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Report

Circadian rhythms *

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Circadian rhythms are a ubiquitous adaptation of eukaryotic organisms to the most reliable and predictable of environmental changes, the daily cycles of light and temperature. Prominent daily rhythms in behavior, physiology, hormone levels and biochemistry (including gene expression) are not merely responses to these environmental cycles, however, but embody the organism's ability to keep and tell time. At the core of circadian systems is a mysterious mechanism, located in the brain (actually the suprachiasmatic nucleus of the hypothalamus) of mammals, but present even in unicellular organisms, that functions as a clock. This clock drives circadian rhythms. It is independent of, but remains responsive to, environmental cycles (especially light). The interest in temporal regulation – its organization, mechanism and consequences – unites investigators in diverse disciplines studying otherwise disparate systems. This diversity is reflected in the brief reviews that summarize the presentations at a meeting on circadian rhythms held in New York City on October 31, 1992. The meeting was sponsored by the Fondation pour l'Étude du Système Nerveux (FESN) and followed a larger meeting held 18 months earlier in Geneva, whose proceedings have been published (M. Zatz (Ed.), Report of the Ninth FESN Study Group on 'Circadian Rhythms', *Discussions in Neuroscience, Vol. VIII, Nos. 2 + 3*, Elsevier, Amsterdam, 1992). Some speakers described progress made in the interim, while others addressed aspects of the field not previously covered.

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* These manuscripts were presented at a FESN Study Group Follow-up Meeting on 'Circadian rhythms', held in New York City on October 31, 1992

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1. Temperature, seasonality and circadian rhythms

Irving Zucker, Theresa M. Lee and Norman F. Ruby

Prominent seasonal changes in circadian rhythms (CRs) have been documented in fish, birds and mammals. The period (τ) of the free-running activity CR and the times of activity onset relative to dawn or dusk change markedly over the course of the year[36]. One might suppose that seasonal variations in day length, temperature, humidity and food availability induce these responses. Chronobiology is, however, replete with counterintuitive causalities: seasonal changes in some CRs are endogenous; they persist for several years even when environmental conditions are held constant[89].

During the spring and summer, golden-mantled ground squirrels are homeothermic and defend body temperatures (T_b) of 37°C; during the autumn and winter heterothermic phase, T_b declines to 1–2°C above ambient temperature (T_a). Squirrels kept at a T_a of 23°C maintain a T_b of 37°C for half the year and decrease their T_bs intermittently to 24°C during the remaining 6 months[37]. A constant T_a is, therefore, compatible with marked fluctuations in T_b. We sought to explain seasonal variations in CRs within this framework. Specifically, we asked whether seasonal changes in τ of the free-running CR are caused by endogenous seasonal reductions in T_b. This conjecture was upheld: when T_a was increased and decreased by 10°C during the squirrels' heterothermic phase, the τ of the activity CR became shorter and longer, respectively (Fig. 1)[37]. Identical changes in T_a were without effect on τ during the squirrels' homeothermic phase. T_a appears to influence squirrel CRs when it effects changes in T_b. A similar relation obtained in Siberian hamsters: τ of the CR changed only when animals displayed torpor (Thomas, Jewett and Zucker, unpublished observations).

When tissue temperature increases by 10°C reaction rates of many biological processes double or triple, but circadian time measurement changes relatively little

