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Research Article

Single-cell recordings from chick pineal glands in vitro reveal ultradian and circadian oscillations

J. Schenda and L. Vollrath*

Institute of Anatomy, Johannes Gutenberg-University, D-55099 Mainz (Germany), Fax + 49 6131 39 23 719, e-mail: vollrath@mail.uni-mainz.de

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Abstract. Evidence is clear that each melatonin-producing cell in the chick pineal gland contains a circadian oscillator that continues to function in vitro, resulting in a prominent day/night rhythm of melatonin secretion. The aim of the present investigation was to examine whether the circadian organization of the gland has an electrophysiological correlate. To this end, single-cell recordings were made from isolated chick pineal glands kept in vitro under a light/dark cycle of 12:12 h, identical to that of the donors, or under continuous light or darkness. In all the glands investigated, a very small percentage of cells exhibited sodium-dependent spontaneous spike activity with a mean frequency below 10 Hz. The cells revealed rhythms with periods in the 15- to 60-min range and, additionally, exhibited ultradian and circadian rhythms in firing, with periods of 10.75 ± 1.06 h and 26.25 ± 1.26 h (mean $\pm$ standard deviation), respectively. Most of the cells exhibited circadian rhythms with higher activity during daytime than at night, showing that the electrical activity and melatonin rhythm were out of phase. Under constant light or darkness, the circadian rhythm persisted. When the light/dark cycle of the donors was phase-advanced by 5 h, the cells revealed complete entrainment. We discuss whether the cells, albeit small in number, could function as a secondary ultradian/circadian oscillator contributing to the ultradian/circadian organization of the gland.

Key words. Pineal gland; chick; electrical activity; chronobiology; circadian rhythm.

Ample evidence exists that the chick pineal gland contains a light-sensitive circadian oscillator that continues to oscillate in vitro, leading to low melatonin formation during the day and high synthesis at night [1–3]. Even small parts of the gland or dispersed cells oscillate [4–8], suggesting that each melatonin-producing cell has its own oscillator. That this is indeed the case has recently been established when singly cultured pinealocytes were shown to secrete melatonin in a circadian fashion [9].

Electrophysiological studies have demonstrated that the avian pineal gland contains spontaneously active cells [10–17] of unknown significance. To gain some insight into their function, we have carried out electrophysiological investigations on chick pineal glands in vitro to examine how the recorded electrical activity relates to the well-established circadian rhythm of melatonin synthesis. As most of the electrophysiological studies on the avian pineal gland have involved the use of multiunit recordings that give little insight into the electrical behaviour of individual cells and have been short term, we carried out single-cell recordings for up to 48 h to determine the behaviour of the electrically active cells over longer periods of time. Although isolated chick pineal cells kept in vitro have, as yet, not been shown to exhibit action potentials, whole-cell patch-clamp

* Corresponding author.

recordings have revealed that Na^+ currents are expressed in conjunction with several kinds of outward current [18], indicating that action potentials probably occur.

Materials and methods

Thirty-eight newly hatched male chickens were kept for 2–42 days under standard laboratory conditions with a 12-h light/12-h dark rhythm (LD 12:12), lights on at 0600 hours clock or zeitgeber time (ZT) 0 h, temperature 22 ± 2 °C, and food and water ad libitum. Three additional chickens were kept under an advanced day/night cycle for 2 weeks, with lights on at 0100 hours and lights off at 1300 hours. All the animals were decapitated under mild ether anaesthesia. The pineal glands were quickly removed, transferred into a temperature-controlled superfusion chamber at 36 °C and continuously superfused with carbogenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) at a rate of 0.5 ml/min. The ACSF contained (in mmol/l) 124 NaCl, 5.0 KCl, 1.24 KH_2PO_4, 1.3 $MgSO_4$, 0.9 $CaCl_2$, 26.0 $NaHCO_3$ and 10.0 glucose (osmolarity 280–290 mosm/l, pH 7.4). The sodium channel blocker tetrodotoxin dissolved in ACSF was superfused at a concentration of 3 µmol/l during the light phase only.

