

# Olfactory bulb neurons express functional, entrainable circadian rhythms

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## Abstract

Circadian pacemakers drive many daily molecular, physiological and behavioural rhythms. We investigated whether the main olfactory bulb is a functional circadian pacemaker in rats. Long-term, multielectrode recordings revealed that individual, cultured bulb neurons expressed near 24-h oscillations in firing rate. Real-time recordings of *Period1* gene activity showed that a population of cells within the bulb expressed synchronized rhythmicity starting on embryonic day 19. This rhythmicity was intrinsic to the mitral, and not the granule, cell layer, entrainable to physiological temperature cycles and temperature compensated in its period. However, removal of the olfactory bulbs had no effect on running wheel behaviour. These results indicate that individual mitral/tufted cells are competent circadian pacemakers which normally synchronize to each other. The daily rhythms in gene expression and firing rate intrinsic to the olfactory bulb are not required for circadian patterns of locomotion, indicating that they are involved in rhythms outside the canonical circadian system.

## Introduction

The mammalian retina and suprachiasmatic nucleus (SCN) of the hypothalamus regulate a variety of physiological and behavioural processes with periods close to 24 h. These two tissues fulfil the standard criteria for a circadian pacemaker, their daily rhythms synchronize to (and persist in the absence of) environmental cycles and show little change in their period over the physiological range of temperatures (Klein *et al.*, 1991; Herzog & Huckfeldt, 2003; Lee *et al.*, 2003; Tosini & Fukuhara, 2003). We recently reported that the isolated olfactory bulb (OB) expresses a circadian rhythm in gene transcription (Abe *et al.*, 2002). It is not known whether this intrinsic rhythmicity in gene transcription has a function at the levels of cellular physiology or behaviour and whether the OB retains the properties common to other circadian pacemakers. This study investigates whether the OB, like the retina and SCN, acts as a circadian pacemaker and participates in entrainment of the circadian system.

The laminar organization and postnatal maturation of the OB has facilitated extensive studies on its development and plasticity. This study correlates the development of intrinsic circadian function with differentiation in the OB. Using a bioluminescent reporter of *Period1* (*Per1*) gene activity, we found that, by embryonic day (E) 19, a population of OB cells expressed coordinated circadian rhythmicity in the rat OB. Using long-term multielectrode recording, we found that individual mitral, but not granule, cells can act as independent circadian pacemakers driving rhythms in firing rate. Removal of the OB showed that rhythms intrinsic to the OB were not required for normal circadian patterns of locomotion.

## Materials and methods

### Animals

Wild-type and homozygous *Per1-luciferase* (*Per1-luc*) transgenic Wistar rats (Yamazaki *et al.*, 2000) were maintained in the Hilltop animal facility at Washington University and fed *ad libitum* (lights on at 07:00 h and off at 19:00 h, 22 °C). Proestrous females were paired with a male overnight. Pregnancy was confirmed within 3 h of lights on the following morning by the presence of sperm in the vaginal smear or a copulatory plug. Embryonic day 1 and postnatal day (P) 1 were defined as the day after successful mating and the day of birth, respectively. Gestational age was confirmed based on published morphological guidelines (Altman & Dittmer, 1962).

On the desired gestational day, pregnant mothers were anaesthetized using 0.2% Avertin 30–60 min before lights off. Pups were quickly removed and decapitated. Rats older than 10 days of age were anaesthetized by CO<sub>2</sub> inhalation prior to brain removal. Embryonic OBs were dissected with scalpels and neonatal OBs were sectioned with a vibratome (coronal, 300 µm) in cold Hanks buffered saline (H6136; Sigma, St Louis, MO, USA). The OB sections used were from bregma, 5.7–6.7 mm (Paxinos & Watson, 1998). In some cases, the mitral or granule cell layers were separated with scalpels along the division of the mitral cell layer (including the external plexiform layer) and the granule cell layer. Experiments on animals less than 12 days of age used both sexes and experiments on older animals used males. All procedures were approved by the Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

### *Per1* expression

*Per1* gene activity was assessed by measuring bioluminescence from cultured tissues harvested from *Per1-luc* transgenic rats using methods similar to those in previous reports (Yamazaki *et al.*, 2000; Abe *et al.*, 2002). Tissue explants were placed on membrane inserts

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(Millicell-CM; Millipore, Bedford, MA, USA) with 1 mL of Dulbecco's modified Eagle's medium (D5030; Sigma) supplemented with 10 mM Hepes (H0887; Sigma), 2.2 mg/mL NaHCO<sub>3</sub> and 0.1 mM beetle luciferin (E1601; Promega, Madison, WI, USA). Each culture was sealed in a Petri dish, maintained at 36 °C in darkness and its bioluminescence was continuously monitored for 5–7 days with a photomultiplier tube (HC135–11MOD; Hamamatsu Corp., Shizouka, Japan).

For temperature entrainment experiments, temperature cycles were imposed by changing the incubator set point at 12-h intervals. Culture temperature changed at a rate of 2.5 °C/h so that a 1.5 °C shift was complete within 40 min. Temperature ranged by less than 0.3 °C without circadian variation at each set point.

### Multielectrode recordings

Long-term firing rate patterns were recorded from cells dispersed onto multielectrode arrays (60 electrodes, 200 µm spacing, 10 µm tips; Multichannel Systems, Reutlingen, Germany). The OBs of two pups or the SCN from eight pups (P1–P7) were pooled, dispersed and grown according to published methods (Herzog *et al.*, 1998). The OB (~90 000 cells/array) or SCN (~4000 cells/array) cells were cultured at 37 °C in 5% CO<sub>2</sub>/95% air for 2–4 weeks. Culture chambers were then covered with a fluorinated ethylene-propylene membrane and transferred to a recording incubator (Potter & DeMarse, 2001). Extracellular voltage signals from 60 electrodes were chosen from among four cultures and simultaneously recorded for at least 5 days. Action potentials larger than a user-defined voltage were digitized and a time-stamped, 2-ms cut-out of each spike was stored to the hard drive (MC Rack software; Multichannel Systems). Spikes from individual neurons were discriminated offline using principal component-based cluster analysis (Offline Sorter; Plexon Inc., Dallas, TX, USA) and the discharge rate of each neuron was determined in 10-min bins using Neuroexplorer software (Plexon Inc.).