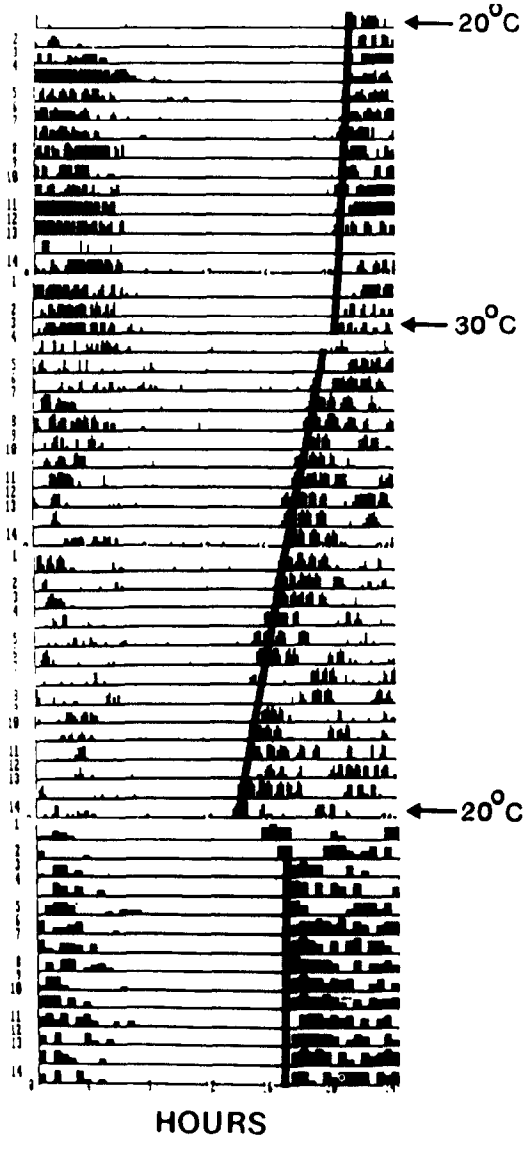

Fig. 1. Decreases and increases in circadian τ in response to 10°C changes in T_a of a squirrel kept in constant dim light. The heavy lines on the activity record were added to facilitate visual estimation of τ (ref. 37).

(temperature compensation)[72]. The findings summarized above do not challenge this principle; they do indicate, however, that when fluctuations in T_a induce marked changes in T_b, the impact on CRs is substantial. It has not yet been determined whether the circadian pacemaker in the suprachiasmatic nucleus (SCN) is directly responsive to temperature. The deemphasis of temperature as a significant influence on CRs is unjustified and based on misinterpretation of temperature-compensation dogma.

The importance of the SCN for circadian organization of mammals is widely acknowledged; its role in generation of the T_b CR has been both championed and disputed on the basis of ablation studies in rats. Species that display daily torpor provide a favorable model system to address this question; the amplitude

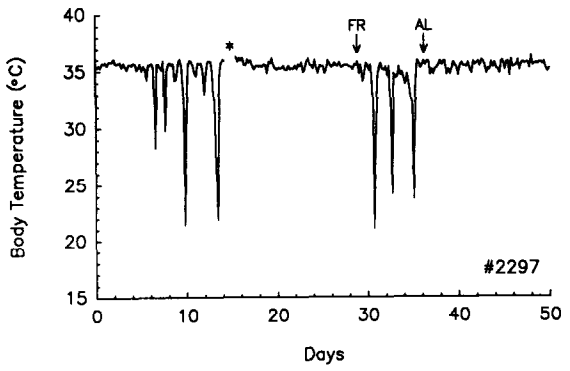

Fig. 2. Elimination of daily torpor in a hamster after ablation of the SCN (*); torpor was reinstated during food restriction (FR) and absent during resumption of ad libitum (AL) feeding[63].

of their T_b CRs is an order of magnitude greater than that of homeothermic mammals. Torpor bouts of Siberian hamsters typically endure for 4–8 h, during which T_b declines from 37° to 20°C. Torpor bouts are entrained by the light–dark (LD) cycle and free-run in constant illumination[64]. Torpor, which typically is manifested during the winter, can be induced by maintaining animals for several months in short day lengths or at any time of year by curtailing food availability. Ablation of the SCN (SCN_x) eliminated torpor in short day hamsters (Fig. 2)[63]. Torpor was, however, reinstated when animals were food restricted (Fig. 2)[63]. The temporal structure of torpor bouts was severely disrupted in SCN_x hamsters; entrainment to the LD

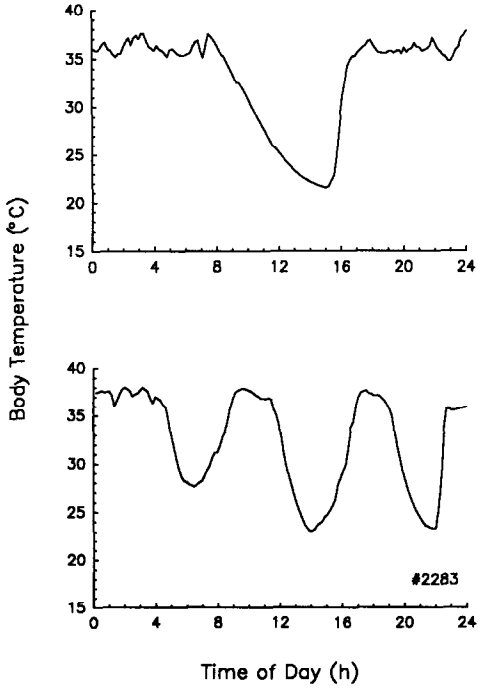

Fig. 3. Representative preoperative (top) and postoperative T_bs from a food-restricted SCN_x hamster that expressed multiple torpor bouts per 24 h after surgery. Lights were on from 08.00 to 16.00 h.

cycle and the coherence of the free-running rhythm were eliminated. The occurrence of several torpor bouts per day in SCN_x but not intact animals was notable (Fig. 3)[63]. The SCN is not essential for the expression of torpor but plays a crucial role in its temporal organization[63].

