From the Pineal Gland to the Central Clock in the Brain: Beginning of Studies of the Mammalian Biological Rhythms in the Institute of Physiology of the Czech Academy of Sciences

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Summary

The Institute of Physiology of the Czech Academy of Sciences (CAS) has been involved in the field of chronobiology, i.e., in research on temporal regulation of physiological processes, since 1970. The review describes the first 35 years of the research mostly on the effect of light and daylength, i.e., photoperiod, on entrainment or resetting of the pineal rhythm in melatonin production and of intrinsic rhythms in the central biological clock. This clock controls pineal and other circadian rhythms and is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. During the early chronobiological research, many original findings have been reported, e.g. on mechanisms of resetting of the pineal rhythm in melatonin production by short light pulses or by long exposures of animals to light at night, on modulation of the nocturnal melatonin production by the photoperiod or on the presence of high affinity melatonin binding sites in the SCN. The first evidence was given that the photoperiod modulates functional properties of the SCN and hence the SCN not only controls the daily programme of the organism but it may serve also as a calendar measuring the time of a year. During all the years, the chronobiological community has started to talk about "the Czech school of chronobiology". At present, the today´s Laboratory of Biological Rhythms of the Institute of Physiology CAS continues in the chronobiological research and the studies have been extended to the entire circadian timekeeping system in mammals with focus on its ontogenesis, entrainment mechanisms and circadian regulation of physiological functions.

Key words

Pineal • Melatonin • AA-NAT rhythm • Light entrainment • Photoperiod • SCN clock

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Effect of light on the melatonin metabolism in the pineal gland

After I had defended my CSc. (PhD) thesis (*Urea formation in rats during postnatal development*) in 1966, my then boss Professor Jiří Křeček, Head of the Department of Developmental Physiology and Pathophysiology of the Institute of Physiology of the then Czechoslovak Academy of Sciences, brought to my attention a paper of Fiske *et al.* [1] on the effect of light on the weight of the pineal in the rat. I became interested in the pineal gland as I knew that newborn rats opened their eyes only at the age of 14 days after birth and I was curious to learn whether the opening of their eyes and thus perception of light might affect their pineals.

At the end of sixties it has been already clear that the mammalian pineal is a secretory organ [2]. In 1958, Lerner *et al.* [3] isolated a factor from bovine pineal glands that lightened amphibian melanophores and named it melatonin. In 1959, Lerner *et al.* [4] described the melatonin structure as N-acetyl 5-methoxytryptamine. It is said that on reading of the isolation and characterization of melatonin, Julius Axelrod, the later Nobel Prize winner for his work on the release, reuptake and metabolism of catecholamines, considered that the enzyme machinery requested for such a synthesis within the body did not exist [5]. However, between 1960-1968

he and his coworkers then proceeded to demonstrate the existence of all elements of the pathway within the pineal gland, summarized in a review in *Science* in 1974 [6]. According to the review, the amino acid tryptophan is hydroxylated by tryptophan-5-hydroxylase to 5-hydroxy-tryptophan which is then decarboxylated by aromatic L-amino acid decarboxylase to 5-hydroxy-tryptamine, i.e., serotonin. Serotonin is acetylated by N-acetyl-transferase (NAT), nowadays characterized as arylalkylamine N-acetyltransferase (AA-NAT) [7], to N-acetylserotonin which is then methylated by hydroxyl-indole-O-methyltransferase (HIOMT) to melatonin [5]. In 1963, Quay [8] described the daily rhythm in the melatonin precursor serotonin in the rat pineal, with high levels during the day and low levels at night. In 1970, Klein and Weller [9] found a robust daily rhythm in the N-acetyltransferase (NAT) activity in the rat pineal, with the nighttime values 100 fold higher than the daytime ones. The end of the pathway leading to melatonin synthesis is shown on Fig. 1A [5] and the daily rhythm in N-acetyltransferase activity on Fig. 1B [10].

During my first studies on the effect of light on the pineal gland I found that following exposure of rats to a sudden light at night, the pineal serotonin content increased within 13 min from low nighttime to high daytime levels and stayed high at light (Fig. 2) [11]. The rapid serotonin increase on light at night suggested that the pineal melatonin metabolism might respond to the sudden light at night almost instantaneously. I was quite surprised by these results as at that time I did not know anything about the biological clock and how an environmental light might affect and reset it. The concept of the biological clock was mostly introduced at the symposium "The Biological Clock" organized in Cold Spring Harbor in 1960. At that symposium it has been accepted that daily rhythms are endogenous and not just a passive response to environmental cycles of light and darkness and hence that they must be driven by an endogenous clock or pacemaker [12, 13]. Our paper on the rapid serotonin rise on light was published in 1971 and one year later, in 1972, Klein and Weller [14] and independently Deguchi and Axelrod [15] reported that a sudden light at night induced an almost instantaneous decline of the pineal N-acetyltransferase (NAT) activity. Both groups cited our previous work on the serotonin rise on light [11]. Apparently, when the NAT activity declined on light, serotonin as its substrate might increase.

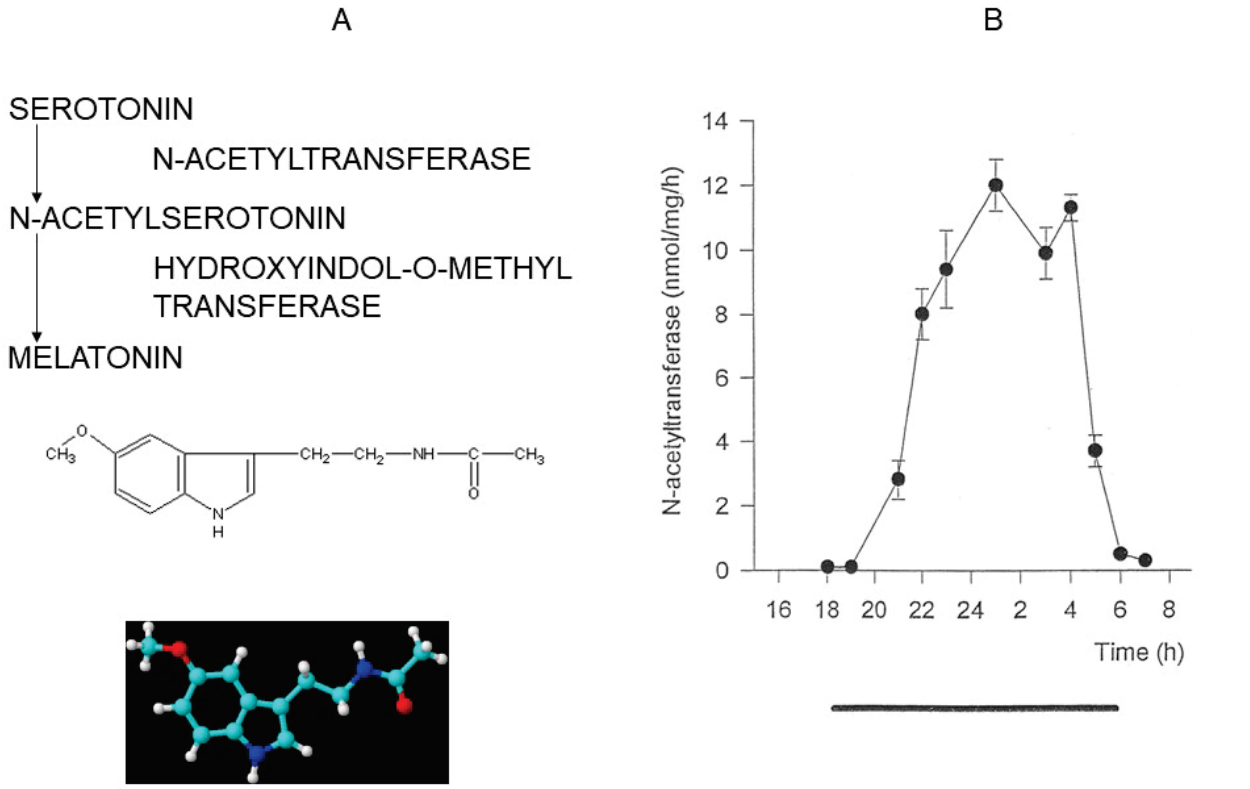

Fig. 1. A: End of the pathway leading to melatonin synthesis in the pineal gland [5]. **B**: The circadian rhythm in N-acetyltransferase activity in the rat pineal gland. The black bar under the abcissa indicates the duration of the dark period. Data were taken from [10].

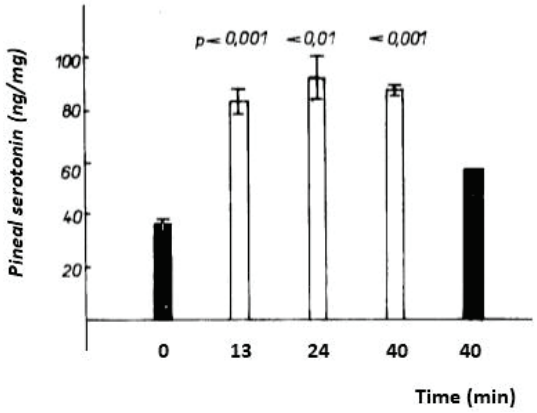

Fig. 2. The effect of a sudden light at night on the pineal serotonin content. Rats maintained in LD 12:12 were exposed to light at night after 4 h in darkness and killed after 13, 24 and 40 min on light (open columns). Some of the rats were left all the time in darkness and killed at the beginning or at the end of the experiment (dark columns). Numbers under the abcissa denote time in min since the start of the experiment. Data were taken from [11].

