*=7), among them 12±4 (≈1%) PDH-ir somata. By raising one group of cockroaches in an 11 h:11 h L:D cycle and another group in a 13 h:13 h L:D cycle, we obtained two populations of animals with significantly different endogenous periods in free-running activity rhythms (Page and Block, 1980; Page, 1982). In a total of 179 individuals (*N*=91 for the 11 h:11 h L:D cycle, and *N*=88 for the 13 h:13 h L:D cycle), the left optic lobe was removed. Then, the periods of the free-running locomotor activity rhythms of the one-lobed cockroaches were assessed in running-wheel assays (see Materials and methods). Period lengths of these operated cockroaches were 22.56±0.41 h (*N*=70, 11 h:11 h L:D) and 23.79±0.33 h (*N*=58, 13 h:13 h L:D). Of the 179 animals, 106 (54 in the 11 h:11 h L:D cycle, and 52 in the 11 h:11 h L:D cycle) were selected for further experiments, while 73 cockroaches (41%) were excluded because of a lack of stable rhythmic locomotor activity rhythms. Altogether, 87 (82%) of the 106 operated animals, which survived in running-wheel assays for more than 14 days (3.0±1.5 months), were evaluated further, and, if they did not die before, were processed for immunocytochemistry. We focused on long-term analysis of locomotor activity records of transplanted and control animals to search for recovery of rhythmic activity. Thus, we favoured long-term survival at the expense of fast and frequent immunocytochemical analysis.