2. Aging, feedback and the circadian clock

Fred W. Turek

Age related changes have been documented in endocrine, metabolic and behavioral circadian rhythms in a variety of animal species, including humans[7]. These changes include alterations of the period length, amplitude and/or phase of many circadian rhythms. Support for the hypothesis that age-related changes in the expression of circadian rhythms reflect, at least in part, the aging of the circadian clock itself come from studies demonstrating that metabolic and neuropeptidergic changes occur in advanced age in the suprachiasmatic nucleus (SCN) of the hypothalamus, the location of a master circadian pacemaker in mammals[71,78].

The underlying causes for age-related changes in the circadian system remain unknown. Recent findings demonstrating that changes in the activity–rest rhythm can have major feedback effects on the circadian clock that regulates this rhythm[74], coupled with the observations that the activity–rest cycle is disrupted in old age[7], have led us to investigate whether the feedback effects of the activity–rest cycle on the circadian clock may be altered in advanced age.

Acute exposure of old hamsters (16–28 months of age) to two different stimuli (i.e., benzodiazepines or a dark pulse on a background of constant light), which are known to induce phase shifts in the circadian clock of young hamsters (2–6 months of age) by inducing activity at times when the animals are normally inactive or showing little activity, has very little, if any, phase-shifting effect on the circadian clock (Fig. 4). In contrast, old hamsters remain sensitive to the phase shifting effects of stimuli that are not associated with any change in the activity–rest state, such as protein synthesis inhibitors or pulses of light. In addition, we have recently found that while the circadian rhythm of locomotor activity of young hamsters can be entrained by daily injections of a short-acting benzodiazepine, this response is lost or attenuated in old animals.

Taken together, these results indicate that the circadian system of old animals becomes selectively unresponsive to synchronizing signals mediated by the activity–rest state. Previous age-related changes in circadian rhythmicity that have been observed in mammals, including humans, might be related to weakened coupling or feedback between the activity–rest cycle and the circadian clock.

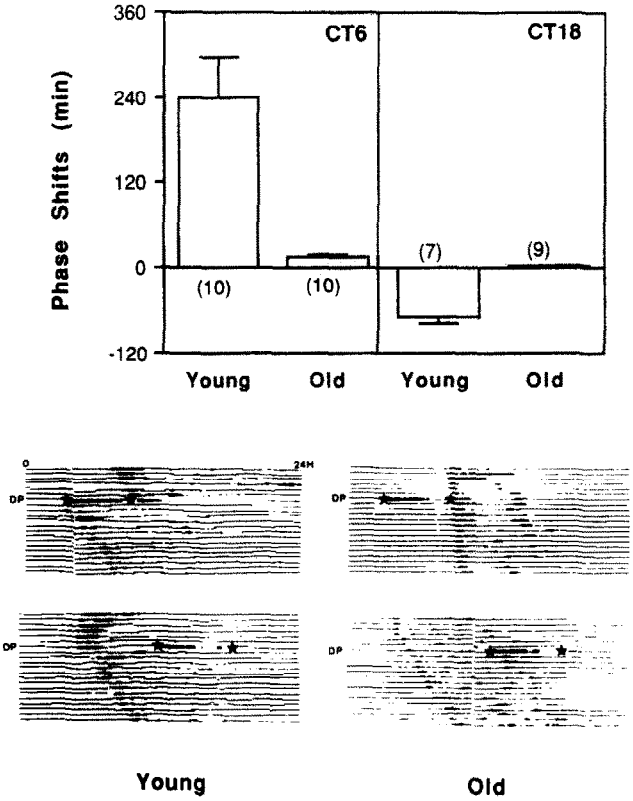

Fig. 4. Mean (±S.E.M.) phase shifts in the activity rhythm of young and old hamsters maintained in LL that were subjected, beginning at either CT 6 (left panel) or CT 18 (right panel) to a 6-h dark pulse. A value above the solid line indicates an advance in the onset of locomotor activity, a value below indicates a delay. The values in parentheses indicate the number of trials for each group of animals. The mean phase shift in the activity rhythm in response to the dark pulse was significantly greater ($P < 0.001$) in young than in old hamsters at both circadian times tested. Bottom: representative sections from the wheel-running activity records of two young (left panels) and two old (right panels) hamsters housed in LL before and after they were subjected to a 6-h dark pulse beginning at CT 6 (top panels) or CT 18 (bottom panels). The day of the dark pulse is designated by 'DP' at the left of each record with the exact beginning and end of the dark pulse designated by two stars. Reproduced with permission from O. Van Reeth et al.[76].