The temperature inside the recording chamber was recorded with six thermocouples (PT-6 with Thermes-16; Physitemp Instruments, Inc., Clifton, NJ, USA). All temperature sensors were calibrated against a National Institute of Standards and Technology (NIST)-calibrated thermometer (15043 A; Fisher Scientific, Chicago, IL, USA). Long-term recordings showed that the temperature of the culture medium did not fluctuate with time of day and ranged by less than 0.2 °C from the set point.

### Morphological measurements

The diameter of OB cells cultured on electrode arrays ( $n = 2$  cultures harvested from two animals) was measured from linearly calibrated, digitized images of living cultures (Retiga 1350EX; QImaging, Burna, Canada) using Northern Eclipse software (Empix, North Tonawanda, NY, USA). Cells growing near the electrode array were randomly selected, the arborization of their dendrites was characterized and the diameter of their soma was measured at the widest point.

### Locomotor activity measurements and olfactory bulbectomy

Male rats (3 weeks of age) were individually housed in cages (32 × 20 × 17 cm high; Nalgene) with a 17.8-cm diameter running wheel. Cages were placed in ventilated chambers illuminated by fluorescent bulbs for 3 h/day ( $3.9 \times 10^{17}$ – $6.9 \times 10^{18}$  photons/s/cm<sup>2</sup> at the bottom of the cages; F30T12-SP41-RS; General Electric, USA). Wheel-running activity was recorded in 6-min bins (Clocklab; Actimetrics, Evanston, IL, USA). After 7 days, rats were anaesthetized (75 mg/kg ketamine and 0.5 mg/kg medetomidine i.p.) and mounted in a stereotaxic frame (David Kopf, Tujunga, CA, USA). Rats were either bulbectomized through a 3-mm hole above the OB using suction

( $n = 10$ ) or sham operated ( $n = 6$ ). Wheel revolutions were recorded for 13 days under the same light schedule (lights on at 07:00 h and off at 10:00 h), then for 21 days after delaying the light schedule by 6 h (lights on at 13:00 h) and, finally, for another 19 days after advancing the light schedule by 6 h (lights on at 07:00). The onsets and duration of daily activity were determined using a 6-h activity template in Clocklab. The average onset of activity relative to the time of lights on (termed the phase angle of entrainment) was determined from the 3 days prior to surgery and during the last 3 days in each of the three light schedules. The time to re-entrain following the shift in the light schedule, calculated to the nearest day, started on the day of the shift and ended on the day when stable entrainment was observed. At the end of the locomotor activity recordings, rats were killed by CO<sub>2</sub> overdose to confirm the bulbectomy.

### Data analysis

The phase, period and amplitude of the bioluminescence rhythms were determined as previously reported (Abe *et al.*, 2002). Briefly, a 24-h running average was subtracted from the raw data to reduce trends in the baseline that occur over subsequent days. A 3- and 24-h running average were calculated from the detrended dataset. The phase of peak bioluminescence between the crossings of these two smoothed lines was used as the phase marker for each cycle of the recorded rhythms. All times were reported in Zeitgeber time (ZT) relative to the animal's prior light cycle such that ZT0 was the time of light onset in the animal colony (07:00 h) and ZT12 was the time of light offset (19:00). The period and amplitude of *Per1-luc*, electrical and behavioural rhythms were also determined using chi-square periodogram analysis (Clocklab). The temperature coefficient, a measure of the rate of change of a reaction as a function of temperature, was calculated using the equation:

$$Q_{10} = (\tau_1/\tau_2)^{10/(T_2-T_1)}$$

where  $\tau$  is the culture's period and  $T$  is temperature. Statistical comparisons of phase, period and amplitude were analysed by a one-way ANOVA followed by the least significant difference (LSD) posthoc test.

## Results

### Ontogeny of *Per1* expression in the rat olfactory bulb

*Per1-luc* measurements showed the coordinated expression of the *Per1* gene in a population of OB cells. Circadian rhythms in *Per1-luc* activity were apparent in the OB of rats as young as E19 (Fig. 1a; Table 1). Neither the period (one-way ANOVA,  $F_{4,29} = 2.3$ ,  $P > 0.05$ ; Fig. 1b) nor amplitude (one-way ANOVA,  $F_{4,29} = 3$ ,  $P > 0.05$ ; Fig. 1c) of rhythmic OB cultures depended on age. However, the time of peak bioluminescence relative to the previous light schedule was age dependent (one-way ANOVA,  $F_{4,29} = 34.5$ ,  $P < 0.0001$ ; Fig. 1d). While E19 OB peaked in the late subjective night (around ZT21), P1 OB peaked at midday (around ZT6) and, by 1 week postnatal, the OB reached its adult phase peaking near dusk (around ZT12). This phase agrees with published results on the time of peak *Per1* expression in the adult OB (Abe *et al.*, 2002). These data suggest that a developmental programme regulates circadian phase in a group of OB cells over the first week of life until it assumes its adult phase.