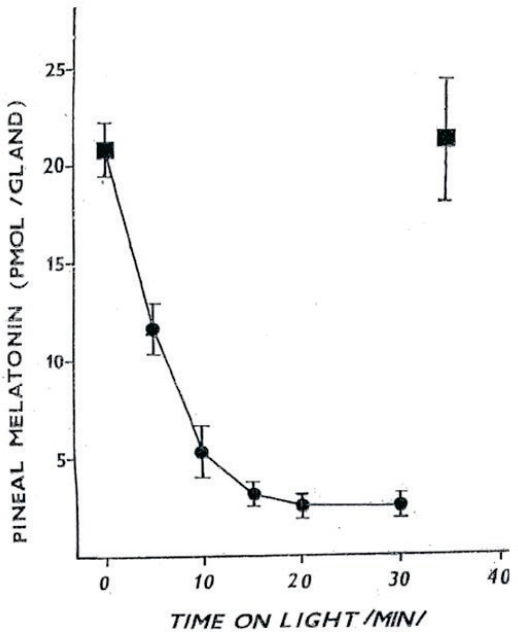

Fig. 3. The effect of a sudden light at night on the pineal melatonin content. Rats maintained in LD 12:12 were exposed to light at night after 5 h in darkness and killed at different times on light (circles). Some of the rats were left in darkness and killed at the beginning and end of the experiment in darkness (squares). Data were taken from [17].

Later, radioimmunoassays (RIAs) for melatonin were developed. In 1978, using a modified Arendt´s RIA [16], we showed with Lennart Wetterberg and his coworkers for the first time the rapid decline of the pineal melatonin content (Fig. 3) and of the serum melatonin concentration following exposure of rats to a sudden light at night [17, 18]. The pineal melatonin decreased rapidly on light with a half- time around 5 min and reached the lowest level already after 20 min. The melatonin concentration in serum fell precipitously on light as well, in a manner almost identical to the drop in the pineal melatonin, only with a 5 min time lag [17]. The half-time for the pineal melatonin decline correlated well with the half-time of 3-5 min reported for the decline in the pineal NAT activity after exposure of rats to light at night [14]. It thus seems that the rapid changes in the pineal melatonin content are due to changes in the pineal NAT activity. Indeed, the evening rise and the morning decline in the pineal NAT activity and in the pineal melatonin content occur at the same time, be it in pineals of rats or Djungarian hamsters [19]. Hence, the NAT rhythm in the pineal drives the rhythm in the melatonin synthesis at least in the two above mentioned species.

The above mentioned finding showed that the pineal NAT activity [14] as well as the pineal melatonin content [17] declined very rapidly when rats were exposed to light for the whole time interval before killing. Such a precipitous drop suggested that even a very brief exposure of rats to light at night, such as for 1 min, after which rats would continue to be in darkness, could trigger a decrease in NAT activity and in melatonin content to low levels. This was indeed the case. Only a 1 min exposure to light 5 hours after the evening onset of darkness caused a rapid decline in NAT activity and in melatonin content with a half-life less than 5 min, the same as in rats exposed to light 5 hours after the evening onset of darkness and then left on light [20].

We then explored, together with Jiří Vaněček, effect of 1 min light pulses applied at night on the pineal rhythm in NAT activity as the indicator of the nocturnal melatonin production. Importantly, the NAT rhythm in the rat pineal has two well-defined phase markers, namely the time of the evening NAT rise (E) and the time of the morning NAT decline (M) (see Fig. 1B). Rats maintained under a 12 h of light and 12 h of darkness regime (LD 12:12) were either left unpulsed or they were exposed to a 1 min light pulse at different night times before or after midnight and then they were released into darkness (Fig. 4). After the 1 min light pulse before midnight the NAT activity, after an initial decline, increased anew; after the pulse applied after midnight, the activity also rapidly declined, but it stayed low for at least another 8 hours in darkness [21-25]. We were fascinated by the sharp boundary between the effect of light pulses before and past midnight. I must admit that I am fascinated by the boundary even now and I wonder whether we have a good explanation for it.

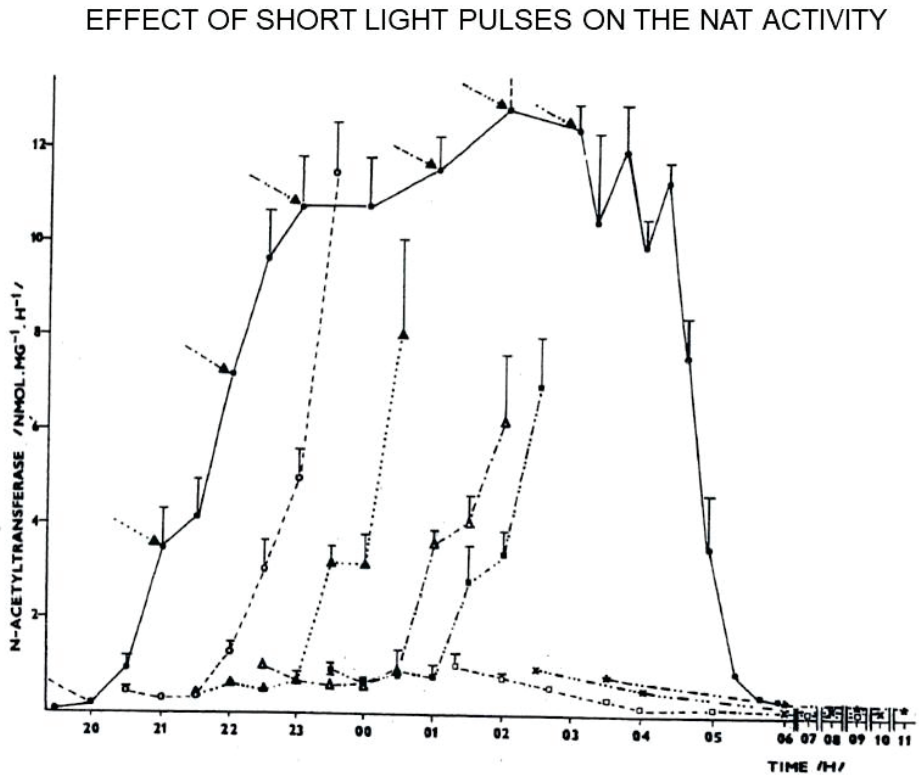

Fig. 4. The N-acetyltransferase (NAT) rhythm in the course of the night when 1 min light pulses were presented. Rats maintained in LD 12:12, with lights on from 06 to 18 h, were either unpulsed (filled circles) or exposed to a 1 min light pulse at 20 h (open circles), or at 21 h (filled triangles), or at 22 h (open triangles), or at 23 h (filled squares), or at 01 h (open squares), or at 02 h (crosses), or at 03 h (asterisks) and from that time on they were kept in darkness until they were killed. Arrows indicate times of the pulse presentation and point to the NAT activity in darkness at the moment of the light pulse. Data were taken from [21,22] and the picture was modulated from that in [23].

Entrainment or resetting of the rhythm in melatonin production by light

In non-periodic environment, e.g. in constant darkness, circadian rhythms free run with a period close, but not identical with 24 h. With the 24 h day, they are synchronized or entrained by an environmental light-dark cycle. Light experienced during the subjective night may reset circadian rhythms to another endogenous time [12, 13]. We thus wanted to find out whether a 1 min light pulse might entrain or reset the NAT rhythm. First, we looked on the effect of the 1 min light pulse administered before midnight on the NAT rhythm on day 0, when rats were pulsed, or on day 1 and 4 after the pulse. Following the pulse, the rats were released into constant darkness (Fig. 5). The pulse at 21 h delayed the evening NAT rise (E) the same night while the morning NAT decline (M) was phase-delayed just slightly. However, after 1 and 4 days, the evening rise and the morning decline were phase-delayed almost to the same extent (Fig. 5A). Fig. 5B shows phase delays of the evening NAT rise (E) and of the morning decline (M) read from the Fig. 5A and other similar figures and plotted as a function of time when pulses were applied [10, 22]. The picture reveals that phase delays of E on day 0 might be quite large while those of M were only small. However, on day 1 and 4, phase delays of M were almost the same as those of E. It appears that at the beginning the pulse in the first half of the night phase delays primarily the evening NAT rise (E), however, within one day E and M are phase-delayed almost to the same extent. Hence, the whole NAT rhythm may be phase-delayed within one day [10, 22, 23, 26, 27].

We got a very different picture when we looked on the effect of the 1 min light pulse administered after midnight (Fig. 6). Following the 1 min light pulse administered at 03 h, the NAT activity immediately declined as if the morning decrease were phase-advanced. On day 1 after the pulse, only the morning decline (M) was phase-advanced, but not yet the evening rise (E). Only after 4 days, E started to be phase-advanced as well, though still to a lesser extent than M (Fig. 6A). Fig. 6B shows phase shifts of the evening NAT rise (E) and of the morning decline (M) read from the Fig. 6A and other similar figures and plotted as a function of time when 1 min light pulses were applied. The picture reveals that on day 1 after administration of the 1 min light pulse, only the morning NAT decline (M) was phase-advanced but not yet the evening NAT rise (E). Four days after the pulse presentation, there were also phase advances of E, but still substantially smaller than those of M [10, 22, 23, 26, 27].