Single-cell recordings obtained using glass micropipettes filled with 3 mol/l KCl were carried out in a darkened room in a small light-proof chamber (< 0.1 lx). In most experiments, the glands were exposed to a lighting schedule identical to that of the donors; however, it should be noted that the light was provided by an Osram Xenophot HLX 64634 EFR 15-V, 150-W bulb and was transmitted via glass fibre optics, yielding a light intensity of 6.4 klx near the surface of the gland. The spectrum of the light source is given in figure 1. In some experiments, the effects of 15-min light (same light intensity as above) during the dark phase and of 15-min darkness during the light phase were tested. In addition, recordings were made under constant light or constant darkness. For technical reasons, the recordings were always started during the light phase. When short-term recordings were made, several cells per gland were usually investigated. In the long-term recordings lasting up to 48 h, only one cell per gland was monitored. The signals recorded were amplified and fed into an oscilloscope and personal computer for data acquisition and on- and off-line analysis. Short-term rhythms were analysed by spectral analysis (SPSS on a VAX computer). Frequency changes following pulses of light/darkness were analysed using the Wilcoxon signed-rank test ($p < 0.05$). The presence of ultradian and circadian rhythms was studied by means of cosinor rhythmometry [19, 20]. In each case, the period length, acrophase, mesor, amplitude and correlation coefficient were determined for the best-fitting curve. The cosinor data for the various experimental groups, expressed as means $\pm$ standard deviation (SD), were analysed using Student's t-test following verification that the data were normally distributed (Kolmogorov-Smirnov test). The data obtained under constant lighting conditions were related to circadian time (CT), CT 0 (zero) being the onset of the light phase in the donors. The experiments were carried out in line with local and state guidelines and conformed with the 'Ethical principles and guidelines for scientific experiments on animals' of the Swiss Academy of Medical Sciences.

Results

In each of the chicken pineal glands studied in vitro, a few spontaneously active cells with clear action potentials were demonstrable (fig. 2a,b). The firing rates ranged from < 0.1 to 20 Hz, with a predominance between 1 and 3 Hz. The spikes appeared to be sodium channel driven, since the application of the sodium channel blocker tetrodotoxin ($n = 4$) abolished spike activity completely and irreversibly. In all cases, a short increase in firing was noted immediately prior to inhibition (fig. 2c), reminiscent of nerve cells in the central nervous system [21, 22]. A detailed analysis of 31 cells, each one recorded for a minimum of 1 h, revealed that 30% ($n = 9$) fired regularly, with no prominent changes. Seventy percent of the cells ($n = 22$) exhibited considerable variation in their discharge rates and patterns, lasting for 15–60 min (fig. 3). Saw-toothed-like discharge patterns (fig. 3a,c) were noted in 30% ($n = 9$) of the cells. Here, the discharge rates increased steadily for about 10–20 min (fig. 3c), and occasionally for 60 min

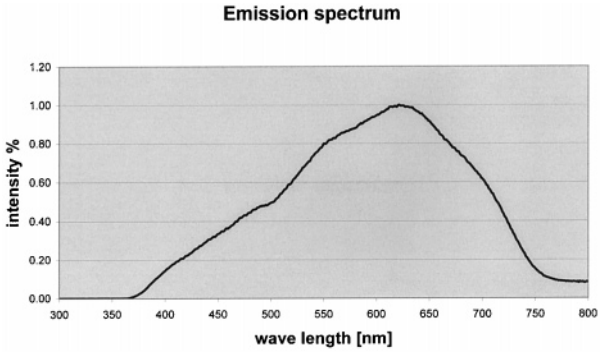

Figure 1. Emission spectrum of the light source to which the pineal glands were exposed. Note that the λ_{max} of the chick pineal photopigment pinopsin is at 470 nm. Hence, from the 6400 lx applied, only part of the intensity is effective.