### Circadian firing patterns of olfactory bulb neurons

To address whether rhythms in *Per1* transcription correlate with function in individual neurons, we recorded daily patterns of spontaneous firing rate from bulb neurons cultured on multielectrode arrays. The OB neurons from P1–P7 grew readily on the arrays. After 3 weeks

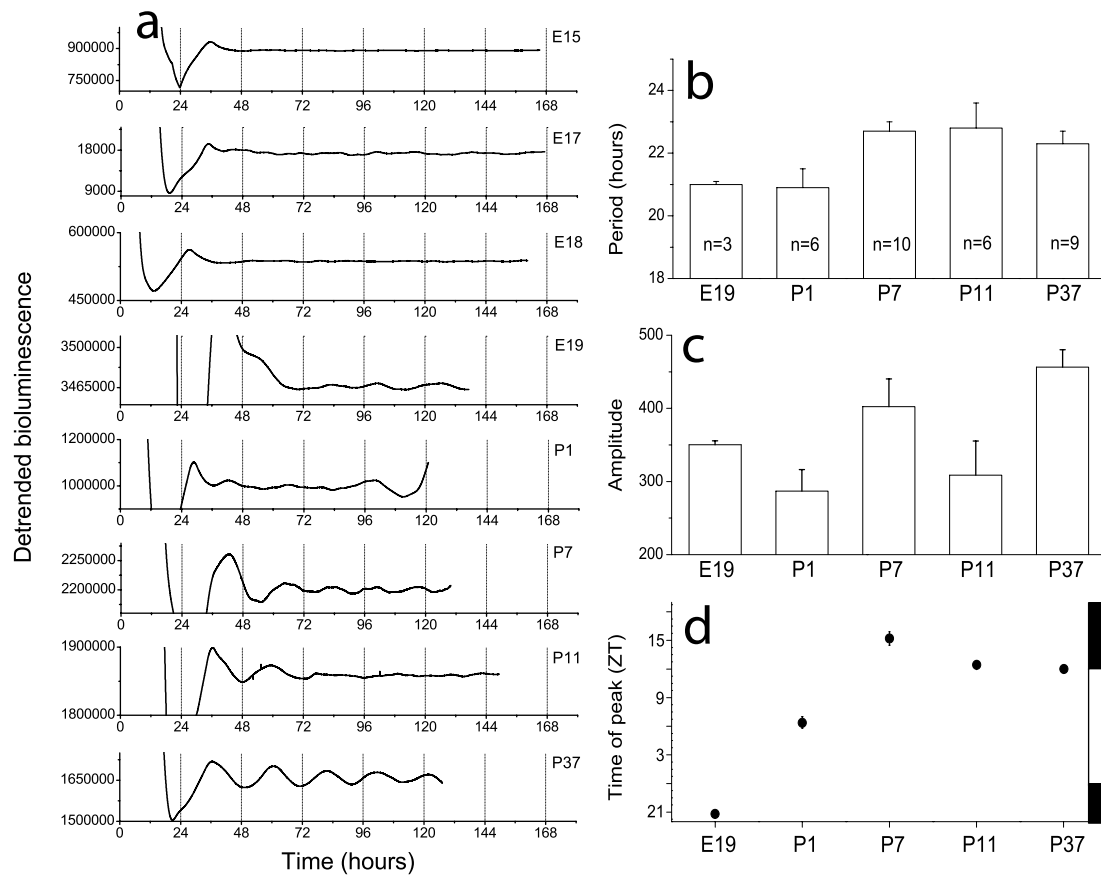


FIG. 1. Circadian patterns of bioluminescence recorded from olfactory bulb (OB) explants taken from rats carrying a *Period1-luciferase* (*Per1-luc*) transgene. (a) Daily oscillations of *Per1* gene activity were present in the OB by embryonic day (E) 19. (b and c) The average period and amplitude of rhythmic OB cultures did not depend upon developmental age. (d) From E19 to postnatal day (P) 7, the *Per1-luc* expression in the OB peaked progressively later until it reached its mature phase around 1 week postnatal. Phase is given in Zeitgeber time (ZT) where ZT0 is dawn in the animal colony (07:00 h) and ZT12 is dusk (19:00 h). Open and filled bars indicate times of light on and off. Error bars show SEM.

*in vitro*, two morphological cell types were apparent, small bipolar cells with a diameter of  $11.1 \pm 0.2 \mu\text{m}$  ( $n = 50$  cells from two cultures; mean  $\pm$  SEM) and pyramidal-shaped neurons with many processes and a diameter of  $33.4 \pm 1.0 \mu\text{m}$  ( $n = 50$  from two cultures; mean  $\pm$  SEM). These small and large cell types are consistent with the descriptions of cultured granule and mitral/tufted cells, respectively (Trombley & Westbrook, 1990).

We were able to reliably discriminate the firing patterns of 35 OB and 30 SCN neurons for at least 3 days (average of  $4.9 \pm 0.5$  days, range of 3–10 days for OB cells from three cultures and  $12.7 \pm 1.4$  days,

TABLE 1. Percentages (and numbers) of olfactory bulb explants which were rhythmic at different ages

Age	Percentage (n)
E15	0 (6)
E17	0 (7)
E18	0 (3)
E19	100 (3)
P1	100 (5)
P7	100 (10)
P11	100 (6)
P37	100 (9)

E, embryonic day; P, postnatal day.

3–20 days for SCN cells from three cultures). Approximately half of the recorded neurons (16 OB and 14 SCN) expressed circadian firing patterns. The peak-to-peak amplitudes of their biphasic action potentials were  $101.6 \pm 7.1 \mu\text{V}$  (mean  $\pm$  SEM) for OB neurons and  $67.0 \pm 3.0 \mu\text{V}$  for SCN neurons.

In general, rhythmic OB neurons showed similar peak firing rates to SCN neurons (0.4–4.2 Hz for OB neurons and 0.4–3.1 Hz for SCN neurons) but higher spontaneous activity during their daily trough and more variability in their amplitude and cycle-to-cycle onsets (compare Fig. 2a and c). The average periods of OB ( $22.8 \pm 0.7$  h) and SCN ( $24.9 \pm 0.9$  h) firing rate rhythms were similar to those seen in *Per1-luc* rhythms ( $23.0 \pm 0.7$  h for OB and  $24.5 \pm 0.3$  h for SCN, mean  $\pm$  SEM); the periods of the OB tended to be shorter than those of the SCN but did not reach significance (Fig. 3;  $P = 0.06$ , Student's *t*-test).