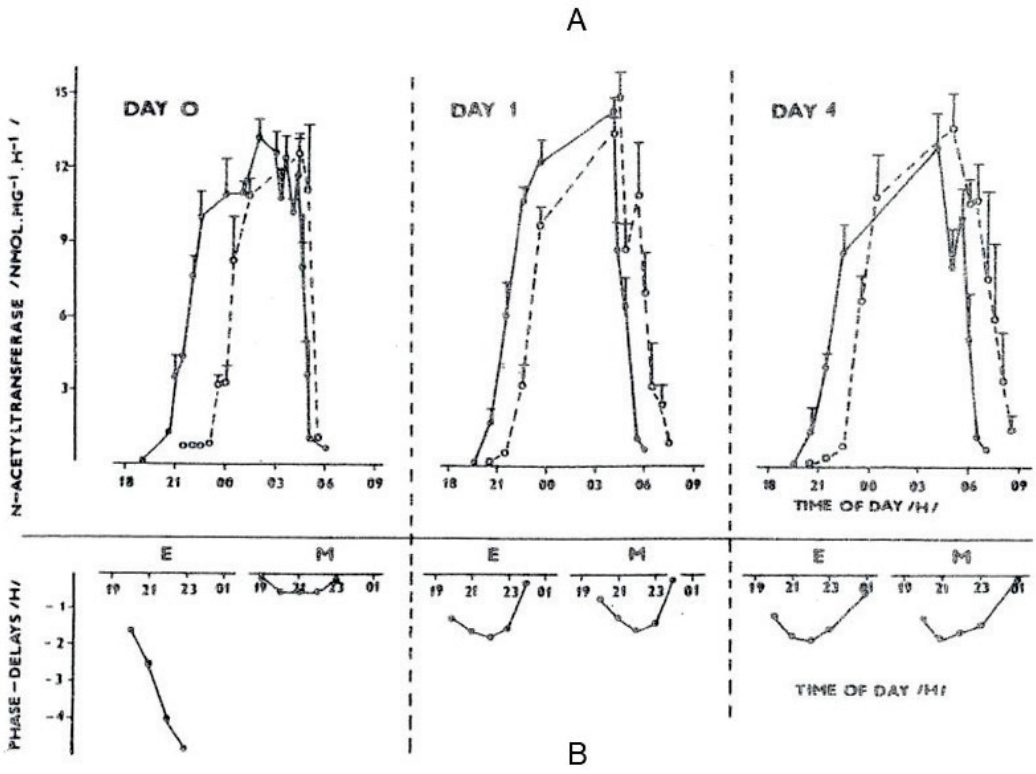

Fig. 5. Phase delays of the N-acetyltransferase (NAT) rhythm after 1 min light pulses applied before midnight. **A**: The NAT rhythm after presentation of a 1 min light pulse at 21 h. Rats maintained in LD 12:12 with lights on from 06 h to 18 h, were either exposed to a 1 min light pulse (open circles, broken line) or left unpulsed (filled circles, full line). Thereafter, they were released into constant darkness and killed during the night when they were pulsed (day 0), or 1 (day 1), or 4 days (day 4) after the pulse presentation. **B**: Phase delays of the evening NAT rise (E) and of the morning NAT decline (M) during the night when rats were pulsed and after 1 and 4 days. Phase shifts were determined at the level of 3 nmol/mg.h of the NAT activity from Fig. 5 A and other similar figures and they were plotted as a function of time when the pulses were presented. Phase delays were plotted with the sign -. The abcissa denotes time of the pulse administration. Data were taken from [10, 22, 23].

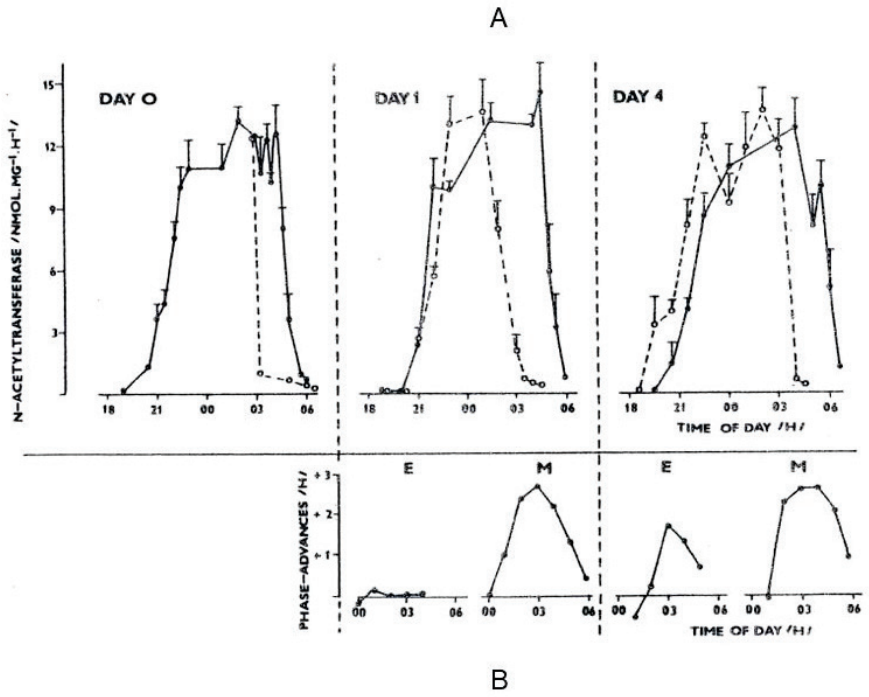

Fig. 6. Phase advances of the N-acetyltransferase (NAT) rhythm after 1 min light pulses applied after midnight. **A**: The NAT rhythm after presentation of a I min light pulse at 03 h. Rats maintained in LD 12:12, with lights on from 06 to 18 h, were either exposed to the 1 min light pulse (open circles, broken line) or left unpulsed (filled circles, full line). Thereafter, they were released into constant darkness and killed during the night when they were pulsed (day 0), or 1 day (day 1), or 4 days (day 4) after the pulse presentation. **B**: Phase shifts of the of the evening NAT rise (E) and of the morning NAT decline (M) during days 1 and 4 were determined at the level of 3 nmol/mg.h of the NAT activity from Fig. 6A and other similar figures and they were plotted as a function of time when the pulses were applied. Phase advances were plotted with the sign +, phase delays with the sign -. The abcissa denotes time of the pulse presentation. The data are taken from [10, 22].

Experiments with light pulses at night showed for the first time that such short light pulses as was the 1 min light pulse might entrain the pineal rhythm in melatonin production. Before, a longer exposure to light at night had been used to entrain or reset mammalian circadian rhythms. The fact that the 1 min light pulse may phase shift mammals into a different endogenous time seems to me still as science fiction. Another important piece of knowledge is that delays of the evening NAT rise (E) and of the morning NAT decline (M) are accomplished almost within one cycle and hence the NAT rhythm as a whole is phase-delayed within one cycle. Advances of the morning NAT decline are accomplished also within one cycle, in contrast to the then dogma that several transients cycles are necessary before phase advances are accomplished. Transient cycles are, however, necessary for the complete phase advancing of E.

From the above mentioned facts it is possible to deduce that rats might better adapt to delaying of the light-dark cycle, i.e. to a simulated westward time-zones transition, than to advancing of the light-dark cycle, i.e. to a simulated eastward time-zones transition. And it is indeed so. After an 8 h delay of a light -dark (LD) 12:12 cycle by lengthening of one light period by 8 h, the pineal N-acetyltransferase rhythm adjusted to the delay shift almost within one cycle. In contrast, after an 8 h advance of the LD cycle accomplished by shortening of one dark period by 8 h, the NAT rhythm adjusted to the advance shift within 5 cycles only; during the first 2-3 cycles the rhythm was abolished [28]. However, when the 8 h advance of the LD cycle was accomplished by twice delaying the LD cycle during two consecutive nights by 8 h, the NAT rhythm persisted and attained its original waveform by 2 days earlier than under the former advance of the LD cycle [29]. Finally, non-parallel phase shifts of E and M point to a possibility of a complex pacemaker driving the pineal NAT rhythm [22, 24], such as was proposed by Pittendrigh and Daan [30] for the circadian pacemaker driving the rhythm in the locomotor activity.

With 1 min light pulses, we could phase shift the NAT rhythm by 2-3 h at most. A question arose whether a longer light exposure at night might phase shift mammalian circadian rhythms by more than 3 h. Rats maintained in LD 12:12 experienced a prolongation of the light period into the evening and night hours and thereafter they were released into darkness (Fig. 7). Fig.7A shows that on day 0, the prolonged light till 22 h and 02 h, respectively, phase delayed primarily the evening NAT rise (E), but not so much the morning decline (M). After prolongation of the light period till 02 h, the phase delay of E was about 8 hours. On day 1, both the rise and decline were phase-delayed almost to the same extent, i.e., by about 6 hours at most (Fig. 7B) [31]. Thus, it is possible to phase delay the whole NAT rhythm by prolongation of the light period into the night by as much as by 6 hours within one cycle [23, 26, 27, 31]. Next we studied response of the NAT rhythm to an earlier light onset in the morning. Rats maintained in LD 12:12 experienced either the usual morning lights-on at 06 h or an advance of the morning lights-on to 01 h or to 23 h. After the advance of the morning light onset, the NAT activity immediately declined (day 0).

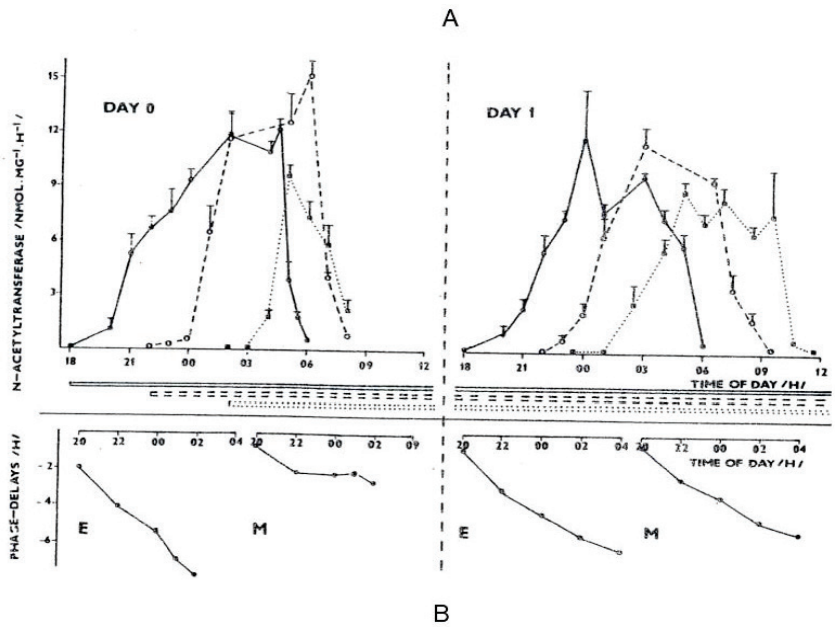

Fig. 7. Phase delays of the N-acetyl-transferase (NAT) rhythm after delays of the evening lights off. **A**: The NAT rhythm during the night when the lights-off was delayed (day 0) and during the next night (day 1). Rats maintained in LD 12:12, with lights on from 06 h to 18 h, were subjected either to the expected lights-off (filled circles, full line), or to a delay of the lights-off till 22 h (open circles, broken line) or till 02 h (filled squares, dotted line), respectively. Thereafter, they were released into darkness. Lines under the abcissa indicate dark periods. **B**: Phase delays of the evening NAT rise (E) and of the morning NAT decline (M) of the experimental animals relative to the control ones determined at the level of 3 nmol/mg.h of the NAT activity from Fig. 7A and other similar figures. The abcissa denotes time of the onset of darkness on day 0. Data were taken from [31].