3. Functional organization of the circadian timing system

Robert Y. Moore

3.1. Introduction

The mammalian circadian timing system (CTS) is a set of neural structures that functions to provide a temporal organization for physiological processes and behavior to promote adaptation and survival. The components of the CTS are as follows: (1) photoreceptors and projections of retinal ganglion cells that form entrainment pathways; (2) circadian pacemakers; and (3) efferent pathways that couple the pacemakers to effector systems displaying circadian function.

3.2. Entrainment pathways

Light is the principal *Zeitgeber* for entrainment. Light affects a specific subset of retinal photoreceptors[18]. These photoreceptors are connected to a subset of ganglion cells that projects as a retinohypothalamic tract (RHT) to the suprachiasmatic nucleus (SCN), the adjacent anterior hypothalamic area and retrochiasmatic area and to the lateral hypothalamic area[25]. The RHT is sufficient to maintain entrainment; transection of the RHT results in a loss of entrainment[24]. The RHT forms as collaterals of optic nerve axons that traverse the optic chiasm and optic tract to innervate a subdivision of the thalamic lateral geniculate complex, the intergeniculate leaflet (IGL). The IGL contains a population of neuropeptide Y (NPY) neurons that projects in a separate pathway, the geniculohypothalamic tract (GHT), to the SCN[52]. IGL neurons are also GABA-containing (Fig. 5)[51].

What is the function of these pathways? Stimulation of the RHT produces changes in the phase of free-running rhythms with a phase response curve (PRC) very similar to that of light[45]. In contrast, stimulation of the GHT or direct application of NPY to the SCN, results in phase changes with a PRC that is quite different from light or RHT stimulation[52]. This PRC also can be obtained from a series of disparate stimuli that share the common feature of inducing locomotor activity[52]. Ablation of the IGL eliminates activity-induced phase changes[52] indicating that the IGL-GHT system is involved in mediating feedback regulation of the circadian pacemaker. This suggests that, contrary to generally held views, pacemaker homeostasis is accomplished by an interaction of visual and feedback input to the pacemaker. Much of the feedback regulation

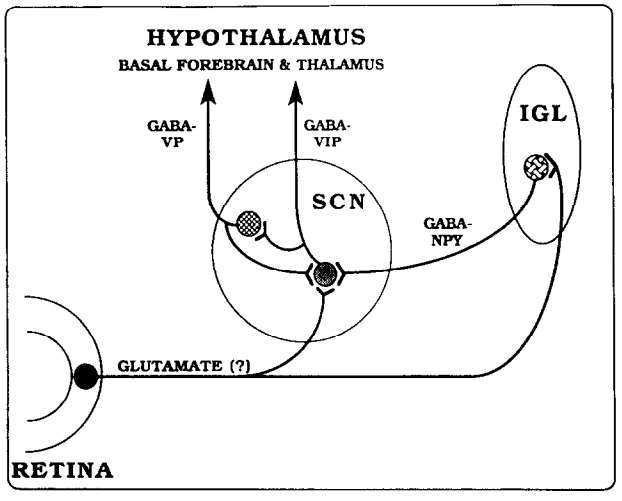

Fig. 5. Diagram showing the organization of the circadian timing system. Retinal ganglion cells, thought to contain glutamate, project to both the SCN and IGL. One population of SCN neurons, containing VIP and GABA, receives input from both the retina and the IGL. A second population contains VP and GABA and is reciprocally connected with the VIP-GABA neurons. Both project mainly to the hypothalamus with some projections to basal forebrain and thalamus.

Fig. 6. Photomicrographs showing viral labeling of a population of retinal ganglion cells (A) that projects to the suprachiasmatic nucleus (B) and the intergeniculate leaflet (C) of the lateral geniculate but not to other primary visual nuclei. The Bartha strain of the pseudorabies virus is injected into the eye and is taken up by the selected population of ganglion cells that transmits the virus to the SCN and IGL. Marker bars, 20 μm (A), 150 μm (B), 200 μm (C).

appears to be accomplished through the IGL-GHT pathway but other, direct inputs to the SCN undoubtedly contribute as well.

Although it has appeared likely that the retinal ganglion cell projection to the SCN and IGL derives from a separate subset of ganglion cells that do not project to other major visual centers, this heretofore has not been demonstrated directly. Using a specific neurotrophic herpesvirus, we have found labeling of a subset of retinal ganglion cells with a mean perikaryal diameter of about 15 μm that projects selectively to the SCN and IGL (Fig. 6)[9]. On this basis we would conclude that these ganglion cells are components of the CTS.