Individual OB neurons showed different circadian periods in the same culture. In one culture, the firing patterns of seven OB neurons were discriminated for 4 days. Periods ranged from 18.9 to 25.3 h and the times of peak firing on the first day of recording ranged from 13.9 to 22.1 h. Two cells recorded on two different electrodes 100  $\mu\text{m}$  apart showed a period difference of 2.3 h and peaked 6 h apart on the first day of recording (Fig. 4). These results indicate that the OB is a multi-oscillator structure and that each OB neuron may function as an independent pacemaker.

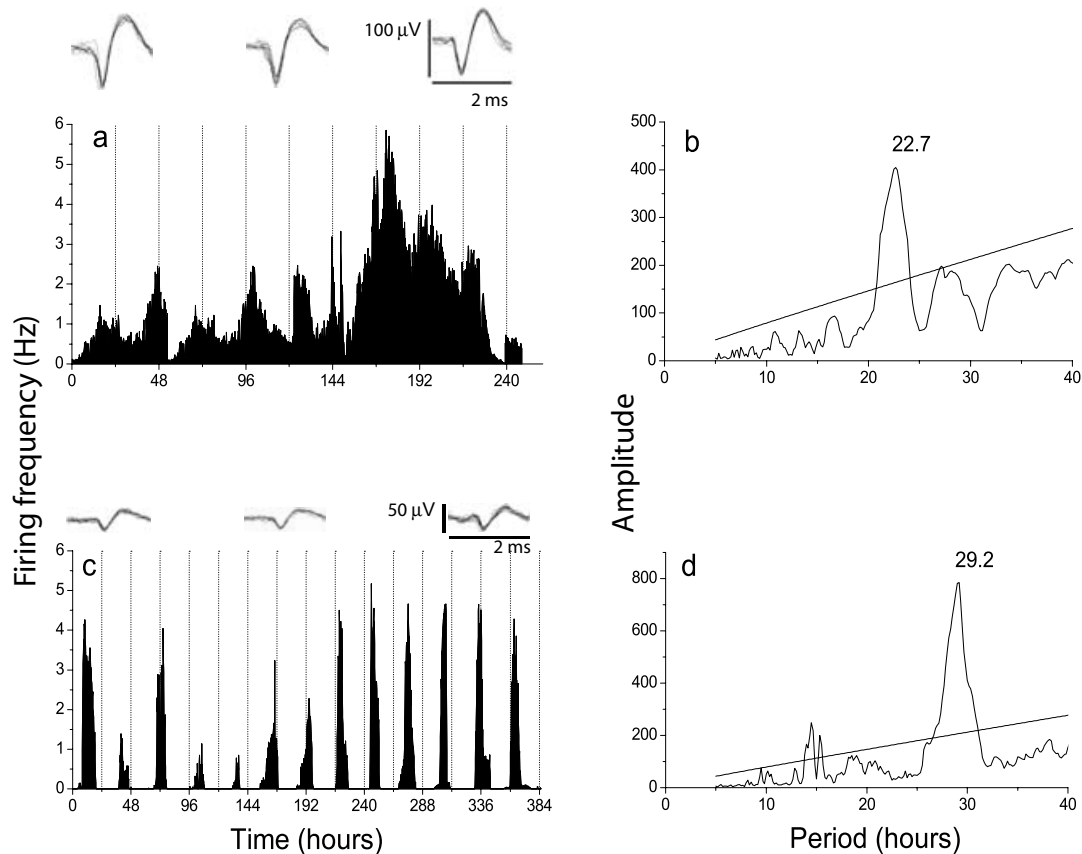


FIG. 2. (a) Circadian rhythm in electrical activity of a representative olfactory bulb (OB) neuron. Insets show 10 superimposed action potentials recorded at different times over 10 days to illustrate the reliable discrimination of the activity of a single OB cell. (b) Chi-squared periodogram analysis of the firing pattern from the cell in (a) shows a dominant frequency at 22.7 h. (c) Circadian firing pattern of a suprachiasmatic nucleus (SCN) neuron recorded for 16 days on a different multielectrode array. Insets show small action potentials typical of SCN neurons. The period estimate for this cell shown in (d) was 29.2 h.

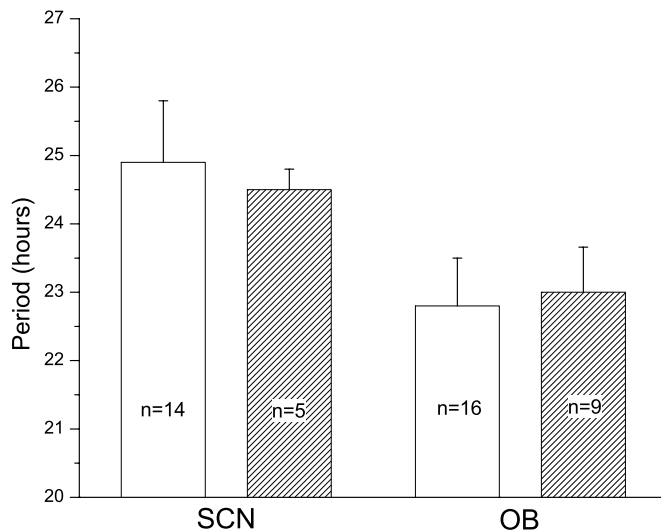


FIG. 3. The average periods of firing rate (open bars) and *Period1-luciferase* (*Per1-luc*) activity (hatched bars) for cultured suprachiasmatic nucleus (SCN) and olfactory bulb (OB) were similar. Period estimates were made from firing patterns of cells dispersed on postnatal day (P) 4–6 and cultured for 16 days and from *Per1*-driven bioluminescence of tissues explanted and recorded beginning on P37. While there was a tendency for the SCN to have a longer period than the OB, period differences were not significant ( $P = 0.06$ , Student's *t*-test). The periods of *Per1-luc* and electrical rhythms did not differ for either the SCN or OB ( $P > 0.1$ , Student's *t*-test). Error bars show SEM.