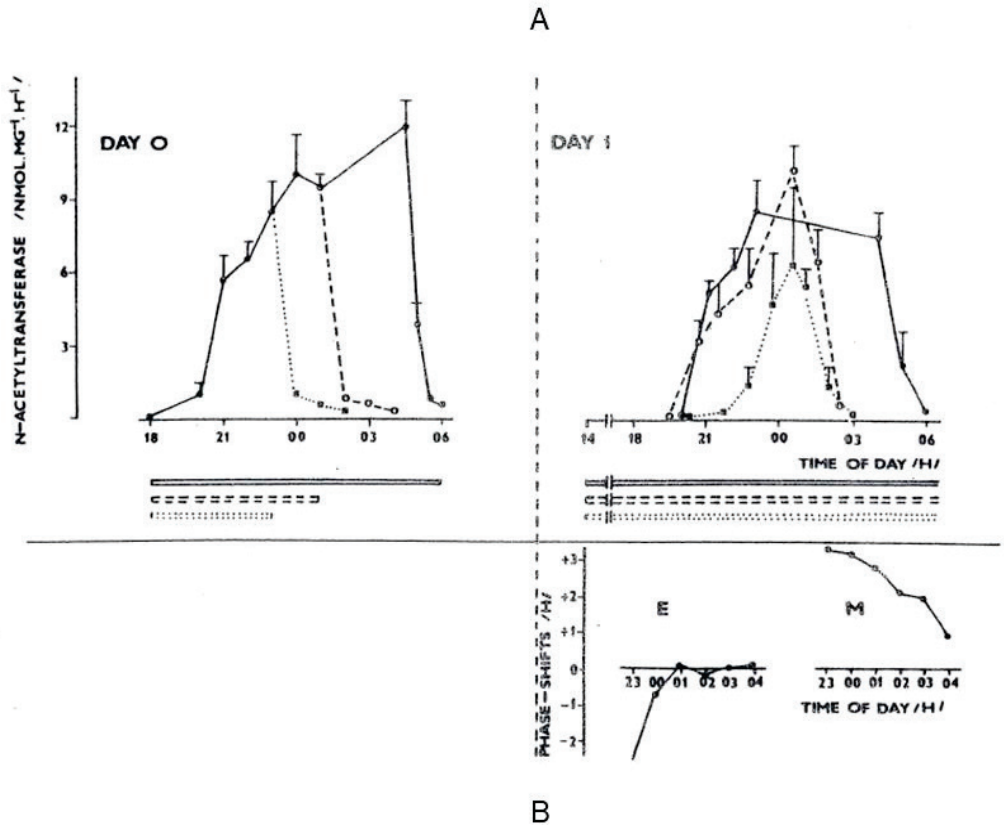

Fig. 8. Phase shifts of the N-acetyltransferase (NAT) rhythm after bringing forward the morning lights-on. **A**: The NAT rhythm during the night when the lights-on was brought forward (day 0) and during the next night (day 1). Rats maintained in LD 12:12, with lights on from 06 h to 18 h, were subjected to the usual lights-off at 18 h and later that night either to the usual morning lights-on at 06 h (filled circles, full line), or to an advance of the lights-on to 01 h (open circles, broken line), or to 23 h (filled squares, dotted line), respectively (day 0). Thereafter, light was turned off at 14 h and the NAT rhythm was followed during the subsequent night (day 1). Lines under the abcissa indicate dark periods. **B**: Phase shifts of the evening NAT rise (**E**) and of the morning NAT decline (**M**) the next night after bringing forward the morning lights-on, determined at the level of 3 nmol/mg.h of the NAT activity from Fig. 8A and other similar figures. Phase advances are expressed with the sign +, phase delays with the sign -. The abcissa denotes time of the light onset on day 0. Data were taken from [31].

Thereafter, the rats were released into darkness at 14 h and the NAT rhythm was followed in the subsequent night (Day 1, Fig. 8A) [31]. All phase shifts of the morning NAT decline (M) and of the evening NAT rise (E) on day 1 after bringing forward the morning light onset on day 0 are shown in Fig. 8B [31]. After the advance of the morning light onset, only the morning NAT declines (M) were phase-advanced by 3 h at most, but not the evening NAT rises (E) [23, 27, 31]. When the morning light onset was brought forward to before midnight, the evening NAT rise (E) was even phase-delayed and the NAT rhythm waveform and amplitude might change dramatically [31]

Effect of the photoperiod on the rhythm in melatonin production

If the evening light phase delays primarily the evening NAT rise (E) and the morning light phase advances primarily the morning NAT decline (M), we may expect that on long summer days the duration of the elevated melatonin production might be compressed due to the phase delaying effect of the evening light intruding into the late evening hours on E and to the phase advancing effect of the morning light intruding into the early morning hours on M, and on short winter days the duration might be decompressed. And it was indeed the case (Fig. 9B) [32]. On long summer days in June, the duration of the elevated NAT activity and hence of the high melatonin production was by more than 4 hours shorter than the duration on short winter days in December. Similarly, under an artificial LD 16:8 regime, the duration of the elevated NAT activity was shorter than that under the LD 8:16 regime (Fig. 9A) [32]. Hence the phase relationship between the evening NAT rise (E) and the morning NAT decline (M), determining the duration of the nocturnal melatonin production, might transduce the information on a changing daylength, i.e.

on the photoperiod, onto mammals [24, 32]. In 1979, we sent the manuscript on the pineal NAT rhythm in rats under different artificial photoperiods and in natural daylight in the course of a year to the *Journal of Endocrinology*, but it was rejected with an explanation that the difference in the duration of the nocturnal melatonin production between long and short days was not enough dramatic for showing that the melatonin signal duration might be the sought photoperiodic message. But the difference was dramatic – it was more than 4 hours! In 1980, the manuscript was published in *Neuroendocrinology* [32]. At about the same time, an article on Syrian hamsters appeared, in which a difference in the melatonin signal duration between a short and a long photoperiod was visible, but the authors did not yet recognized the importance of this observation [33]. In 1981, the difference in melatonin signal duration in animals maintained under long days and those maintained under short days was already reported for ewes [34], Djungarian hamsters [35] and white-footed mice [36].

When rats were exposed to six different photoperiods, the NAT rise (E) depended on the photoperiod [23, 37, 38]. The time interval between the onset of darkness and the evening NAT rise (E) increased with the increasing duration of the dark period to more than 6 hours in LD 6:18. In contrast, the time interval between the morning NAT decline (M) and the onset of light was almost constant under all photoperiods, i.e., between 1-2 hours [23, 38]. The NAT rhythm is therefore locked to the morning lights-on rather than to the evening lights-off. It appears that the morning light may entrain the NAT rhythm to the 24 h day and the evening light may serve rather as a photoperiodic signal.

Decompression of the phase relationship between the evening NAT and melatonin rise (E) and the morning decline (M) after a change from a long to a short photoperiod is a gradual process. After a change from LD 16:8 to LD 8:16 accomplished by a symmetrical prolongation of the dark period around midnight, extension of the pattern of the NAT rhythm proceeded mainly into the morning hours and was thus achieved more by phase delays of the morning NAT decline (M) than by phase advances of the evening NAT rise (E) [39]. Extension of the period of the elevated nocturnal NAT activity proceeded more rapidly when the change from LD 16:8 to LD 8:16 was accomplished asymmetrically by prolongation of the dark period into the morning and daytime hours than when the dark period was extended into the evening and late day hours [38, 39]. Whereas the extension of the phase relationship between the evening NAT rise (E) and the morning decline (M) may be a gradual and slow process, compression of the duration by long days may proceed rapidly [18, 32].