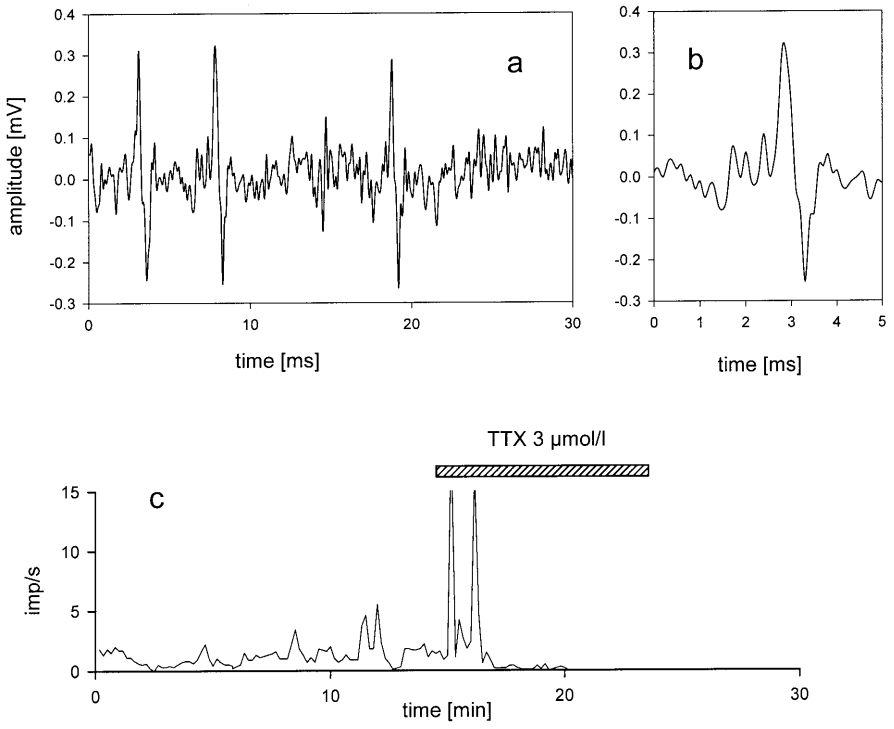

Figure 2. Electrophysiological single-cell recordings obtained during the daytime from a 5-day-old chick pineal gland kept in vitro, showing original recordings of action potentials (*a*, *b*) and a spike/time histogram (*c*). In the latter, superfusion of the sodium channel blocker tetrodotoxin (TTX) abolishes the spike activity completely and irreversibly, revealing that the action potentials are sodium driven.

(fig. 3a), and were followed by an abrupt decrease. Burst activity of identical height (fig. 3b) was seen in 40% ($n = 13$) of the cells. The inter-burst intervals were of equal lengths in a given cell, but differed between cells, ranging from 15 to 60 min. Visual inspection of the data plots did not reveal any obvious age-related changes.

Continuous recordings for up to 48 h revealed that, apart from some exceptionally high peaks or phases with a number of peaks (especially during phases of high activity), the recorded data relative to ZT or CT exhibited a sinusoidal shape. Application of cosinor rhythmometry to all the long-term recordings showed that all but one cell (see below) exhibited statistically significant ($p = 0.0001$) ultradian or circadian rhythms. The fitted curves had a correlation coefficient of 0.583 ± 0.17 SD.

Recordings from glands kept under an LD 12:12 lighting regime comparable to that to which the donors had been exposed revealed that the firing rates of all cells investigated ($n = 8$) exhibited long-term rhythms with 3- to 10-fold fluctuations in amplitude. Six of the 8 cells had periods in the circadian range. Five exhibited higher discharge rates during photophase than scotophase (fig. 4a; 'day-active cell'), with no distinct change in firing at the light/dark and dark/light transitions. The period length was 26.25 ± 1.26 h and the calculated acrophases occurred at ZT 6.04 ± 1.9 h. The mesors and amplitudes were 0.56 ± 0.07 and 0.45 ± 0.11, respectively. One cell (fig. 4b) was distinctly more active at night than during the day. Its firing activity followed the ambient lighting conditions relatively closely, with several ups and downs during scotophase, and the highest firing rates near its end. The period length was 21.5 h, with acrophase occurring at ZT 21.1 h. Two cells exhibited firing rhythms in the ultradian range (period length 10.75 ± 1.06 h; acrophase at ZT 7.89 ± 2.16 h; see fig. 4c).