3.3. Pacemakers

The SCN are pacemakers in the mammalian brain[45]. The evidence for this conclusion is as follows: the RHT terminates in the SCN[25]. Ablation of the SCN abolishes most, if not all, circadian function[45]. In isolation, both in vivo and in vitro, the SCN maintain a circadian rhythm in neuronal activity[45]. Transplantation of fetal SCN into the third ventricle of arrhythmic, SCN-lesioned hosts restores circadian rhythmicity with a period characteristic of the donor[59]. The SCN contains two subdivisions: one is characterized by a population of vasoactive intestinal polypeptide-producing neurons and receives most of the RHT and GHT input; the second is characterized by a population of vasopressin-producing neurons and receives very limited visual input[45]. Both sets of SCN peptide-producing neurons also appear to be GABA-producing (Fig. 5)[51]. The SCN subdivisions appear to be comprised of individual circadian oscillators that become coupled by interconnections to form a network with pacemaker function.

3.4. Efferent pathways

The efferent projections of the SCN are largely to adjacent anterior hypothalamic areas with relatively restricted projections to basal forebrain and midline thalamus (Fig. 5)[77].

3.5. Circadian timing system organization

The CTS has three identified sets of component nuclei, the retina, SCN and IGL. The retinal ganglion cells projecting to the SCN and IGL are a distinct subpopulation of ganglion cells that does not project to other visual centers[9]. Like other ganglion cells, however, they appear to produce glutamate or a closely related excitatory transmitter (Fig. 5)[45]. IGL neurons produce either GABA and NPY and project to the SCN or GABA and enkephalin and project to the contralateral IGL[52]. The IGL-GHT system appears to integrate photic with non-photic entraining input to participate in pacemaker homeostasis[52]. The neurons of the SCN are also GABA-producing[51] and, hence, the output of the CTS provides a cyclic inhibitory influence on systems that express circadian function.

4. Neurophysiology and neuropharmacology of the suprachiasmatic nucleus in culture

Majid Mirmiran, Nico P.A. Bos and Gerdien C. Koster-Van Hoffen

4.1. Introduction

An overwhelming body of evidence indicates that the suprachiasmatic nucleus (SCN; the so-called biological clock) of the anterior hypothalamus is involved in the generation and synchronization of circadian rhythms[32,45,81]. The following results suggest that the SCN is indeed capable of endogenous rhythm generation with a periodicity of around 24 h in the absence of neuronal input/output connectivities: (1) in vivo, circadian rhythms of electrical activity remain in the neuronally isolated SCN island; (2) after SCN transplantation it only takes a few days before the circadian rhythms reappear in an arhythmic SCN-lesioned animal (this is not long enough for the reestablishment of the neuronal input/output connections); interestingly, the rhythm period is defined by the donor SCN and not by the original inherited host rhythm; (3) in vitro, neuronal discharges, 2-deoxyglucose uptake, protein synthesis and peptide production/release show circadian rhythms in acute or chronic SCN explants.

Earlier studies using acute SCN slices taken from animals sacrificed during the subjective day and night showed that neuronal discharges are high by day (peak at CT 6–8) and low at night[67]. In a semi-chronic culture preparation[16] were able to show high- and low-rhythmic levels of vasopressin release from SCN explants in the medium over several days. A recent

study suggests that such rhythms are even present in dissociated SCN cells taken from newborn rats[53].

One of the difficulties of interpreting data obtained in vitro from acute adult SCN slices is the presence of degeneration and acute hypoxia. In order to overcome this problem and develop a suitable model for studying neuronal mechanisms underlying the generation of circadian rhythms within the SCN, we cultured organotypic SCN explants (taken from 12-day-old rat pups) in a chemically defined medium over a period of several weeks[5]. The morphology of the SCN remains more or less intact in this preparation except for the elimination of retinohypothalamic fibers and the SCN input/output from the rest of the brain. Starting 1–4 weeks after culturing, we were able for the first time to record continuously from single SCN neurons over a period of 36–66 h. Twenty-two cells were recorded (each from a different explant) and 17 neurons showed high- and low-activity levels with a periodicity ranging from 16–32 h. Eleven of these cells had a circadian period of 20–25 h[5]. There was no indication, however, of a connection between high/low firing levels and the time of day (Fig. 7).

In a recent microiontophoretic study of our cultured SCN we were also able to characterize three types of SCN neurons: spontaneously *silent* (glutamate responsive), *irregular* and *regular*. We could not find any specific distribution pattern of these different types[6]. Glutamate excited the majority of the regular and 60% of the irregular cells. GABA inhibited both the spontaneous and the glutamate-evoked activity of more than 90% of all three types of SCN neurons. MK-801 partially blocked glutamate responses. Of the nine SCN neurons tested for the effects of MK-801 on spontaneous activity only one showed a reduction in firing rate. This suggests that even if the neuronal discharges within the SCN are synaptically mediated, their spontaneous activity does not depend on activation of NMDA receptors. Another interesting finding of this microiontophoretic study was that the magnitude of glutamate response varies inversely as a function of the spontaneous firing frequency of regular SCN neurons. It was also found that *N*-acetylaspartylglutamate (NAAG) directly increases firing rate or potentiates glutamate responses in several of the SCN neurons studied (Figs. 8 and ref. 6).