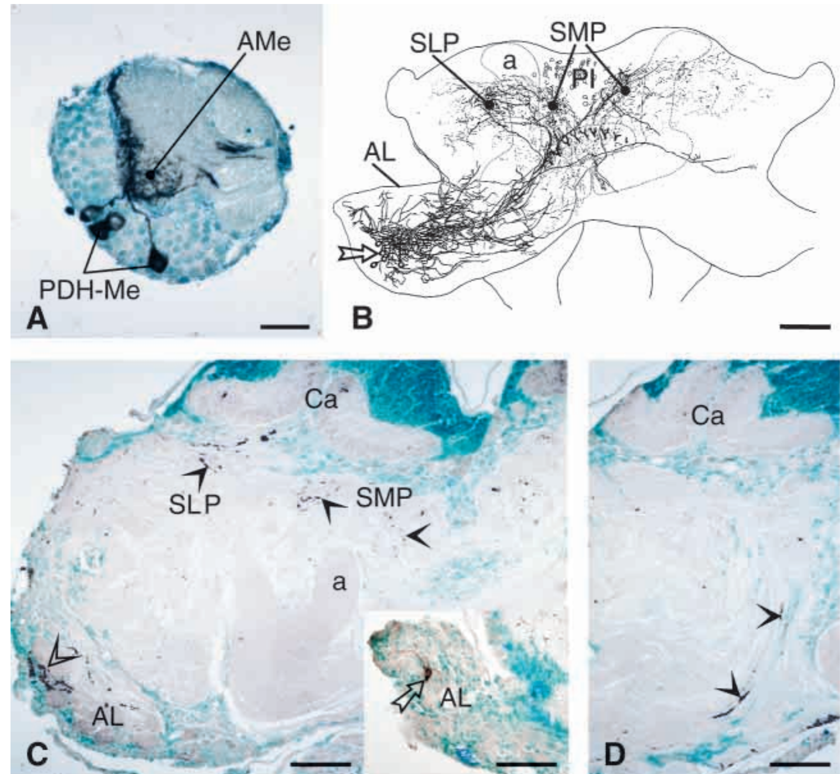

Fig. 4. PDH-immunoreactivity in an accessory medulla (AMe)-explant as used for transplantations (A) and in the central brains of two postoperatively rhythmic cockroaches (B–D). (A) In the 10-μm paraffin section of an excised AMe-graft, two large- and two medium-sized PDH-ir medulla neurons (PDH-Me) send processes into the AMe. Counterstaining with methylene blue shows unstained somata next to the PDH-Me. (B) Reconstruction of PDH-immunoreactivity in the brain of a postoperatively rhythmic cockroach (animal ID 11/16; Tables 2, 3). Three large- and two medium-sized grafted PDH-ir cells in the antennal lobe (AL; arrow) project *via* new routes to original arborisation sites in the superior medial and superior lateral protocerebra (SMP and SLP, respectively). Faintly stained PDH-ir neurons in the pars intercerebralis (PI) give rise to spotted staining in the protocerebrum, which can be clearly distinguished from regenerated fibres. a, alpha lobe. (C) Frontal brain section of the animal (animal ID 13/84; Tables 2, 3) in Fig. 2 with regenerated PDH-ir arborisations in the SMP and SLP (arrowheads) and antennal lobe (AL, open arrowhead). Inset: grafted large PDH-ir soma in the anterior AL (arrow). Ca, calyces of the mushroom bodies. (D) A more posterior slice of the same brain shows regenerated fibres invading the protocerebrum *via* the antenno-glomerular tract (arrowheads). Scale bars: 50 μm (A), 200 μm (B), 100 μm (C,D).

In a first set of transplantation experiments, we exchanged the remaining AMe between cockroaches of different endogenous periods (*N*=7). Animals were donors and hosts at the same time; thus, the graft was implanted into the space of the host's removed AMe (Fig. 1A). Two of the seven animals regained circadian locomotor activity (Tables 1, 2) four weeks after the transplantations. Immunocytochemistry in one of these specimens revealed PDH-ir somata (*n*=2) at the transplantation site as well as regenerated PDH-ir fibres in the midbrain (data not shown). Because it was difficult to unequivocally distinguish implanted PDH-ir neurons from remaining host PDH-ir cells, we continued with ectopic transplantations. In 45 experiments, the AMe-graft was inserted into the right antennal lobe of arrhythmic cockroaches, which had both optic lobes removed (Fig. 1B). In one control group (*N*=22), the remaining optic lobe was removed without further transplantations. In another control group (*N*=20), the remaining optic lobe was removed and grafts of medulla tissue

Table 1. *Correlation between regained rhythmic activity and regenerated central PDH-ir projections*

	Rhythmic			Arrhythmic		
AMe transplantations (*N*=45)	13 (29%)			32 (71%)		
Controls (*N*=42)	5 (12%)			37 (88%)		
	+PDH	–PDH	No histol.	+PDH	–PDH	No histol.
AMe transplantations	4	0	9	10	8	14
Controls	1	0	4	2	19	16

N represents the number of rhythmic and arrhythmic operated and control animals according to Rhythm-Detector evaluation (see Materials and methods). The number of AMe transplantations comprises 7 transplantations into the optic lobe and 38 into the antennal lobe. +PDH and –PDH indicate the number of cockroaches with or without PDH-ir somata and regenerated PDH-ir fibres in the central brain, respectively. No histol. represents the number of specimens without histological examination.

Table 2. *Correlations between pre- and postoperative rhythmicity*

Animal ID	Initial light condition (h:h)	τ forerun (h)	τ donor (h)	τ post-op. (h)	Duration of rhythmic activity (days)	Onset of rhythmic activity (days)
AMe-transplantations						
11/16	11:11	22.4	23.9	23.2	14	44
11/18	11:11	22.6	23.8	20.4	7	54
11/23	11:11	22.4	23.8	23.9	5	99
11/47	11:11	22.2	23.8	23.6	6	16
11/49	11:11	23.0	23.8	27.8	5	19
11/68	11:11	22.7	23.8	23.2	9	25
11/79	11:11	23.1	23.8	27.9	9	15
13/01*	13:13	23.8	22.6	23.4	12	29
13/06*	13:13	23.8	22.6	23.4	11	25
13/05	13:13	23.9	22.6	23.2	12	17
13/21	13:13	23.5	22.6	20.8	5	148
13/84	13:13	24.2	22.6	23.7	7	102
13/87	13:13	23.3	22.6	28.2	5	10
Controls						
11/59	11:11	22.6	23.8	22.1	6	106
11/64	11:11	22.9	23.8	20.9	7	57
13/39	13:13	23.8	22.6	24.1	13	56
13/46	13:13	22.2	22.6	28.5	7	47
13/58	13:13	23.7	22.6	20.6	6	46