To clarify whether the firing rhythms were endogenous or exogenous, glands from donors kept under LD 12:12 were recorded under constant light (LL) or constant darkness (DD), in some instances after a 5-h phase-advance (see table 1). The cells recorded under LL did not differ from the LD12:12 cells as far as period and acrophase were concerned (fig. 4d). However, the cells from the non-phase-advanced glands exhibited significantly elevated mesors and amplitudes. The cells recorded under DD did not reveal statistically signifi-

cant differences compared with the LD12:12 and LL cells. Nevertheless, cosinor analysis revealed much shorter periods, i.e. of around 18 h (table 1, fig. 4e) and, in the case of the cell from the non-phase-advanced gland, a much higher mesor and amplitude.

Cells from 5-h phase-advanced animals/glands revealed that, under LL, the acrophases did not differ significantly from those of the LD12:12 and the non-phase-advanced cells, suggesting that the cells were fully entrained to light. Under DD, the acrophase of the cell differed considerably from the non-phase-advanced cell (table 1). However, since, according to cosinor analysis, the rhythm of this cell was not very pronounced, whether the cell was fully entrained to light remained uncertain.

Following exposure of isolated pineal glands to 15-min pulses of light during the night or darkness during the day, most cells investigated revealed immediate, but short-lasting (ca 10 s) effects only (fig. 5). Following a light pulse given to 12 cells, 7 cells were stimulated ($p < 0.05$; fig. 5a), 3 cells were inhibited ($p < 0.05$) and 2 cells did not respond. A dark pulse increased the firing frequency in 7 out of 13 investigated cells ($p < 0.05$), in 2 of which, termination of the dark pulse was followed by a short stimulatory off response (fig. 5b). Two cells responded with decreased activity ($p < 0.05$) and 4 cells showed no effect.