#### Circadian oscillations localize to the mitral/tufted cell layer

Within the OB, mitral/tufted and granule neurons are known to fire action potentials (Trombley & Westbrook, 1990; Chen & Shepherd, 1997; Cang & Isaacson, 2003). To determine which cell types express circadian rhythms, we recorded *Per1*-driven bioluminescence and electrical activity from isolated cell layers of P7 OB. All explants of the mitral cell layer expressed robust circadian rhythms in *Per1* activity ( $n = 6$ ; Fig. 5a) with an initial peak at ZT12.5  $\pm$  0.3 h and an average period of 21.5  $\pm$  0.7 h. In contrast, explants of the granule cell layer did not express circadian rhythmicity ( $n = 6$ ; Fig. 5b). Similarly, five neurons recorded from a dispersal of mitral/tufted cells showed circadian rhythms in firing rate for at least 3 days with a period of 20.1  $\pm$  0.6 h (mean  $\pm$  SEM; Fig. 5c), while no rhythmic neurons were found in cultures prepared from the granule cell layer (Fig. 5d). We did not determine the percentage of rhythmic mitral cells. These results indicate that circadian oscillations probably arise within the mitral/tufted cells of the OB.

#### Temperature entrainment of circadian rhythmicity in the olfactory bulb

Circadian pacemakers are only useful to the organism if they can synchronize to local time. To determine whether the OB can entrain to daily cues, we exposed OB explants to a 1.5  $^{\circ}$ C temperature cycle for 8 days (12 h : 12 h, 36.8  $^{\circ}$ C : 35.3  $^{\circ}$ C, warm at ZT0). The OB cultures synchronized their rhythmicity to these cycles (Fig. 6a). Cultures,

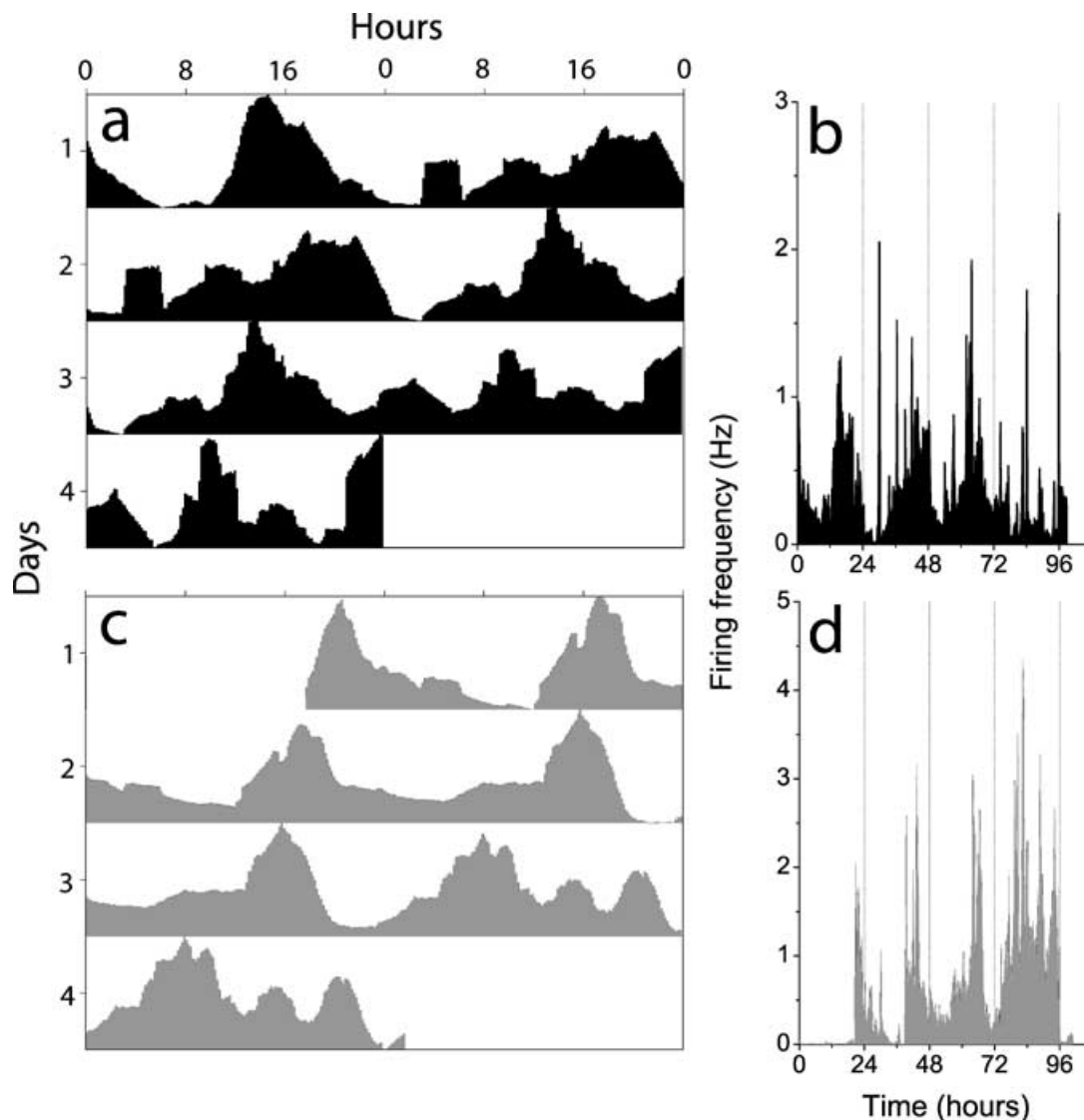


FIG. 4. Olfactory bulb neurons in the same culture can express different circadian periods. (a and c) Actograms for the firing patterns shown in (b and d), respectively. The two cells, recorded on electrodes 100  $\mu\text{m}$  apart, had periods of 22.8 h (top) and 20.5 h (bottom).

which peaked at approximately ZT12.3 in the first day of the temperature cycle, rapidly shifted to peak at approximately ZT20.1 in the second day of the temperature cycle ( $n=4$ , Fig. 6b). All cultures continued to peak just prior to the cool-to-warm transition for the next 7 days. Following the temperature cycle, OB cultures peaked approximately 24 h after the last cool-to-warm transition and oscillated with an average period of  $21.9 \pm 0.6$  h (mean  $\pm$  SEM). Results were similar in cultures taken from P1 and P19 animals ( $n=2$  at each age). These data indicate that the OB can entrain to physiological temperature oscillations and that this intrinsic entrainability develops early in the ontogeny of the OB.