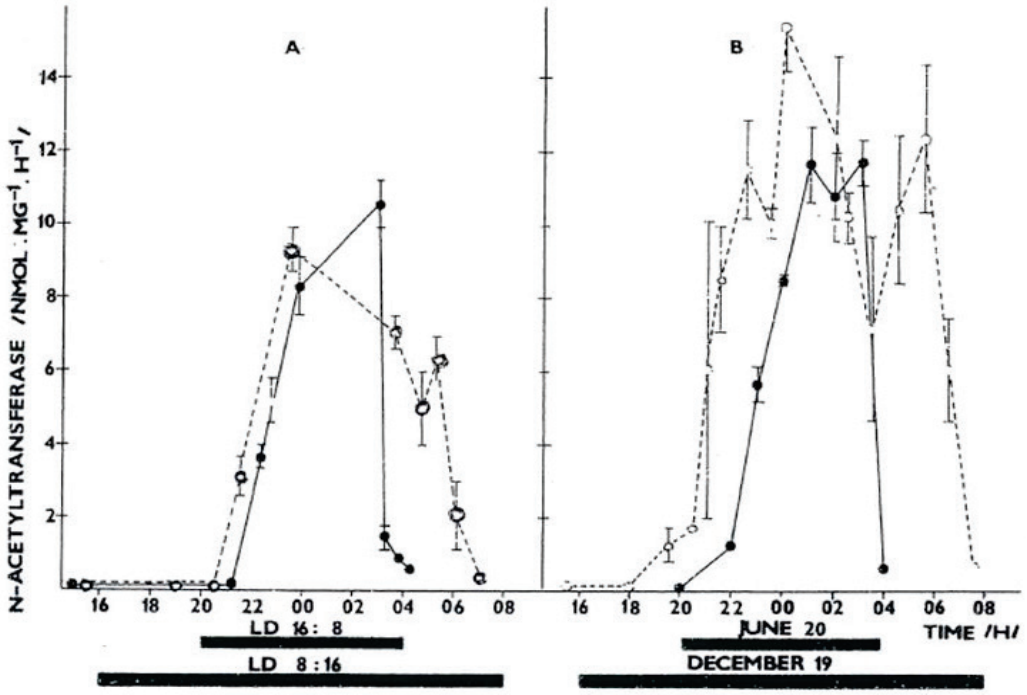

Fig. 9. The N-acetyltransferase rhythm in rats maintained under artificial light-dark regimes (**A**) or in natural daylight (**B**). Filled circles represent rats kept for 5 weeks in LD 16:8 (A) or rats kept in natural daylight and killed on June 20 (B). Open circles represent rats maintained for 5 weeks in LD 8:16 (A) or rats maintained in natural daylight and killed on December 19 (B). Data were taken from [32].

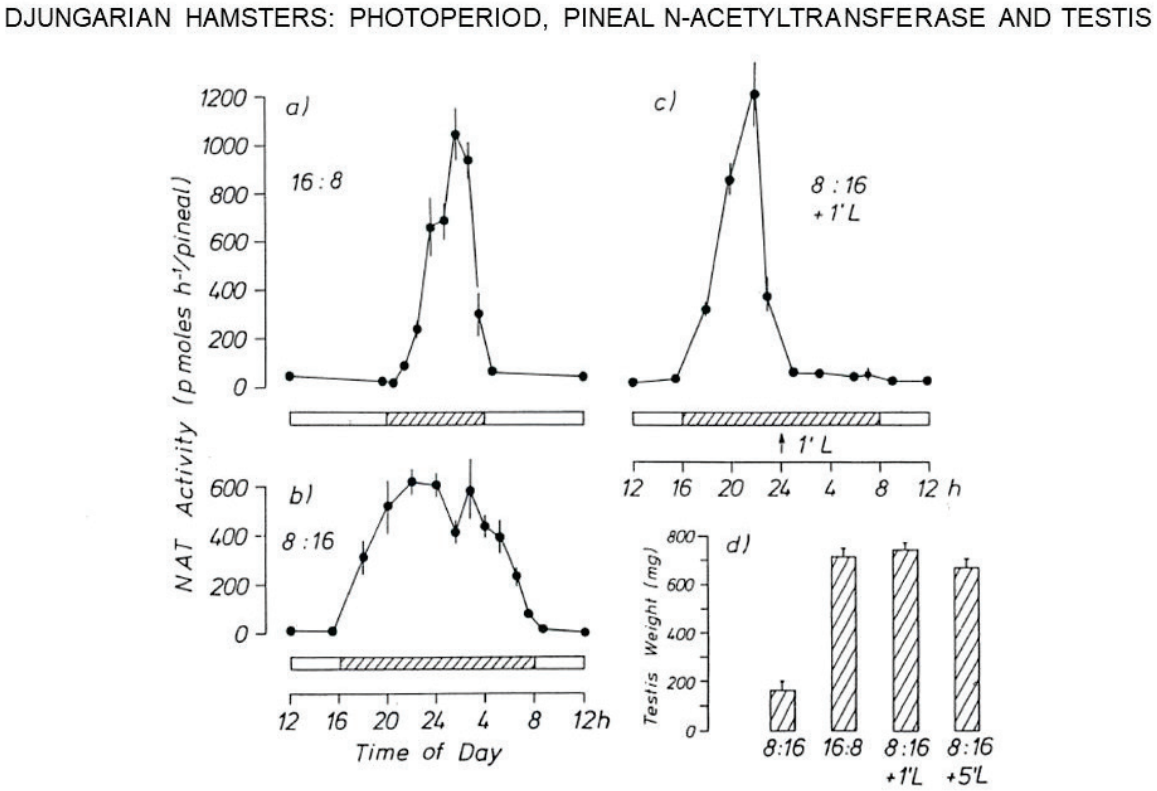

Fig. 10. Diurnal rhythm of N-acetyltransferase (NAT) activity in pineals of Djungarian hamsters under different photoperiods (**a-c**) and effects of such photoperiods on testicular recrudescence (**d**). NAT activity profile **a**) in long photoperiods (LD 16:8); **b**) in short photoperiods (LD 8:16); **c**) in short photoperiods in which the dark period was interrupted by 1 min of light each night. **d**) Testis weight after 45 days in the photoperiods indicated. Open horizonal bars in (a-c) represent light, hatched bars darkness. Note similarity of patterns in (a) and (c) and similarity of effect of LD 16:8, LD 8:16 + 1 min light and LD 8:16 + 5 min light in 3(d). Data were taken from [35].

In all mammals so far studied the melatonin signal duration changes according to a season of the year. However, in humans the melatonin signal duration appears to depend just slightly on the ambient photoperiod and only at higher latitudes, e.g., at 60 °N in Sweden [40] and at 68 °S in Antarctica [41]. In the temperate zone 50 °N, we found no difference in the duration of elevated nocturnal melatonin concentration between summer and winter in plasma of healthy urbanized people [18, 42]. Only in winter the melatonin rhythms were phase-delayed by about 1.5 hours as compared with summer patterns. However, at 50 °N in summer, exposure of human subjects to a natural 16 h bright light photoperiod phase-advanced the morning salivary melatonin decline and shortened thus the nocturnal melatonin signal by 2 hours relative to the winter patterns of the same subjects followed under a combined artificial and natural light 16 h photoperiod [43]. The data suggest that natural summer days experienced from sunrise till sunset and not winter days with a combined artificial and natural light photoperiod evoke a true long days response of the human circadian system.

Duration of the nocturnal melatonin production as the photoperiodic signal?

We have mostly used the rat as a model animal. However, rats are only marginally photoperiodic under normal conditions, that means that they do not respond to a change in daylength, i.e., in the photoperiod, by a change in gonadal size, body weight or pelage color. To find out whether the melatonin signal duration might be indeed the sought photoperiodic signal, we joined our forces with Klaus Hoffmann from the Max Planck Institute in Andechs who was an expert on photoperiodism and had as model animals the photoperiodic Djungarian hamsters. In these hamsters short photoperiods induce regression of testes and accessory glands [44] and the nocturnal melatonin synthesis is also driven by the pineal NAT activity as in rats [19]. When Djungarian hamsters were maintained in

short, LD 8:16 days, the duration of the nocturnal elevated NAT activity and hence of the high nocturnal melatonin production was long and the testis weight was low (Fig. 10) [35]. When Djungarian hamsters were maintained either in a long, LD 16:8 photoperiod or in a short, LD 8:16 photoperiod with the dark period interrupted by 1 min of light in the middle of night, the duration of the nocturnal NAT activity was short and the testis weight was high. Apparently under short days, with a 1 min light pulse in the middle of the dark period, the Djungarian hamsters perceived the pulse already as a dawn. Results of our study indicated that duration of the nocturnal melatonin formation might be indeed the photoperiodic signal [35].

Our next study brought similar conclusions [45]. Djungarian hamsters maintained on a regime of 16 h of light and 8 h of darkness per day (LD 16:8) were transferred to a LD 8:16 regime either by extending the dark period into the morning hours till noon (A) or by bringing forward the evening dark onset to the afternoon hours till noon (B). Under the schedule A, extension of the compressed state of the nocturnal melatonin signal proceeded more rapidly and regression of testes and accessory glands proceeded with a higher velocity than under the schedule B [45]. The different rate of gonadal regression after different ways of transition into the same short photoperiod might be due to the different rate of decompression of the melatonin pattern.

Two years after our first study on photoperiodism in Djungarian hamsters had been published [35], Carter and Goldman brought a direct proof that duration of the nocturnal melatonin signal was indeed the proper photoperiodic message [46, 47]. They found that melatonin infusion to young pinealectomized Djungarian hamsters for 8 or more hours per day induced a gonadal regression similar to that induced by short days whereas the infusion for 4-6 hours per day stimulated a gonadal growth similar to that induced by long days. When Bruce Goldman sent reprints of these two papers to Klaus Hoffmann, he wrote there: “You had it all the time right, Klaus”.

However, when considering the importance of the melatonin signal duration in conveying the photoperiodic message, a question arises whether an absolute value of the duration is important or whether rather a change in the duration is important. To solve this question, we maintained Djungarian hamsters under a long, LD 16:8 photoperiod and we transferred them to another long, but a shorter LD 14:10 photoperiod. We also maintained Djungarian hamsters under a short, LD 8:16 photoperiod, and we transferred them to a long, LD14:10 photoperiod [18, 48]. Finally, both groups were under the same, LD 14:10 photoperiod (Fig. 11). Melatonin profiles of both groups under LD 14:10 were the same and consequently the melatonin signal duration was the same as well, regardless of whether the animals came from the long or from the short photoperiod. However, the reproductive organs of Djungarian hamsters responded to the same signal duration in a different way depending on whether they came from the long or from the short photoperiod (Fig. 12). When the male Djungarian hamsters came to the LD14:10 photoperiod from the LD 16:8 photoperiod, they responded to shortening of the photoperiod and hence to lengthening of the original melatonin signal duration by a decrease in reproductive organs weight, whereas when they came to the LD 14:10 photoperiod from the LD 8:16 photoperiod, they responded to the lengthening of the photoperiod and hence to shortening of the melatonin signal duration by a dramatic increase in reproductive organs weight. This finding showed for the first time that mammals might read rather a change in the melatonin signal duration than the absolute value of the duration [18, 48].