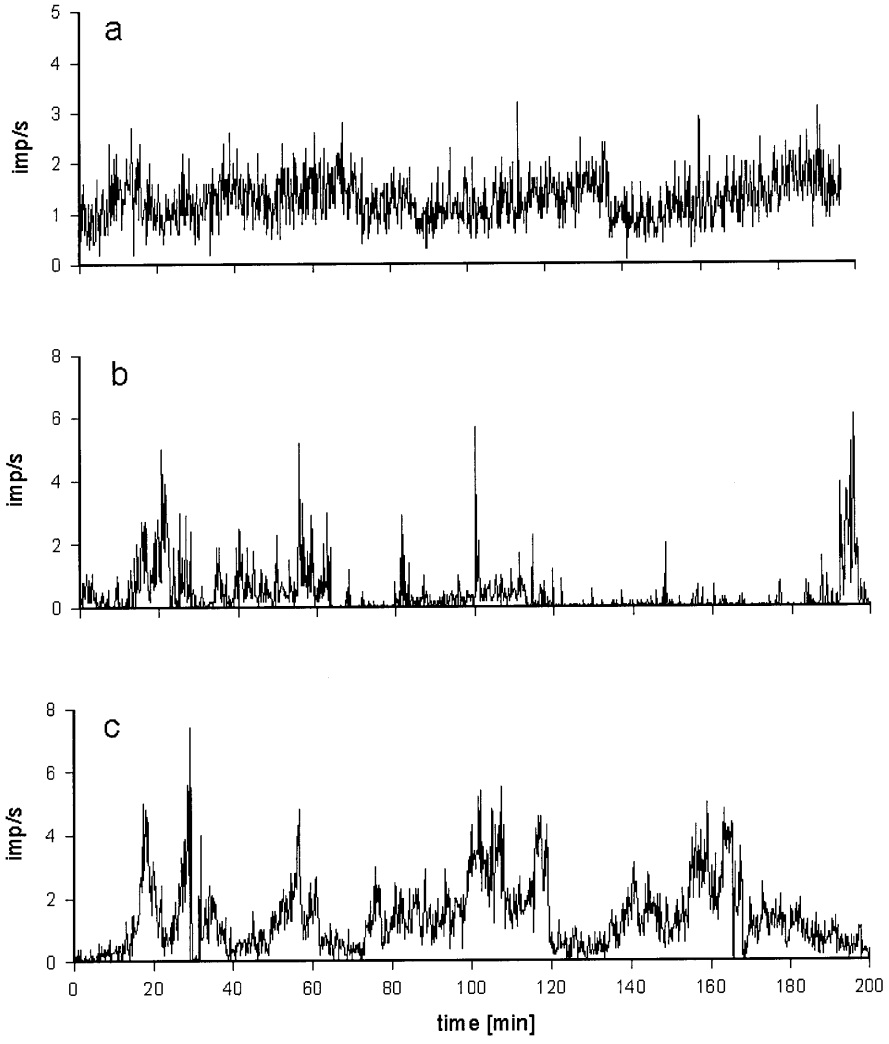

Figure 3. Spike/time histograms of cells exhibiting oscillations in their discharge patterns. Some cells showed saw-toothed oscillations (*a*, *c*) with continuously increasing firing rates followed by an abrupt decrease. Other cells exhibited regularly repeated bursts with a relatively constant inter-burst interval (*b*).

Discussion

The present study demonstrates for the first time, in an avian pineal gland kept in vitro, electrically active cells with clear ultradian and circadian firing properties. The firing frequency below 10 Hz is in agreement with data obtained in sparrow pineals in vitro [11]. Results from other studies are not directly comparable because either no spontaneous activity could be recorded [23] or multi-unit and not single-cell activity was investigated [12–15]. It may be argued that the firing observed in our study was not endogenous but was caused by degenerating intrapineal sympathetic fibres and the resulting leakage of noradrenaline. That the firing may be principally affected by noradrenaline was shown in pigeon pineal glands in which the microiontophoretically administered substance stimulated, inhibited or did not affect the firing [24]. However, since the electrical activity we observed was uniform and followed distinct rhythmic patterns (see below), we conclude that, in our material, noradrenaline leakage did not cause the electrical events.

The most important finding of the present study is that, in isolated and hence denervated pineal glands, electrically active cells exhibit distinct firing rhythms. This observation, like the continuing circadian synthesis of melatonin in vitro, supports the concept of rhythmic autonomy of the chick pineal gland. In this context, the cells with rhythms in the circadian range are of particular interest because they predominate numerically in our material. The circadian nature has been verified by cosinor rhythmometry ($p < 0.001$), yielding reasonably high correlation coefficients (0.583 ± 0.17 SD) for the best-fitting cosine curves. The following observations are in agreement with the assumption of circadian rhythmicity: (1) the phases of low and high activity do not precisely follow the light/dark phases, (2) the calculated periods are not 24 h but longer (26.25 h), (3) the rhythm persists under constant lighting conditions and (4) the rhythm can be phase-advanced by exposing the donors to a phase-advanced lighting cycle.

Abundant evidence is available that the chick pineal gland is sensitive to light in vitro [1, 2, 6, 25]. Since the rhythms of the cells from glands exposed to LD 12:12 exhibit periods longer than 24 h, the time cues provided by the LD cycle are not apparently sufficient to set the