List of all animals that regained circadian rhythmic locomotor activity, as revealed by Rhythm-Detector analysis (see Materials and methods). Animals were raised in 11 h:11 h L:D or 13 h:13 h L:D and received accessory medulla (AMe)-grafts from donor animals of the opposite L:D cycle. τ forerun: free-running circadian period lengths (τ) in hours for mono-lobectomised animals assessed before the transplantation/control experiment. τ donor: τ of donor animals. τ post-op.: τ after transplantation/control experiment. The duration of the free-running rhythmic activity episode was determined on the activity histogram plots. Onset of rhythmic activity was counted from the day of the operation until the first day of rhythmic activity. *Animals received AMe-graft into the right optic lobe; all other animals received AMe-graft into the right antennal lobe.

next to the AMe were transplanted into the host's antennal lobe.

After transplantation or control surgery, in most cases the locomotor activity was disrupted for several days and then became arrhythmic. The amounts and patterns of arrhythmic locomotor activity largely varied between individuals as well as within the postoperative life span of a single individual. Rhythmic locomotor activity returned in several animals but often became arrhythmic again. Based on these observations, we developed a new method for an automated search of shorter rhythmic episodes in long data records with rather strict standards for rhythmicity, to avoid biased judgement of periodicity (see Materials and methods).

Applying this analysis, a total of 18 (21%) of the 87 animals examined regained rhythmic locomotor behaviour (Tables 1, 2). Among the rhythmic animals were 13 cockroaches with AMe transplants and five controls (Tables 1, 2; Figs 2, 3). Significantly more animals regained

rhythmicity in the transplantation group (29%) *versus* the control group (12%), as tested with a single-tailed two-by-two frequency table, which involves a χ^2 test with one degree of freedom (χ^2=3.82, P<0.05, d.f.=1). As a second test, we applied a G-test of association, which compared the distributions of values between the AMe-transplanted and control groups. We assumed the results of the control operations as the predicted values for the AMe transplantations, if the transplantations would have no effect. Therefore, we would expect six rhythmic animals in the 45 AMe transplantations. However, the frequency of rhythmic animals differed significantly from those predicted by the control operations (G=7.44, P<0.05, d.f.=1).

The regenerated rhythmic behaviour of all these animals differed in at least one of the following criteria from rhythmic behaviour of normal animals: (1) rhythmicity was only transiently maintained (Table 2), (2) the onsets of locomotor activity were more variable, (3) phases of rhythmic activity were sometimes interrupted by bursts of continuing activity, (4) the amount of activity often fluctuated from one circadian day to the next, (5) unusual period lengths sometimes occurred (Table 2) and (6) rhythmic activity phases were often introduced by long bursts of activity (Fig. 3A). No correlation was detectable between the periods of donors and hosts (Table 2).

Of the 45 AMe transplantations, 22 (49%) animals could be examined histologically before they died. Of these, PDH-ir somata (n=1–5) in the antennal lobe and PDH-ir midbrain arborisations were observed in all of the cockroaches that regained rhythmicity after transplantation of the AMe (N=4; Tables 1, 2; Fig. 4B–D). Among the transplanted PDH-ir neurons, mostly two of their three size classes – the medium-sized (12–16 μm) and large (>16 μm) – but only one of the small (<12 μm) class PDH-ir somata were found (Table 3). Additionally, 10 (31%) of the 32 arrhythmic AMe-transplanted animals expressed PDH-ir terminals in the midbrain (Table 4). In all rhythmic animals examined (including one rhythmic control animal) regenerated PDH-ir fibres arborised in the superior medial and superior lateral protocerebra (SMP and SLP, respectively; N=5), but PDH-immunoreactivity in the ventro- or inferior lateral protocerebra or in the posterior optic tubercles was not found in all rhythmic animals (Table 4). In the AMe-implanted antennal lobes, we did not find any AMe-like neuropil structure retained from the implanted tissue, but regenerated PDH-ir fibres in the antennal lobe showed varicosities.

Regained rhythmicity in the one control animal, which could be histologically examined, also correlated with the presence of regenerated PDH-ir arborisations in the midbrain (Tables 2–4). The PDH-ir somata were found in the antennal lobe, which was implanted with medulla tissue, as well as in the stump of one sectioned optic lobe. Moreover, two other arrhythmic control animals each had one PDH-ir soma in an optic lobe stump. Because three of the 22 histologically examined controls exhibited PDH-ir neurons, the expected error rate for these difficult control surgeries was 14%.