#### Temperature compensation of circadian rhythms in the olfactory bulb

Circadian pacemakers also show relatively little change in their period at different constant temperatures. To determine if the OB is temperature compensated, we compared *Per1-luc* rhythms at 30  $^{\circ}\text{C}$  ( $n=10$ ) and 36  $^{\circ}\text{C}$  ( $n=9$ ). The circadian period of OB slices was slightly longer at 30 than at 36  $^{\circ}\text{C}$  ( $23.7 \pm 0.5$  h and  $21.6 \pm 0.4$  h, respectively;

mean  $\pm$  SEM). This equates to a  $Q_{10}$  of 1.16 which is within the range seen for other circadian systems. In contrast, the amplitude of rhythmicity had a  $Q_{10}$  of 0.67 over the same temperature range ( $274.8 \pm 15.9$  at 30  $^{\circ}\text{C}$  and  $348.6 \pm 15.5$  at 36  $^{\circ}\text{C}$ ). These results suggest that circadian period, but not amplitude, is temperature compensated.

#### Removal of the olfactory bulbs has no effect on locomotor behaviour

Indirect evidence has implicated the OB in the regulation of daily rhythms in locomotor activity. Removal of the bulbs lengthens the free running period in mice and hamsters (Possidente *et al.*, 1990; Lumia *et al.*, 1992) and delays photic re-entrainment in male *Octodon degus* (Goel *et al.*, 1998). We assessed the necessity of the OB for normal locomotor patterns in bulbectomized rats. Although rats without their OBs tended to synchronize to a delayed light schedule approximately 1.1 days sooner than intact rats ( $P=0.08$ ), we found no significant effect of bulbectomy on the phase angle of entrainment, rate of re-entrainment to advances or delays in the light schedule or amount

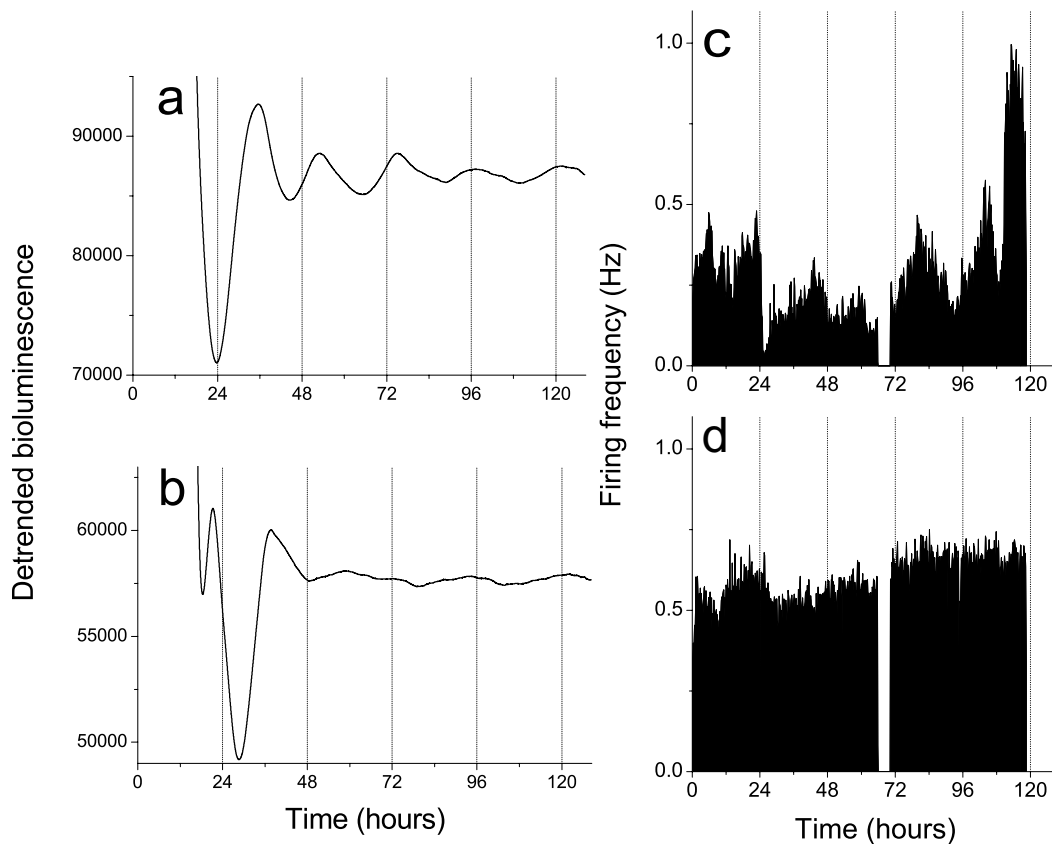


FIG. 5. Explant cultures of (a) the mitral cell layer showed *Period1-luciferase* circadian rhythms while (b) the granule cell layer did not. Similarly, cells dispersed from (c) the mitral cell layer showed firing rate circadian rhythms while (d) cells from the granule cell layer did not. Data collection was briefly interrupted around 72 h for a medium change in (c and d).

of daily activity (Table 2 and supplementary figure Fig. S1 – see Supplementary material section). These data suggest that, in rats, the OB is not required for photic entrainment or period regulation of locomotor rhythmicity.