Assuming that the SCN is capable of generating circadian rhythms independently of the rest of the brain and/or body, it is of interest to know how this is done. There are several hypotheses: (1) the *pacemaker* hypothesis: independently and/or in concert a group of pacemaker cells generate the clock output rhythm; and (2) the *network* hypothesis: the interaction between pacemaker and non-pacemaker cells (a small percentage of SCN cells are pacemakers) is the key factor in the generation of a precise cicadian rhythm within the SCN. It is important to keep the contribu-

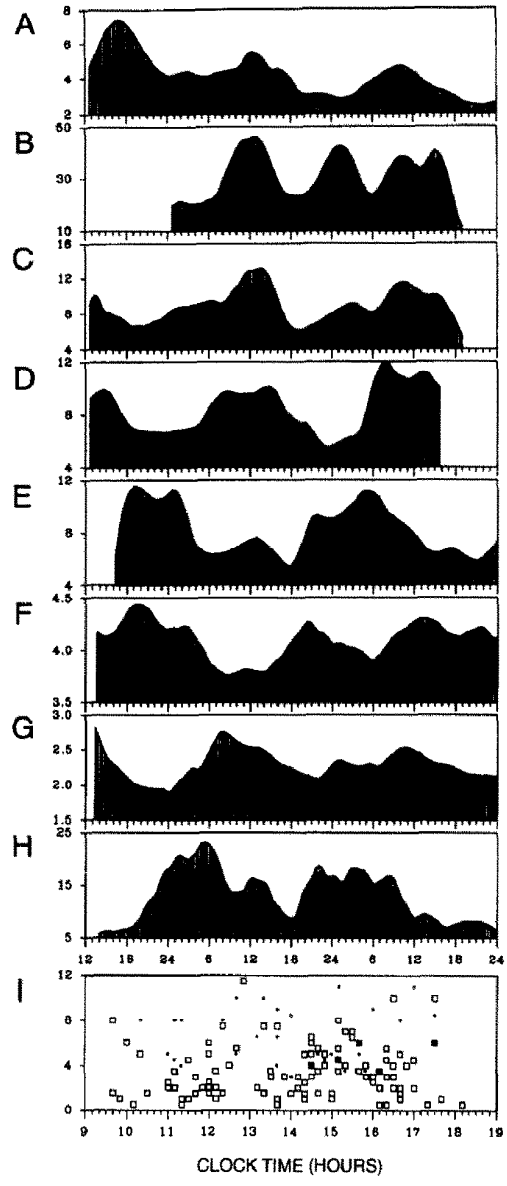

Fig. 7. Spontaneous discharges (HZ) of cultured neurons continuously recorded as a function of time of day are shown (A–H). Each record shows a single SCN neuron continuously recorded from a different explant, except for B, which is the multiple unit recording of the same explant as in C. The recording shown in H is also a multiple recording of another SCN explant. Note that apart from the main circadian rhythmicity also activity peaks with a periodicity lower or higher than the mean are found[5]. This is very pronounced when the multiple neuronal recording in B is compared with the single neuronal data discriminated from the same record in C. These results are in accordance with the *multiple sub-oscillators model of the SCN* in which due to a lack of synchronization (by light or other internal Zeitgebers) the pacemaker and non-pacemaker cells gradually drift apart as a function of in vitro culturing. This hypothesis is supported by the fact that 8-h samplings on several successive days (using 5-min registrations) gave no indication of high- or low-activity levels as a function of the time of day (I). In I, individual regular SCN neuronal firing rates are indicated as dots, while irregular cells are shown as open squares.