Table 3. *Distribution of soma sizes of transplanted PDH-ir neurons in postoperatively rhythmic animals*

Animal ID	Soma sizes (μm)
11/16	20, 20, 13, 15, 23
11/18	20
13/01*	22, 16
13/39	11
13/84	18, 19

Most transplanted PDH-ir somata in postoperatively rhythmic animals belong to the medium-sized (diameter, 12–16 μm) and large (diameter, >16 μm) PDH-ir medulla somata. The control animal 13/39 also has four leftover PDH-ir medulla somata in the left optic lobe stump belonging to the larger somata. *Animal received graft into the right optic lobe.

Discussion

To examine whether the AMe is the circadian pacemaker of the cockroach *L. maderae*, we transplanted AMe grafts into optic lobe-less arrhythmic hosts. Here, we show that the ectopically transplanted AMe-grafts restore transient circadian rhythmic activity in optic lobe-less cockroaches. In addition, all of the histologically examined rhythmic animals showed PDH-ir regeneration into original target areas in the superior protocerebrum. Thus, our study demonstrates for the first time that the AMe contains the circadian pacemaker of the cockroach *L. maderae*. Future studies have to test whether other additional neurons, neighbouring the PDH-ir neurons, are responsible for the control of circadian locomotor rhythms.

Complete arrhythmicity after removal of both optic lobes was repeatedly demonstrated in *L. maderae*, as in other cockroaches (Roberts, 1974; Sokolove, 1975; Lukat and Weber, 1978; Page, 1982; Stengl and Homberg, 1994), crickets (Loher, 1972; Tomioka and Chiba, 1984; Abe et al., 1997) and wetas (Waddell et al., 1990). Thus, re-established rhythmicity after AMe transplantation in cockroaches without optic lobes strongly suggests that the transplanted AMe-grafts indeed contained the circadian pacemaker. This is supported by the significant difference in the number of rhythmic animals in the transplantation group *versus* the control group. Furthermore, only the presence of circadian rhythmicity argues for the presence of an intact circadian clock, but the absence of circadian locomotor rhythms does not prove the lack of an intact circadian clock (Stengl and Homberg, 1994; Stengl, 1995). This is also shown by the occurrence of 41% apparently arrhythmic cockroaches with one intact circadian clock in the foreruns of the locomotor activity assays of the current study. Thus, the presence of successfully transplanted PDH-ir neurons in arrhythmic animals does not weaken the conclusion that the transplanted tissue contains the circadian clock. In addition, the selective transplantation of medulla control tissue next to the AMe, but within the predicted pacemaker location according to Sokolove (1975), restored rhythmicity in arrhythmic animals significantly less often than did AMe transplants. With an error rate of 14%, we also transferred

PDH-ir neurons during our control transplantations, or single PDH-ir medulla neurons were accidentally left in the remaining stump after optic lobe excision. This is not surprising because PDH-ir somata (and possibly other neurons of the AMe) are sometimes not directly beneath the AMe but slightly dislocated towards the medulla or lobula, where we set our cut. Thus, it is likely that in all of the 12% rhythmic controls, rhythmicity was generated by accidentally transferred or leftover AMe neurons, as shown by immunocytochemistry in three control animals (Table 4).

Thus, because cockroaches with intact circadian pacemakers sometimes show only short or no periods of rhythmicity, any episode of clear rhythmicity indicates that these animals contain circadian pacemakers, while arrhythmicity allows no final conclusion about the presence of an intact clock. Since no clear, objective measures for transient rhythmicity in long data sets had been published before, we took great care to develop new software and standards to distinguish rhythmic from arrhythmic episodes in long data sets. Because different analysis methods such as MESA and χ^2-periodogram analysis, as well as a subjective judgement by eye, confirmed our own software we consider our analysis program to be very reliable. In addition, because rather strict criteria were used for the distinction of rhythmicity *versus* arrhythmicity, we very likely underestimate the number of rhythmic animals in the transplantation group.