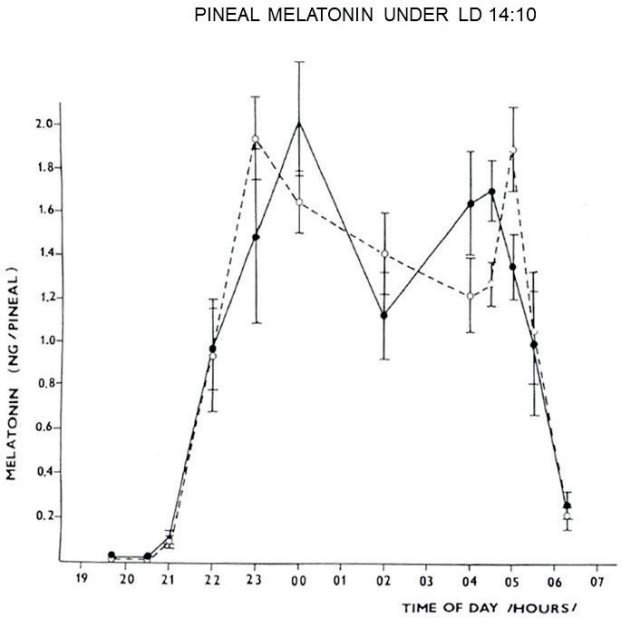

Fig. 11. Profiles of the rhythm in pineal melatonin concentration in Djungarian hamsters under LD 14:10. Djungarian hamsters maintained under LD 16:8 (closed circles) or under LD 8:16 (open circles) were transferred to LD 14:10 and killed 10 weeks later. Data were taken from [48].

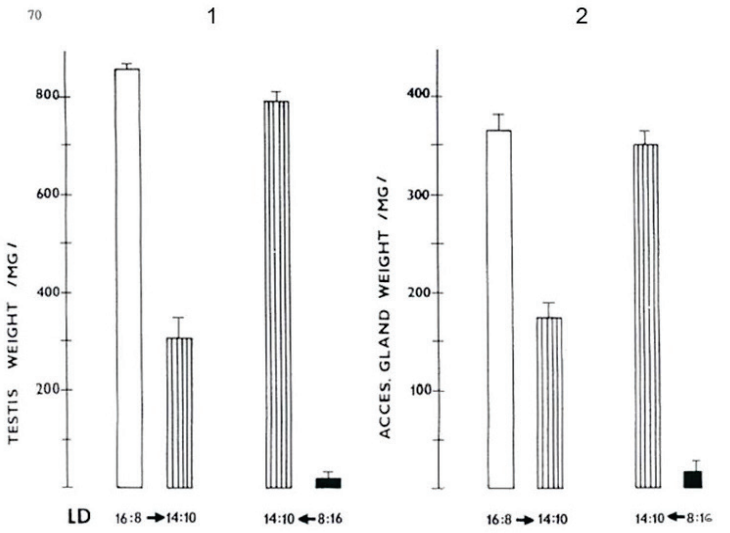

Fig. 12. Testis (**1**) and accessory gland (seminal vesicles, coagulating glands and ampullary glands) (**2**) weight in Djungarian hamsters under LD 14:10. Djungarian hamsters maintained in LD 16:8 (open bars) or in LD 8:16 (closed bars) were transferred to LD 14:10 and killed 10 weeks later (stripped bars). Data were taken from [48].

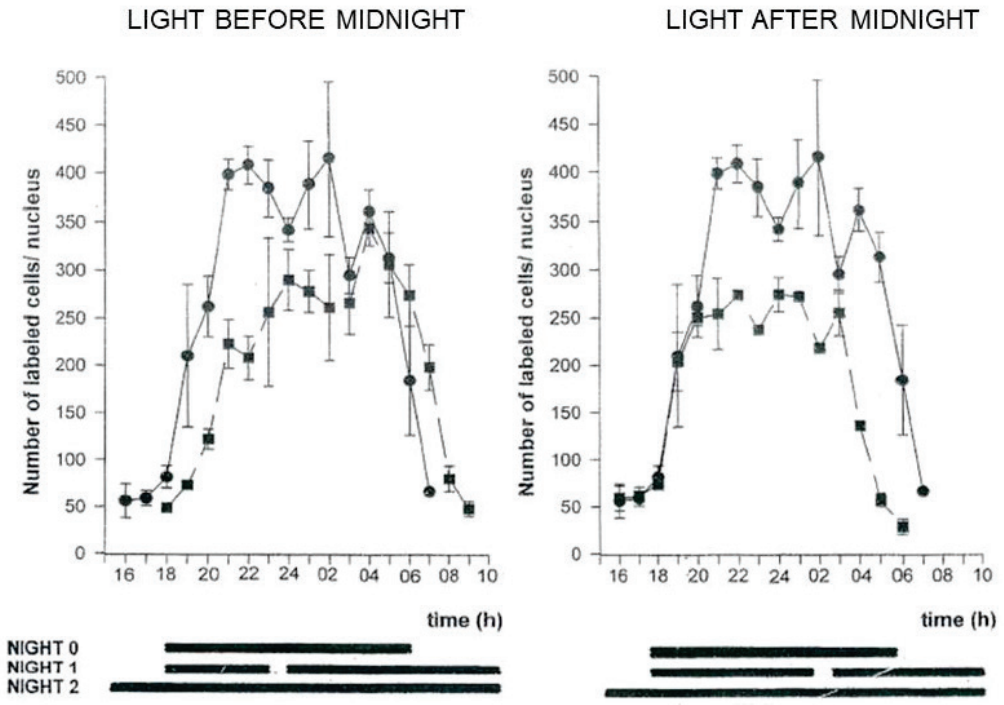

Fig. 13. Resetting of the suprachiasmatic nucleus (SCN) rhythm in light induced c-Fos immune-reactivity. Rats maintained in LD 12:12 (night 0) were either untreated (circles) or exposed to a 1 h of light (squares) from 23 to 24 h before midnight (left) or from 02 to 03 h after midnight (right) (night 1) and then they were released into darkness. The next night (night 2), they were exposed to a single 30 min light pulse at different nighttimes, returned to darkness and killed 30 min later for c-Fos immunoreactivity determi-nation. Filled bars under the abcissa indicate dark periods. Data were taken from [58].

Possible effect of the photoperiod on the circadian pacemaker in the suprachiasmatic nuclei (SCN)

Under longer days, the discrete entrainment of the NAT rhythm is accomplished by smaller phase shifts than under shorter days [18, 49]. Rats maintained under a long, LD 18:6 photoperiod or under a short, LD 6:18 photoperiod were exposed to a 1 min light pulse at different times of night, then they were released into darkness and the next night phase shifts of the evening NAT rise (E) and of the morning NAT decline (M) were determined. In rats maintained under the long, LD 18:6 photoperiod, E was phase-delayed by at most 0.5 h, while in those maintained under the short, LD 6:18 photoperiod, E was phase-delayed by as much as by 2.8 h. Similarly, M was phase-advanced by at most 1.9 h under LD 18:6, but by as much as by 3.5 h under LD 6:18 [49]. Smaller phase shifts under the long than under the short photoperiod point to a possibility that the compressed E-M phase relationship under long days does not allow greater phase shifts [18, 49]. Difference in the magnitude of phase shifts between long and short days might indicate that a state of the pacemaker controlling

the NAT rhythm depends on the photoperiod. Hence, we decided to study the effect of the photoperiod on the circadian pacemaker itself.

The circadian pacemaker or the central biological clock controlling the pineal N-acetyltransferase rhythm [50] as well as other circadian rhythms, e.g., the locomotor activity rhythm or the rhythm in corticosterone formation in the adrenals is located in two suprachiasmatic nuclei (SCN) of the anterior hypothalamus [51, 52]. The SCN consists of two subdivisions, which are morphologically and functionally distinct, namely of the ventrolateral (vl) part called the core and of the dorsomedial (dm) part called the shell [53, 54]. Cells of the ventrolateral SCN receive direct photic signals from the retina and at night respond to them by expression of immediate early genes (IEGs) [55,56]. Cells of the dorsomedial SCN exhibit spontaneous rhythms and their resetting requires communication with the light sensitive cells of the ventrolateral SCN [54].

Effect of light and photoperiod on the ventrolateral SCN

Together with Alena Sumová we tried to find out how photic resetting of the SCN intrinsic rhythmicity proceeds *in vivo* [58]. As a marker of the SCN intrinsic rhythmicity, we used the circadian rhythm in photic induction of IEGs, namely of c-fos [55, 56, 57]. Rats maintained in LD 12:12 were either left untreated or experienced a 1 hour light exposure either before or after midnight (Fig. 13). Then they were released into darkness and the next night they were exposed to a 30 min light pulse at different night times in order to get a profile of the SCN rhythm in the light-induced c-Fos immunoreactivity [58]. Following the 1 hour light exposure before midnight, the next night both the evening rise (E) and the morning decline (M) of c-Fos immunoreactivity were phase-delayed, however, E was delayed slightly more than M. After the 1 hour light exposure after midnight, only the morning decline in c-Fos immunoreactivity was phase-advanced the next night, but not the evening rise (E) [58]. It appears that the light exposure in the first half of the night might phase delay primarily the evening rise in c-fos photoinduction (E), while the light exposure in the second part of the night phase-advanced primarily the morning decline (M), as it was the case with the pineal NAT rhythm [23, 26, 31].