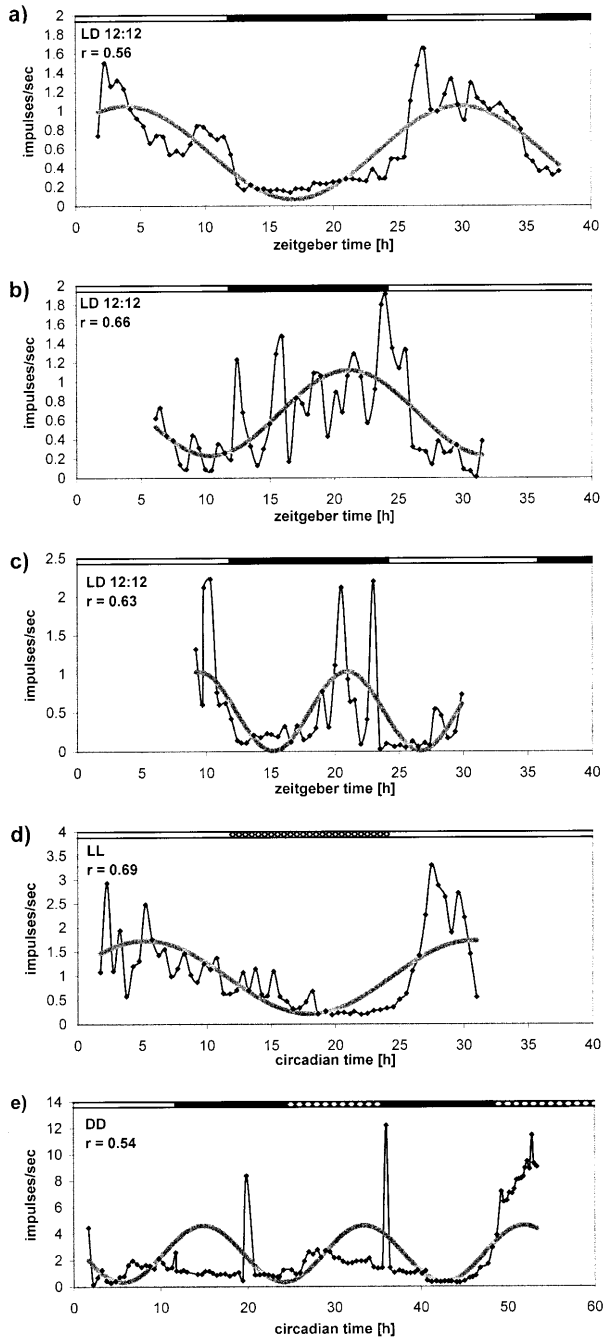

Figure 4. Long-term recordings of five cells from chick pineal glands kept in vitro and exposed to various lighting schedules, as indicated in the graphs. The discharge rates were averaged over half an hour and plotted against time and subjected to cosinor rhythmometry. In each graph, the best-fitting cosine curve and the correlation coefficients (r) are shown. The white/black horizontal bars symbolize lights on/off; the dotted bars indicate subjective day or subjective night. (*a*) Cell exhibiting a higher firing frequency during the daytime than at night ('day-active' cell; from a 20-day-old donor); this cell type was numerically predominant in the material under study. (*b*) Cell exhibiting an activity pattern inverse to that in *a*; in the present material, this night-active cell was found only once (21-day-old donor). (*c*) One of the two cells recorded exhibiting a biphasic rhythm with a first activity maximum during the day and two peaks at night (14-day-old donor). (*d*, *e*) Day-active cells recorded under LL (*d*, 17-day-old donor) or DD (*e*, 36-day-old donor) showing that the circadian rhythm persists under constant lighting conditions. Note that, under DD, the period is distinctly shorter than under LD 12:12 or LL.

Table 1. Cosinor rhythmometry of single cells exhibiting circadian or ultradian firing activity.