Because the lack of circadian rhythmicity in cockroaches without optic lobes is well established by lesion experiments from different laboratories (Roberts, 1974; Sokolove, 1975; Lukat and Weber, 1978; Page, 1982; Stengl and Homberg, 1994), the return of rhythmicity in transplanted animals demonstrated in the present study shows that the transplanted tissue contains circadian pacemaker neurons. However, it does not distinguish which of the transplanted cells are circadian pacemaker cells. The correlation between the presence of regenerated PDH-ir processes in original target areas in all histologically examined rhythmic animals suggests a role for PDH-ir neurons as circadian pacemaker candidates. But, because we focused on long-term behavioural analysis at the expense of histological examination, only five of the cockroaches with regained rhythmicity could be examined histologically. Thus, we cannot draw a statistically significant conclusion about the cellular nature of circadian pacemaker neurons within the AMe transplants. But it is likely that at least

Table 4. *Distribution of regenerated PDH-ir fibres in the central brain of postoperatively rhythmic and arrhythmic animals*

	Number of PDH-ir somata			Regenerated PDH-ir fibres			
Animal ID	Total	Implantation site	Left OL stump	SMP	SLP	VLP and ILP	POTu
Rhythmic animals							
11/16	5	5	0	+	+	–	+
11/18	1	1	0	+	+	+	–
13/01	2	2*	0	+	+	–	–
13/84	3	3	0	+	+	–	+
13/39†	5	1	4	+	+	+	+
Arrhythmic animals							
11/02	8	6*	2	+	+	+	+
11/04	4	3*	1	+	+	–	+
11/06	3	0*	3	+	+	+	+
11/19	4	4	0	+	+	+	–
11/24	?	?	?	–	–	+	–
11/75	?	?	?	+	+	+	–
13/14	2	2	0	+	+	–	+
13/71	?	?	?	+	+	+	–
13/72	3	2	1	+	+	+	+
13/86	?	?	?	+	+	+	+
13/62†	1	0	1	+	+	+	+
13/80†	1	0	1	+	+	+	–

All of the five animals that regained circadian locomotor activity and could be histologically examined showed regenerated PDH-ir fibres in the superior median and superior lateral protocerebrum (SMP and SLP, respectively) but not necessarily in the ventrolateral and inferior lateral protocerebrum (VLP and ILP, respectively) nor in the posterior optic tubercle (POTu). As is known from behavioural assays in wild-type animals, apparent arrhythmicity in running-wheel assays is common and, thus, does not indicate the lack of an intact circadian pacemaker. Thus, it is to be expected that arrhythmic animals also show PDH-immunoreactivity. Additionally, because leftover PDH-ir neurons were found in the optic lobe (OL) stump in at least seven of 44 histologically examined animals, approximately 16% of OL excisions are expected to be incomplete. The control animals are marked with a dagger. *Animals received AMe-graft into the right OL; all other animals received AMe-graft into the right antennal lobe. Question marks represent somata that could not be localized and were apparently lost during the histological procedures.

a subgroup of PDH-ir neurons relays the circadian information to the midbrain because regeneration of PDH-ir neurons to original midbrain targets also correlated with regained circadian activity rhythms after transection of the optic stalk (Stengl and Homberg, 1994).

That the circadian pacemaker is at least partly composed of the PDH-ir neurons is further supported by findings in the fruitfly *Drosophila melanogaster*. In the fruitfly, pigment-dispersing factor is colocalised with the clock proteins PERIOD and TIMELESS in the same circadian pacemaker candidates, the lateral neurons (Helfrich-Förster, 1995, 1998). In addition, PDH is thought to be the crucial circadian output and coupling neuropeptide in insects (Renn et al., 1999; Blanchardon et al., 2001; Taghert, 2001; Reischig and Stengl, 2002). The importance of PDH-ir neurons for circadian activity is further supported by our observation that regained rhythmic activity strictly correlated with regeneration of transplanted PDH-ir neurons into the SMP and SLP, which are the clock's presumed output regions to locomotor centre pathways in wild-type cockroaches as well as in *Drosophila* (Homberg et al., 1991; Renn et al., 1999). Arborisations in the ventrolateral and inferior lateral protocerebrum, or posterior optic tubercle, which are also arborisation sites for PDH-ir terminals in wild-type cockroaches (Homberg et al., 1991), were not necessary for regained locomotor activity. Because rhythmicity resumed within ≥10 days of the operation (Table 2), circadian outputs to locomotor centre pathways appear to rely on regenerated neuronal connections but not on diffusible factors as shown in vertebrates (see Silver et al., 1996).