Consequently, the duration of the interval enabling c-Fos photoinduction at night in the rat SCN might be compressed on long "summer" days with light intruding into the late evening and early morning hours and decompressed on short "winter" days. In collaboration with Bill Schwartz from the university of Massachusetts Medical School we proved that this was actually the case (Fig. 14) [56]. The duration of the interval between the evening rise (E) in c-fos mRNA and the morning decline (M) under long days with only 8 hours of darkness was by about 5-6 hours shorter than that under short days with 16 hours of darkness. We got a similar picture when we used c-Fos immunoreactivity instead of c-fos mRNA as a marker of the rhythm in c-fos photoinduction [56]. The duration represents again the phase relationship between the evening (E) and the morning (M) marker of the circadian rhythm, but this time in the SCN rhythm itself and not in a rhythm controlled by the SCN. Under the short photoperiod, there was a robust correlation between c-fos photoindu-

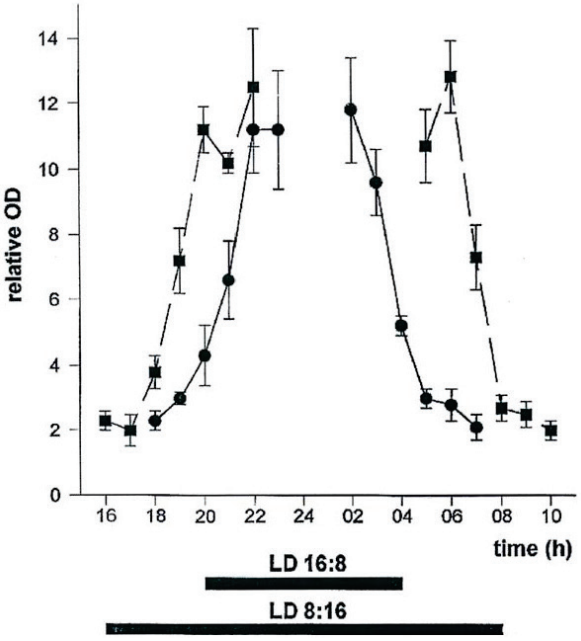

Fig. 14. Light induced c-fos gene expression in the SCN under long and short photoperiods. Rats were maintained under a long, LD 16:8 photoperiod, with lights on from 04 till 20 h, or under a short, LD 8:16 photoperiod, with lights on from 08 to 16 h. On the day of the experiment, the evening onset of darkness was advanced to 12 h or the morning onset of light was delayed to 12 h, respectively, and the rats were exposed to a single 30 min light pulse at different times in darkness. At the end of the pulse, they were killed and phase-dependent photic induction of c-fos mRNA was assayed by *in situ* hybridization in the SCN of rats maintained previously under the long (circles) or under the short (squares) photoperiod. Bars under the abcissa represent original periods of darkness under the long or under the short photoperiod. Data were taken from [56].

ction and the magnitude of phase shifts of the pineal NAT rhythm [59]. Under the long photoperiod, there was also a good correlation between c-fos photoinduction and the magnitude of phase shifts of the intrinsic ventrolateral SCN rhythmicity [60]. Under a very long LD 18:6 photoperiod, even a 5 min light pulse applied around midnight phase delayed the evening NAT rise (E) and phase-advanced the morning decline (M) and at the same time lowered the amplitude of the rhythm in c-fos photoinduction [58]. Apparently, under such a long photoperiod, the E-M phase relationship in the SCN might be so compressed that even only the 5 min light pulse might hit both E and M at the same time. The fact that the photoperiod affects the intrinsic rhythm in c-fos photoinduction in the rat SCN brings the first evidence that the photoperiod modulates the central circadian pacemaker [56]. Hence, the pacemaker may not serve only as a daily program, but also as a calendar, as it was suggested by Pittendrigh and Daan [30]. Importantly, the effect of the photoperiod is not altered by pinealectomy which indicates that the daylength affects the functional state of the SCN circadian pacemaking system directly and not via the pineal melatonin signal [61].

Complex pacemaker in the ventrolateral SCN

The Pittendrigh´s and Daan´s model [30] based on modeling the locomotor activity rhythm in nocturnal rodents anticipates the complex circadian pacemaker consisting of two oscillators or clusters of oscillators, the evening one, E, and the morning one, M, where E controls the evening onset of the activity and is coupled to sunset, while M controls the morning offset of the activity and is coupled to sunrise. Both oscillators interact with each other and their interaction may be affected by the photoperiod [30]. At the time when the model was suggested, the idea of a complex pacemaker underlying circadian rhythms was ingenious as then a prevailing opinion was that the SCN clock in the brain was just a unified single clock, a homogenous population of cells that produced a synchronous daily oscillatory signal. Though the idea of Pittendigh and Daan was ingenious, the two oscillator model was based on splitting of the locomotor activity of hamsters into two activity peaks 180° apart. But this splitting was based just on the existence of the left and right SCN and these „split“ oscillators are not likely to represent E and M because they appear to be functionally equivalent [62].

We thus proposed a similar two-oscilator model, but based on the pineal NAT rhythm [22] and on the SCN rhythm in c-fos photoinduction [56,58]. Non-parallel phase shifts of the E and M phase markers of the pineal NAT rhythm [22] as well as of the rhythm in c-fos photoinduction in the SCN [58] indicate a possibility that the central pacemaker consists of at least two clusters of oscillators, E and M, which interact. The E-M phase relationship is photoperiod-dependent and it determines the subjective night, namely the duration of the nocturnal melatonin signal, the SCN gate for sensitivity to light and the SCN gate for phase shifting circadian rhythms by light. M, coupled to dawn, may entrain the pineal and the ventrolateral SCN rhythms to the 24 h day, while E may serve rather as a photoperiodic signal [56, 62, 63, 64].

More studies support the E-M model. For example, in SCN slices from Syrian hamsters, the circadian rhythm of SCN multiunit neural activity exhibits distinct morning and evening peaks when the slices are cut in the horizontal plane [65]. The morning peak follows the projected dawn, while the evening peak occurs around the projected dusk. The two peaks are differently affected by changes in the antecedent photoperiod. Also in Drosophila, morning and evening oscillators were reported [66,67] as well as in mice [68] and in humans [69,70]. Assuming that E and M oscillators indeed exist, it is not clear whether they are a property of individual SCN cells or instead emerge from an intercellular network interaction. However, as the rhythm in c-fos photoinduction exists only in the ventrolateral, but not in the dorsomedial SCN [71], the assumed E and M oscillators might also exist only in the ventrolateral SCN.

The effect of photoperiod on the intrinsic rhythmicity in the dorsomedial SCN

A question arises whether the circadian rhythmicity of the dorsomedial SCN is also modulated by the photoperiod. Two circadian rhythms are typical for the dorsomedial SCN, the rhythm of arginine vasopressin (AVP) mRNA expression and AVP peptide formation [72] and the spontaneous rhythm in c-Fos immuno-reactivity [53]. The circadian rhythm of AVP mRNA levels in the SCN of rats maintained under a short LD 8:16 photoperiod differed from that of rats maintained under a long LD 16:8 photoperiod. Under the short photoperiod, the morning AVP mRNA rise occurred by about 4 hours later than that under the long one (Fig. 15)

[72]. Similarly, in rats maintained under the short, LD 8:16 photoperiod or under a natural photoperiod in December, the morning rise in c-Fos immunoreactivity in the dorsomedial SCN occurred by about 4 hours later than that in rats maintained under the long, LD 16:8 photoperiod or under a natural photoperiod in June [71,73]. Apparently, the dorsomedial SCN rhythmicity is also affected by the photoperiod and hence the whole SCN state is photoperiod-dependent.

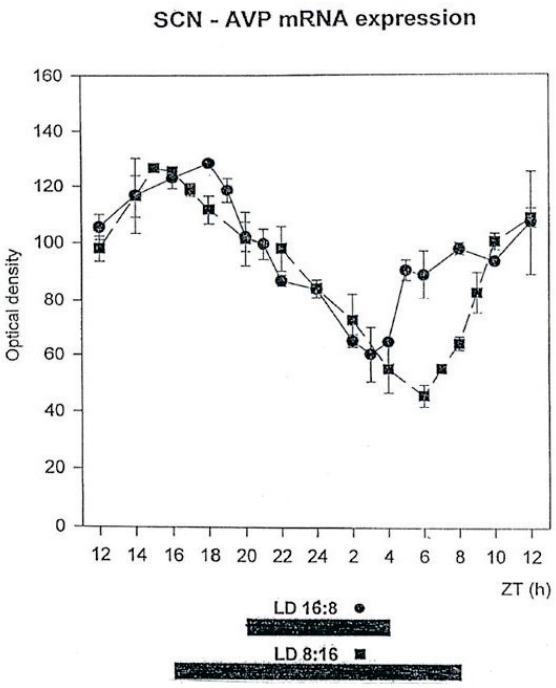

Fig. 15. Daily profiles of arginine vasopressin (AVP) mRNA levels in the SCN of rats maintained either under a long, LD 16:8 (circles) or under a short, LD 8:16 (squares) photoperiod. Bars under the abcissa represent dark periods. Data were taken from [72].

By then, the molecular clockwork in the mammalian SCN has been described, mostly by the groups of Takahashi [74] and Reppert [75]. Eight cloned clock genes are thought to be involved in interacting transcriptional-translational feedback loops that compose the molecular clockwork. We studied the expression of some of the clock genes in the rat SCN under various photoperiods in order to find out whether even the rat molecular clockwork is modulated by the photoperiod. First, we studied the rhythm of a clock gene Per 1 product, PER 1 protein, with the maximum level late in the subjective day and early night and the minimum level in the morning [76]. Under a long, LD 16:8 artificial photoperiod, the interval of elevated PER 1 immunoreactivity was at least by 4 hours longer than that under a short, LD 8:16 photoperiod, due mainly to an earlier PER 1 daytime rise under the long photoperiod [76]. Under a natural photoperiod, profiles of the PER 1 rhythm in summer and in winter resembled those under corresponding artificial photoperiods. Importantly, under all photoperiods, when PER 1 immunoreactivity was elevated, immunopositive cells were localized in the dorsomedial rather than in the ventrolateral SCN [76]. When daily profiles of mRNA of four clock genes, namely of *Per 1*, *Cry 1*, *Bmal 1* and *Clock* were studied under different photoperiods, the photoperiod affected phase, waveform and amplitude of the rhythmic gene expression as well as a phase relationship between their profiles. Under a long photoperiod, the high daytime *Per 1* mRNA expression was lengthened and the high nighttime *Bmal 1* mRNA expression was shortened as compared with the expression under a short photoperiod [77, 78]. Altogether, the whole complex molecular clockwork in the rat SCN appears to be photoperiod dependent and hence it may differ according to the season of the year [77, 78].