Lighting schedule	n	Period (h)	Acrophase (h)	Mesor	Amplitude
LD 12:12	5	26.25 ± 1.26	6.04 ± 1.92 ZT	0.56 ± 0.07	0.45 ± 0.11
LL	3	25.33 ± 0.76	7.05 ± 4.40 CT	0.96 ± 0.01*	0.89 ± 0.20‡
5 h phase advance, LL	2	26.00 ± 0.00	4.04 ± 0.88 CT	0.55 ± 0.13	0.61 ± 0.23
DD	1	18.5	14.90 CT	2.48	2.14
5 h phase advance, DD	1	18	9.90 CT	0.41	0.47

Chick pineal glands were kept in vitro under a schedule of 12 h light and 12 h dark (LD 12:12), constant light (LL) or constant darkness (DD). In some cases, the LD rhythm had been phase-advanced by 5 h. The mesors and amplitudes refer to the firing activity expressed as impulses/s. CT, circadian time; ZT, zeitgeber time.
*, ‡ vs LD 12:12, * $p < 0.001$; ‡ $p = 0.002$.

clock to an exactly 24-h period. Perhaps the rhythm is free running. The sensitivity to light of the chick pineal gland in vitro is also reflected electrophysiologically. Our short-term experiments revealed that, in analogy to the pigeon pineal [26], most cells investigated responded to short pulses of light or darkness. However, the responses were very short-lived, lasting approximately 10 s, suggesting that single acute short light/dark pulses have apparently little or no effect on the circadian rhythm. By contrast, longer-term exposure to an altered lighting regime leads to distinct and permanent changes in electrical activity. Thus, exposure to LL or DD significantly increased the mesors and the amplitudes of the circadian rhythm, pointing to a general activation of the cells under these lighting conditions. Period analysis yielded equivocal results. Whereas the period was apparently unaffected under LL, the two cells studied had a period length of around 18 h under DD, suggesting that DD speeded up the rhythm. A similar observation has been made with respect to the melatonin rhythm in cultured chick pineal cells exposed to constant red light compared with LD [7]. On the other hand, the cells with the 18-h period recorded under DD may belong to the group of ultradian cells observed here with the 10.75-h period and exposure to DD may lengthen the periods in these cells. Perhaps the electrical changes occurring in the first hours of exposure to a new lighting schedule are a sign that the intrapineal circadian oscillators are beginning to phase-shift. We have no explanation for the finding that the mesors and amplitudes increase under constant lighting conditions in the non-phase-advanced glands but not in the phase-advanced glands.

Another aspect needing discussion is that of the night-active cell with a period of 21.5 h that we observed under LD 12:12. A night-active cell has also been recorded in pigeons [26]. In the latter study, only a single long-term recording was made, so we do not know whether night-active cells are an exception in that species, as in the chick, or the rule. As important species differences exist in the pineal gland, extrapolations require caution. In the pigeon pineal, the night-active cell recorded was tonically inhibited by light [26], whereas in the chick, tonic inhibition by light was not observed (this study). In the chick pineal gland, electrically active cells were very rare (this study), whereas in the pigeon, spontaneous activity could be recorded immediately following penetration of the pineal capsule [26], suggesting that here the active cells were abundant. Another difference is that α_2-receptor-mediated mechanisms inhibit melatonin synthesis in the chick [27, 28] and stimulate it in the pigeon [29].

It could be argued that the light intensity applied in vitro (6400 lx) was too high, perhaps resulting in anomalous reactions of the cells and casting doubts on the physiological meaning of the results. At first sight, this seems to be a valid argument. However, the chick pineal photopigment pinopsin has a λ_{max} at 470 nm [30] and light of this wavelength makes up only a small part of the applied light spectrum in our experiments (fig. 1). We therefore assume that the glands were not exposed to abnormally high light intensities.

The precise nature of the spiking cells is currently not known. The gland contains photoreceptor-type secretory pinealocytes that produce melatonin, interstitial cells and rare nerve cells. The pineal photopigment pinopsin [30, 31] is exclusively present in the outer segments of secretory pinealocytes [32], as are the pinopsin-related G proteins [33]. Since the nerve cells do not exhibit immunoreactivity for pinopsin [32] and since photoreceptor cells are well known for not producing action potentials, we hypothesize that the cells described here are nerve cells and that their firing is influenced by the photopigment-containing pinealocytes. In agreement with this hypothesis are the findings that the spiking cells and the nerve cells are both rare and that nerve cells have been demonstrated in the chick pineal gland as early as 3 days after hatching [34]. Another possibility is that the spiking cells belong to the group of melatonin-producing cells. Whole-cell patch-clamp recordings have shown that these cells exhibit Na^+ currents in conjunction with several kinds of outward current [18] suggesting that they are capable of produc-

ing action potentials. That the spiking cells are interstitial cells can be ruled out, because this category of cells comprises fibroblasts and astrocytes, which are not well-known for producing action potentials.