In both the fruitfly and the cockroach, there are different size groups of PDH-ir neurons next to the AMe. In *Drosophila*, small PDH-ir lateral neurons project to the superior lateral protocerebrum, and large PDH-ir lateral neurons appear to connect both optic lobes. In the cockroach, only subgroups of the large- and medium-sized PDH-ir neurons project to the protocerebrum and appear to connect both accessory medullae (Reischig and Stengl, 2002). Because in the current study large- and medium-sized transplanted PDH-ir neurons (Table 3) were found to regenerate to the superior lateral protocerebrum, it is likely that at least these two subgroups of the PDH-ir neurons are circadian pacemaker neurons that can drive circadian locomotor behaviour. This is in contrast to some findings in *Drosophila* indicating that only the small lateral neurons have pacemaker function (Park et al., 2000). However, since in *disco* mutants a single large PDH-ir neuron with aberrant connections to the superior lateral protocerebrum correlates with rhythmic locomotor behaviour (Helfrich-Förster, 1998), in the fruitfly, as in the cockroach, all PDH-ir neurons might be circadian pacemakers.

In contrast to the transplantation studies of Page (1982), our experiments could restore rhythmic behaviour but not period length. The regained period lengths were dissimilar to the donors' periods and ranged from 20.4 h to 28.5 h, closely reflecting the range of periods of non-coupled vertebrate SCN pacemaker neurons *in vitro* (Honma et al., 1998). Our immunocytochemical results indicate that only a few of the transplanted AMe neurons survived in the host's antennal lobe and that the neuropil of the AMe is lost in the host. Apparently, coupling interactions between transplanted AMe neurons that might generate the characteristic period of the wild-type cockroach are strongly reduced or missing. Thus, it is likely that an insect, as well as a vertebrate, circadian pacemaker constitutes its period *via* coupling in an interconnected neuronal network rather than *via* single independent pacemaker neurons (Honma et al., 1998). In addition, we assume that, adjacent to the PDH-ir neurons, other neurons of the AMe are also circadian pacemakers, because no correlation between the number of surviving PDH-ir somata and overt period lengths was observed (Table 2). The different period lengths might possibly indicate varying amounts of coupling between different pacemaker cells in the transplanted grafts (Michel and Colwell, 2001). This further adds to the assumption that the period of the circadian system depends on the period of single pacemaker cells as well as on the coupling between the pacemakers. Furthermore, in hamsters (*Mesocricetus auratus*), it was shown that quality and period lengths of rhythmicity after SCN transplantation are influenced by the number of re-established neuronal connections, the graft volume and the attachment site of grafts (Davis and Viswanathan, 1996; LeSauter et al., 1997). Therefore, the lack of the normal AMe neuropil might explain why regenerated rhythmicity occurred only transiently and why some animals did not regain rhythmic locomotor activity, even in the presence of successfully transplanted PDH-ir neurons. However, it cannot be determined whether these arrhythmic animals lacked a functional clock, since about one-third of non-operated cockroaches with intact circadian clocks did not express circadian locomotor activity in running-wheel assays.

With the exception of the transplantations of whole optic lobes by Page (1982), transplantation of small, defined brain regions containing circadian oscillators succeeded only in vertebrate species (Sawaki et al., 1984; Lehman et al., 1987; Ralph et al., 1990; Grosse and Davis, 1998), thus identifying the suprachiasmatic nucleus as the circadian pacemaker centre controlling locomotor activity in mammals. Only transplantations of embryonic or developing tissue within a narrow time window after birth of the donors succeeded (Romero et al., 1993; Kaufman and Menaker, 1993). Reorganisation of identifiable, fully differentiated central nervous system (CNS) neurons after ectopic transplantation from and into adult animals has not been reported before. Thus, the cockroach is not only an excellent model organism to study the neurophysiology of circadian timing but is also an interesting system for studies of neuronal regeneration after CNS damage or transplantation, because of its dramatic power in repairing severed neuronal connections.

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