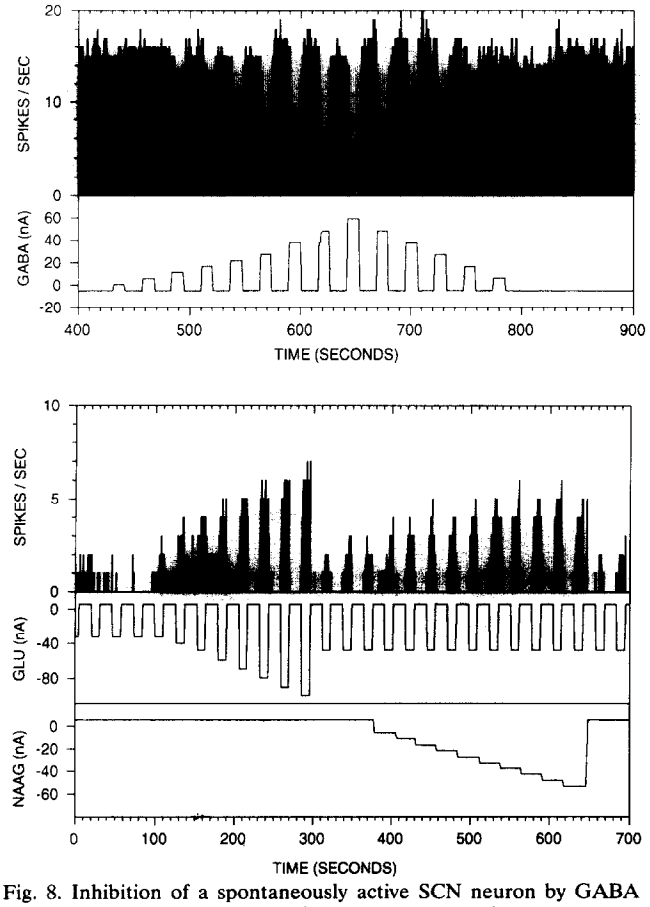

Fig. 8. Inhibition of a spontaneously active SCN neuron by GABA and excitation of another single (spontaneously silent) SCN cell upon microiontophoretically applied glutamate and/or *N*-acetylaspartylglutamate (NAAG).

tion of the glia cells in mind when determining whether (1) and/or (2) is/are *correct*.

4.2. Experimental evidence in favor of the pacemaker hypothesis

1. In vivo infusion of TTX into the SCN disrupts circadian rhythm expression. However, upon drug withdrawal the rhythm phase corresponds to the original phase before drug delivery as if the information about circadian rhythms remains intact in the pacemaker(s) although it is not expressed[65].

2. The presence of circadian rhythms in neuronal discharges of the SCN taken from fetal rat on the last day of gestation despite a very low synapse level[67].

3. In vitro the circadian rhythm of intracellular protein synthesis within the SCN remains intact in the presence of TTX[68].

4.3. Experimental evidence in favor of the network hypothesis

1. Lack of circadian rhythms in 2-DG uptake in the SCN in high Mg^{2+} and low Ca^{2+} in vitro[69].

2. Dissociation of SCN neurons (50%) from two different hamster strains with two different genetically determined circadian rhythms (20 and 24 h) and transplantation to an arhythmic SCN-lesioned hamster result in recovery of the rhythm (around 22 h) in the host which is the mean of the two (and not independent expression of two different rhythms)[60].

3. No circadian rhythm is found in rat 2-DG until 2–3 weeks after birth (when the synaptic density of the SCN has matured)[67,69].

With regard to the pacemaker and network hypotheses, it is important to record SCN neuronal discharges in the absence of synaptic transmission and also to use patch clamp techniques. Furthermore, using only dissociated SCN neurons or glia makes it easier to determine the rhythm generator. As far as the manner in which circadian rhythms are generated within the SCN is concerned, it is also important to know what endogenous SCN neurotransmitters really do. What is the role of, e.g., GABA, which is found abundantly in the internal terminals of the SCN and is co-localized in peptidergic SCN neurons? Our organotypic SCN culture would be a good model for studies aimed at answering these questions.

5. What do suprachiasmatic nucleus transplants do?

Rae Silver and Joseph LeSauter

5.1. Introduction

It is well established that the mammalian suprachiasmatic nucleus (SCN) is a biological pacemaker that provides period and phase information modulating behavioral and physiological responses. The precise location of pacemaker cells and whether or not they are restricted to a locus (loci) within the cytoarchitectonically and peptidergically defined SCN is not known. Evidence of extra-SCN oscillators derives from several lines of research: (1) ultradian rhythms and spectral energy in the circadian range survive complete lesions of the SCN (SCN-X); (2) extra-SCN hypothalamic regions receive direct retinal input; (3) some circadian rhythms, such as anticipatory food responses, survive total SCN-X; and (4) treatment of SCN-X rats with

methamphetamine restores circadian locomotor rhythms. We review our attempts to understand: (a) which circadian rhythms are sustained by pacemaker cells of the SCN itself and which are attributable to extra-SCN oscillators; (b) which functions can be restored by SCN grafts; (c) responses that are similar/different in grafted animals vs those bearing 'SCN islands' vs intact animals; and (d) mechanisms whereby SCN transplants might restore function.