A functional divergence among subpopulations of SCN cells may exist not only along the ventrodorsal, but also along the rostro-caudal axis of the SCN [68, 79]. In mice maintained under a long LD 18:6 photoperiod, daily profiles of two clock genes, namely of Per 1 and of Per 2 expression in the rostral and caudal SCN were desynchronized and the peak of expression in the caudal SCN preceded that in the rostral SCN [80]. Following transition of the mice from the long photoperiod to a short LD 6:18 photoperiod, the Per 1 and Per 2 profiles in the rostral SCN became gradually synchronized with those in the caudal SCN; simultaneously, waveform of the rhythms changed as well [81].

Before leaving the fascinating and complex world of the SCN with its gate for photic sensitivity, which, according to my opinion, has not been sufficiently explained, let´s consider shortly another way of entrainment of the SCN, this time not by light, but by the pineal hormone melatonin.

Entrainment of the rhythm in the pineal melatonin production and of the SCN rhythm in the light-induced c-Fos immunoreactivity by melatonin

In 1987, Jiří Vaněček from our laboratory described for the first time the presence of high affinity melatonin binding sites in the SCN of the rat hypothalamus [82]. The presence of melatonin receptors

in the mammalian circadian pacemaker correlated well with the proposed role of melatonin in entrainment of the SCN controlled rhythms such as are the rhythm in the locomotor activity or in the pineal melatonin production. Melatonin injected at 24 h intervals into free running rats maintained in constant darkness entrained them to the 24 h day [83]. Melatonin given to human volunteers in the late afternoon phase-advanced the onset of their own nocturnal melatonin production [84]. When exogenous melatonin was administered to humans at various times with respect to the time of their own melatonin production and the evening onset of melatonin secretion in dim light was used as a phase marker, maximum phase advances of the marker were found when melatonin was administered in the late afternoon; sporadically, some phase delays also occurred when melatonin was administered in the morning hours [85].

In rats maintained on a regime with 10 hours of light and 14 hours of darkness per day, after a single melatonin injection before the dark onset or after administration of melatonin for 5 successive days or after a 4 day treatment with melatonin and a 1 day withdrawal, the evening NAT rise was phase-advanced relative to that in rats treated with vehicle only; the phase shift was larger after a repeated than after a single melatonin injection [86]. Under all the above mentioned paradigms, the evening NAT rise was phase-advanced significantly, while the morning decline was almost not phase shifted. Melatonin administration for 5 consecutive days phase-advanced the evening NAT rise only in rats maintained under a LD 10:14 or a LD 8:16 photoperiods, but not in those maintained under a LD 12:12 photoperiod [87]. Under the longer photoperiod, the end of the light period exhibited a phase delaying effect on the NAT rise and overrode the phase advancing effect of melatonin. Apparently, light is a stronger entraining agent than melatonin.

Melatonin might act by modulating directly the pacemaking system *via* highly sensitive melatonin receptors in the SCN [82]. The *in vitro* exposure of the rat brain hypothalamic slices to melatonin for 1 hour in the late subjective day phase-advanced the SCN circadian rhythm of neuronal firing rate the next day [88]. However, nothing was known about resetting of a SCN circadian oscillation by melatonin *in vivo*. To fill this gap, i.e., to find out whether melatonin applied *in vivo* resets an intrinsic circadian rhythm in the SCN and how rapidly the resetting is accomplished, we chose as a marker of the SCN intrinsic rhythmicity the rhythm in c-Fos photoinduction [55, 56]. Rats were maintained either under a short, LD 8:16 photoperiod, with the dark period starting at 16 h, or under a long LD16:8 photoperiod, with the dark period starting at 20 h. On the day of the experiment, the dark period started as usually at 16 h in short days, whereas in long days the dark onset was advanced to 16 h as well in order to avoid the masking effect of the evening light [87]. Melatonin and vehicle were administered in the late subjective day and thereafter the rats were exposed to a single light pulse at various times (Fig. 16) [89]. In rats maintained under short days, the evening rise in the light-induced c-Fos immunoreactivity in the melatonin-treated rats was phase-advanced by about 1.4 h relative to the rise in the vehicle-treated animals and by about 1.7 h relative to the rise in the intact animals. In rats maintained under long days, the evening rise in the light-induced c-Fos immunoreactivity in the melatonin-treated rats was phase-advanced by 1.5 h relative to the rise in the vehicle-treated animals and by about 1.2 h relative to the rise in the intact animals [89]. Hence, a single melatonin administration to rats during the late day may advance the evening rise in c-Fos photoinduction be it under short or long days when the masking effect of the evening light is avoided. The data indicate that melatonin administration *in vivo* may instantaneously reset the intrinsic SCN rhythmicity. As the rise in the evening c-Fos photoinduction in the SCN is phase-advanced after melatonin administration, the gate for the SCN sensitivity to light and at the same time the SCN gate for phase shifting of circadian rhythms by light might widen. Extension of the gate might partly explain why melatonin administration accelerated reentrainment of the pineal NAT rhythm after an 8 h advance of the light-dark 12:12 cycle [90].

Towards the circadian timekeeping system in mammals

Apart from the central circadian pacemaker within the SCN, the circadian system may consist of numerous peripheral clocks [91]. It appears that biological clocks are most probably present in all organs, tissues and cells. However, the central clock in the SCN is the only one which is entrained directly by environmental light *via* its connections with the retina. Peripheral clocks are entrained to the outside world mostly *via* yet not fully identified neuronal and humoral pathways from the SCN [92]. Altogether, the central

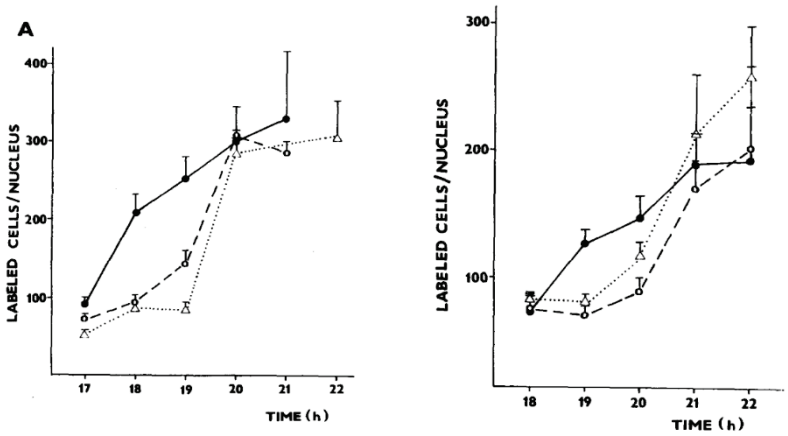

Fig. 16. The effect of melatonin on the evening rise in the light-induced c-Fos immunoreactivity in the suprachiasmatic nucleus of rats maintained originally under a short, LD 8:16 (left) or under a long, LD16:8 (right) photoperiod. On the day of the experiment, the dark period started at 16 h. Rats were either left intact (open triangles, dotted line) or injected with vehicle (open circles, broken line) or with melatonin (closed circles, full line) in the late subjective day, i.e., between 16.30 h and 17.00 h (left) and between 17:30 and 18.00 h (right), respectively. Thereafter, they were exposed to a single 30 min light pulse at various times, returned to darkness and 30 min later they were killed for c-Fos immunoreactivity determination. Data were taken from [89].

SCN clock together with all other clocks and interconnections form a circadian timekeeping system. It is the most integrative system in the body as it integrates all parts to one time, i.e., it keeps all constituent oscillators appropriately phased to each other. Our former Laboratory of Neurohumoral Regulations and nowadays the Laboratory of Biological Rhythms under the guidance of Alena Sumová does not concentrate its attention anymore only to the SCN controlled rhythms in one organ and to the SCN itself, as we had done previously. Instead, it focuses on the circadian system as a whole, namely on central and peripheral clocks and their entrainment by external environment including the mechanism of photic, photoperiodic and non-photic entrainment; on ontogenesis of the circadian system and the mechanisms of how maternal and environmental factors affect the development of the system in mammals; on circadian regulation of physiological function with attention to the circadian regulation of the gastrointestinal tract, the pancreas, the choroid plexus and the hippocampus; on circadian system in humans in health and diseases, mostly on the relationship between the circadian system and neuropsychiatric diseases.

Circadian biology is a marvelous field for research. It teaches us how to live in harmony with the outside day and world. When studying it, you may feel all the time like Alice in Wonderland.

Conflict of Interest

There is no conflict of interest.

Acknowledgement

At the end, I would like to thank my closest collaborators, namely Jiří Vaněček, Alena Sumová and the late Klaus Hoffmann, as well as other collaborators, e.g., Lennart Wetterberg, William J. Schwartz, Martin Sládek, Ivan Hájek, the late Petr Zvolský and Milena Burešová and my students Ludmila Samková, Zdeňka Trávníčková-Bendová, Martin Jáč, Dana Vondrašová-Jelínková and the late Martina Humlová.

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The wonder of the world,
the beauty and the power,
the shapes of things,
their colors, light and shades.
These I saw.
Look ye also while life lasts.

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