## Discussion

### *Olfactory bulb is an independent, functional circadian pacemaker*

Three features conserved among circadian oscillators are their abilities to express near 24-h rhythms in the absence of environmental timing cues, synchronize physiological rhythms to daily cycles and accurately mark time over the range of physiological temperatures. The mammalian OB expresses intrinsic circadian rhythms in gene expression and neuronal firing rate, can be entrained by daily temperature cycles of 1.5 °C and is temperature compensated in its period. These properties are also found in the mammalian SCN and retina (Tosini & Menaker, 1996; Ruby *et al.*, 1999; Herzog & Huckfeldt, 2003). It is not yet known if the multitude of other tissues and cell types which exhibit circadian rhythms in gene expression also fulfil the criteria for a functional circadian pacemaker (Balsalobre *et al.*, 1998; Yamazaki *et al.*, 2000; Abe *et al.*, 2002). It may be that multiple tissues function as semiautonomous timekeepers as in other vertebrates (Underwood, 2001), invertebrates (Page, 2001) and plants (Thain *et al.*, 2000). For example, we recently found that the OB is still rhythmic when explanted from animals that have been behaviourally arrhythmic for over 1 month (Granados-Fuentes *et al.*, 2004). However, it is also possible that some rhythms in gene expression may have no functional

consequence; conversely, some tissues which are rhythmic *in vivo* may lack their own pacemaking ability and be driven by other pacemaking tissues. Regardless, the identification of multiple circadian pacemakers leads to a number of basic questions. Do these pacemakers use a common molecular machinery to generate oscillations and drive physiology? Do they interact *in vivo* to regulate common or distinct behaviours?

Limited evidence indicates that circadian rhythms of the retina, SCN and OB share some of the same molecular bases. Recordings from individual SCN and OB neurons show that the clock regulates neural firing rates and is probably cell autonomous. The periodicities of the SCN and retina are also similarly shortened by the *tau* mutation (Tosini & Menaker, 1996; Liu *et al.*, 1997). Importantly, however, the delayed phase and reduced robustness of OB rhythms, compared with SCN, suggests a functional difference between these oscillators. These differences are probably intracellular, as has been found for the pacemakers in the antennae and lateral neurons of *Drosophila* (Krishnan *et al.*, 2001), but may also reflect differences in intercellular communication.

The similar periods of firing rate and *Per1-luc* rhythms in the SCN and OB suggest a functional relationship between *Per1* expression and electrical excitability. This is consistent with the recent report which correlated levels of *Per1* expression and firing rate of individual SCN neurons (Kuhlman *et al.*, 2003). How the molecular timekeeping mechanism controls diurnal membrane excitability and how membrane potential controls clock gene expression are unknown (McMahon & Block, 1987; Nitabach *et al.*, 2002; Panda *et al.*, 2002; Pennartz *et al.*, 2002).

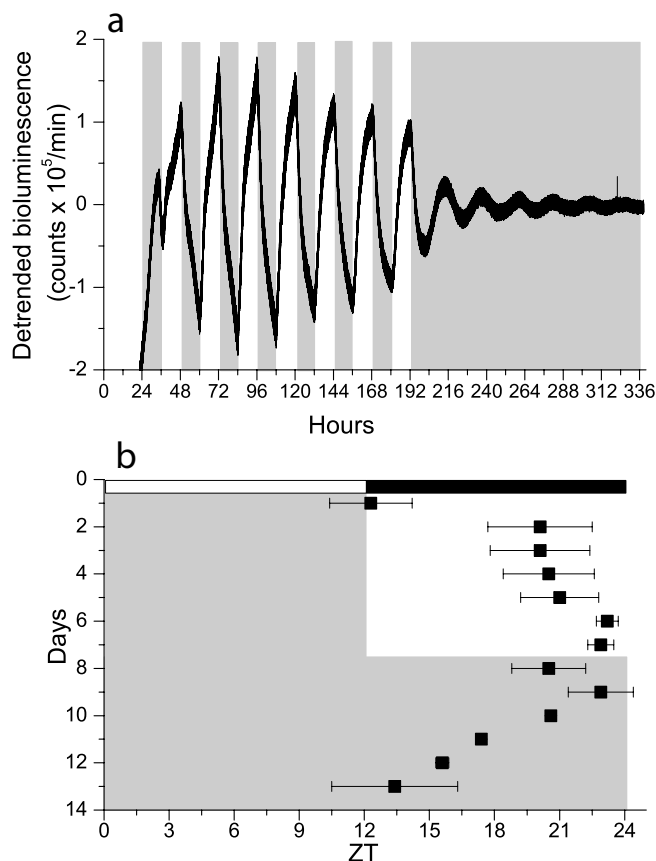


FIG. 6. Temperature cycles entrain the olfactory bulb (OB) *in vitro*. (a) Bioluminescence recording from an OB explant in a temperature cycle of 35.3 °C (white fill) to 36.8 °C (grey fill). *Period1* (*Per1*) gene activity peaked near the cool-to-warm transitions during the 8 days of cyclic temperature. The rhythm free-ran from the last cool-to-warm transition in constant temperature. (b) The peak of OB rhythmicity (■, mean  $\pm$  SEM,  $n = 4$ ) was tightly regulated by the temperature cycle (grey shows warm phases) so that, after 8 days, the OB had delayed to peak approximately 1 h prior to the daily warm phase. Relative to the animals' prior light cycle, the temperature cycle advanced the *Per1-luciferase* rhythm to around subjective dawn (horizontal open and filled bars indicate times of lights on and off in the animal colony, defined as Zeitgeber Time (ZT) 0 and 12, respectively).

#### Rhythmicity and cellular differentiation mature postnatally in the olfactory bulb

The mechanisms that initiate circadian oscillations in any cell type are unknown. Circadian rhythms in the rat OB were first detectable on E19. At this age, many mitral cells have differentiated, grown axons

and begun to receive dendritic input from olfactory sensory neurons (Lopez-Mascaraque & De Castro, 2002). The phase of circadian rhythmicity gradually matures over the first postnatal week. During this time, neuronal precursor cells migrate into the bulb, differentiate into granule and peri-glomerular cells and form synapses with relay neurons (Hinds, 1968; Hinds & Hinds, 1976; Bayer, 1983). This ontogeny is consistent with our localization of circadian rhythmicity to mitral/tufted, and not granule, cells. However, mitral cell differentiation and *Per1* expression in the OB clearly precede the onset of circadian rhythmicity in the OB. Notably, initiation of rhythmicity in the rat SCN has been reported around E19 (Fuchs & Moore, 1980) and its synaptic organization matures over the first 3 weeks postnatal (Lenn *et al.*, 1977). Thus, circadian periodicity develops concurrently in the SCN and OB.