What is the function of the electrically active cells? As they are very rare, their impact on the pineal gland and the organism as a whole is difficult to judge. There is abundant evidence that isolated pinealocytes retain their circadian rhythm of melatonin synthesis in vitro [9, 35]. Hence, the circadian rhythm of the electrical activity does not seem to be a requirement for the melatonin rhythm, especially as the two rhythms are out of phase by 180°. Interestingly, a similar phase relationship exists in the suprachiasmatic nucleus (SCN)/pineal system in mammals, in which the electrical activity of the SCN is high during daytime [36], whereas melatonin synthesis is stimulated at night. Finally, the possibility exists that, under in vivo conditions, the cells represent a secondary circadian oscillator that contributes to phase-shifting processes, either autonomously or under the influence of the SCN-regulated sympathetic nerve fibres that innervate the pineal gland. In this context, the spiking cells may monitor extracellular neurotransmitter and hormone levels in the pineal gland and adapt their firing accordingly, as suggested for the rat [37, 38]. Indeed, the spiking cells in avian pineal glands are principally capable of subserving such a function, as has been demonstrated in the pigeon pineal, in which firing is affected by noradrenaline, acetylcholine, γ-aminobutyric acid, melatonin and 5-methoxytryptophol [24].

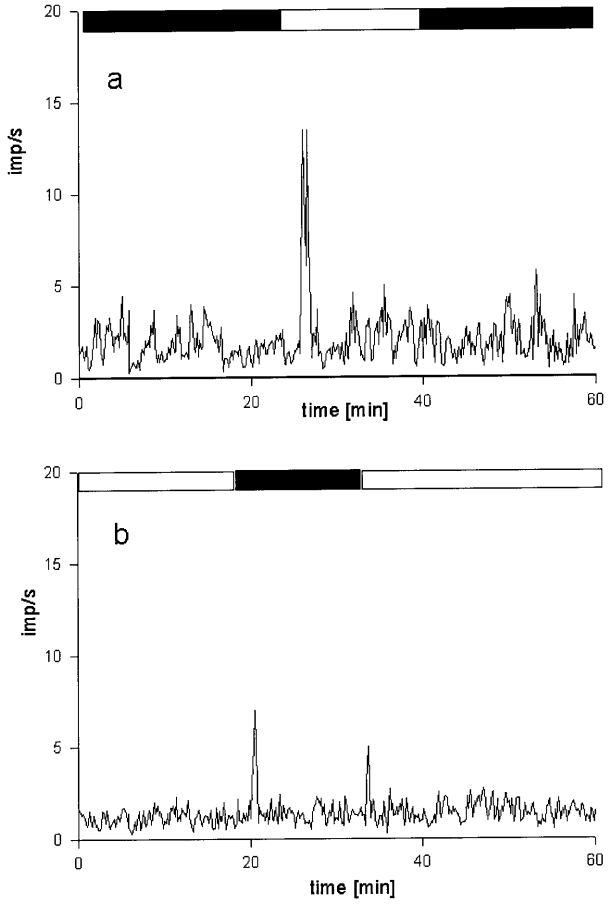

Figure 5. Effects of short light pulses during the night (*a*) and dark pulses during the day (*b*). The spike/time histograms show the responses of two cells. (*a*) The cell responded to the onset of light with a short-lasting burst in firing. Light-off had no effect on the cell. (*b*) Short dark pulses during the day also yielded slight and transitory effects; in the example shown, phasic responses occurred at dark on and off.

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