#### What function does the olfactory bulb circadian oscillator play in behaviour?

The circadian rhythm in firing rate seen in OB neurons indicates that the circadian clock in these cells functions to modulate membrane excitability. This is the first demonstration that firing rate fluctuates with time of day in mitral/tufted cells. The firing rates seen *in vitro* from dispersed OB neurons are similar to the spontaneous rates reported for mitral/tufted cells in anaesthetized rats (cf. Cang & Isaacson, 2003). Although it is not known if *in vivo* OB neurons express daily rhythms in discharge rate, circadian modulation of olfaction has been reported in mammals (Amir *et al.*, 1999; Funk & Amir, 2000) and shown to be intrinsic to the *Drosophila* antennae (Krishnan *et al.*, 1999). The presence of a circadian pacemaker in the OB may, therefore, simply reflect the need for local, temporal regulation of olfactory processing. How the circadian rhythms in transcriptional and electrical activity in the bulb relate to olfaction is unknown.

In addition, the OB may also regulate other circadian pacemakers or coregulate some behaviours with other pacemakers. We found that bulbectomized rats showed no changes in the period or entrainment to light of their locomotor behaviour. This is in contrast to results found in mice, hamsters and *O. degus* implicating the OB in the regulation of period and entrainment of circadian rhythms in locomotion (Possidente *et al.*, 1990; Lumia *et al.*, 1992; Goel *et al.*, 1998). This may reflect species differences. Alternatively, the role of the OB may develop later in adulthood as we found no effects of bulbectomy on rats from 4 to 11 weeks of age, whereas other studies found effects on mice at  $\sim$ 50 days, hamsters at  $\sim$ 22 days and *degus* at 1–2 years of age. This probably reflects a delay in the maturation of the output of the OB circadian pacemaker as we found that *Per1* rhythmicity does not change in phase, period or amplitude after 1 week of age.

It may be that the OB plays a role in olfactory entrainment of the pacemaker in the SCN, e.g. daily exposure to the scent of a conspecific suffices to entrain *O. degus* (Governale & Lee, 2001). Others have

TABLE 2. Bulbectomy (OBX) had no significant effect on entrainment or activity in rats

	Phase angle of entrainment (h)			Rate of re-entrainment (days)		Activity (average counts/day)	
	Lights on at		07:00 h	Rate of re-entrainment (days)		Activity (average counts/day)	
	07:00 h	13:00 h		6 h delay	6 h advance	Daytime	Night-time
OBX ( $n = 10$ )	11.6 $\pm$ 0.5	11.5 $\pm$ 0.5	11.5 $\pm$ 0.2	11.6 $\pm$ 0.4	14.0 $\pm$ 0.4	14.6 $\pm$ 4.8	3300 $\pm$ 1138.4
Sham ( $n = 6$ )	11.7 $\pm$ 0.4	11.5 $\pm$ 0.2	11.3 $\pm$ 0.2	12.7 $\pm$ 0.3	14.7 $\pm$ 0.3	5.2 $\pm$ 1.8	1411 $\pm$ 174.5
Student's <i>t</i> -test	$P = 0.9$	$P = 0.5$	$P = 0.6$	$P = 0.08$	$P = 0.3$	$P = 0.2$	$P = 0.2$

The delay from the daily onset of running-wheel activity to the onset of light (phase angle of entrainment), the number of days required to synchronize locomotor activity following a shift in the light schedule (rate of re-entrainment) and the number of wheel revolutions during the daily bouts of activity (Night-time) and inactivity (Daytime) are expressed as mean  $\pm$  SEM.

noted the robust multisynaptic connections from the OB to the rat SCN (Krout *et al.*, 2002). In flies, social entrainment of the rest–activity cycle has been shown to depend upon olfaction and the antennae (Levine *et al.*, 2002). It will, therefore, be important to determine whether the clocks in the OB (and antennae) play a role in entraining the rhythms in multiple circadian pacemakers.

Finally, the rhythmicity in the OB is likely to be influenced by rhythmicity in the SCN. For example, the daily rhythm in brain temperature is regulated by the SCN (Eastman *et al.*, 1984). This daily temperature rhythm influences circadian timing in the SCN and in peripheral tissues (Brown *et al.*, 2002; Herzog & Huckfeldt, 2003) and, based on the *in vitro* results presented here, is sufficient to entrain rhythmicity in the OB.

Taken together, these results indicate that the circadian system must be modelled as multiple pacemaking tissues which can function semiautonomously from each other. Cells within the mitral cell layer of the OB are probably competent circadian pacemakers regulating their own gene expression and membrane excitability. Their daily rhythms are, like those in the SCN, entrainable to 24-h schedules and temperature compensated but, unlike the SCN, do not play an important role in locomotor behaviours. Future work should reveal the role of circadian pacemaking in the OB on behaviour.

## Supplementary material

The following supplementary figure is available from: [www.blackwellpublishing.com/products/journals/suppmat/EJN/EJN3117/EJN3117sm.htm](http://www.blackwellpublishing.com/products/journals/suppmat/EJN/EJN3117/EJN3117sm.htm).

FIG. S1. Removal of the olfactory bulbs had no significant effect on the circadian locomotor patterns. Actograms show wheel-running activity (black tick marks) of representative sham-operated (a) and bulbectomized (b) rats in double-plotted format. Each horizontal line represents a 48-h period; the second 24-h period is plotted to the right and also below the first. Grey bars indicate the 3 h each day when the lights were on and the arrow marks the day of surgery.

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## Abbreviations

E, embryonic day; OB, olfactory bulb; P, postnatal day; *Per1*, *Period1*; *Per1-luc*, *Per1-luciferase*; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

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