5.2. Where are the pacemakers?

It has been confirmed in many labs that fetal grafts of the anterior hypothalamus which include the SCN can restore free running locomotor rhythms following transplantation into the third ventricle of arrhythmic SCN-X host rats or hamsters, but effective transplants always include extra-SCN tissue. To examine the efficacy of the neural tissue grafts restricted to the SCN in restoring circadian rhythms in SCN-X animals, we first established the oldest effective donor tissue by using SCN grafts taken from animals at postnatal day 1, 3, 5, 7 and 10[62]. Restoration of locomotor rhythmicity following transplantation of postnatal day 1 grafts was as high as that of embryonic grafts and declined thereafter. Using postnatal day 1 donor tissue, we took small 'punches' of the SCN from hypothalamic sections (500 μm) of the anterior hypothalamus, thereby limiting the amount of extra-SCN tissue in the graft. The results to date indicate that such punches are sufficient to sustain circadian rhythmicity. Though a substantial effort remains before we can determine whether any region outside the SCN or whether pacemaker cells restricted to any subregion within the SCN, are effective, this work should define the locus (loci) necessary and sufficient to sustain circadian rhythmicity.

5.3. What responses are restored by suprachiasmatic nucleus grafts?

Not all responses thought to be dependent on the SCN are restored by neural tissue grafts. Rhythms of locomotor activity, drinking and gnawing[39] are restored, but gonadal regression in constant darkness is not. Measures of both gnawing and wheel-running activity in SCN-X hamsters following transplantation indicate that these behaviors reemerge simultaneously, suggesting control by a common pacemaker.

5.4. Responses of grafted animals to pharmacological agents

Melatonin can set the phase of the free-running rhythm in pups of a litter born to SCN-X mothers[10], but has little effect on adult hamsters. We used this age difference in responsiveness to assess the ability of melatonin to set the phase of locomotor activity of SCN-X adult hamsters bearing fetal hypothalamic grafts containing the SCN[61]. Donor anterior hypothalamic tissue was exposed to melatonin or vehicle at one of two circadian phases, starting on embryonic day 8 (E8) during pregnancy and continuing until postnatal day 7 following grafting to an adult SCN-X host. This treatment was effective in setting the phase of the recovered locomotor response in the host animals. Unexpectedly, it also shortened the latency to recover locomotor rhythmicity. There were no effects of melatonin administration in oil injected SCN-X grafted animals, in intact controls, in SCN-X non-grafted animals or in SCN-X animals bearing non-functional grafts. Maternal melatonin may normally synchronize the circadian rhythms of the pups within a litter in a similar way.

Lithium and heavy water (^2H_2O) lengthen the free-running period of circadian rhythms in a variety of mammalian and non-mammalian systems. In SCN-X hamsters which had recovered circadian rhythmicity following implantation of SCN grafts and in intact controls, both of these agents lengthened the period of the free-run without affecting amount of activity[39].

Both triazolam (Tz) and exercise can phase advance free-running locomotor activity rhythms in intact hamsters. Furthermore, Tz increases activity at the time of injection and this appears to be the mechanism whereby Tz produces phase shifts. We compared the phase shifting effect of Tz in grafted and intact animals[8]. In both groups, increases in locomotor activity occurred at the time of Tz injection and most intact, but no grafted animals show a phase advance in response to Tz administration. Given that hamsters bearing SCN grafts have limited neural connections between the host brain and transplanted SCN tissue, the results suggest that a site outside the SCN, with afferents to these nuclei, mediates the phase-shifting effect of Tz and of exercise. In contrast, lithium and ^2H_2O appear to act directly on the pacemaker cells.

5.5. Transplanted suprachiasmatic nuclei vs. those in situ

While SCN grafts restore free-running locomotor rhythms, there are differences in the actograms of grafted animals compared to intact controls. Grafted animals have a less precise onset of locomotor activity and their daily amount of activity is reduced. In intact animals the daily onset is generally more precise than the offset, and the associated activity duration is longer and more intense than the one at the end of the day. In grafted animals the onset and offset of daily activity are often difficult to distinguish. In animals bearing a hypothalamic island created by a knife cut around the

SCN, the precision of the onset of locomotor activity is reduced, as in lesioned animals bearing SCN grafts, but the period of the free-run is not generally altered by the isolation of the SCN from the rest of the hypothalamus and the amount of activity is not reduced[20]. The reduction in amount of activity in lesioned-grafted animals is likely a function of the extent of damage to the hypothalamus. Other differences between intact, lesioned-grafted and 'SCN island' animals suggest that the SCN may regulate circadian rhythms by multiple mechanisms and SCN grafts are sufficient to restore some, but not all functions.

Features of the restored response can be influenced by the condition of the graft at the time of transplantation. While animals bearing whole tissue grafts[38] usually express a free-running period that is longer than 24 h (typical of hamsters), animals bearing dissociated, dispersed cell suspension grafts have a period shorter than 24 h[70]. Possibly, coupling of pacemakers within the graft, location of the grafted tissue or number of transplanted pacemaker cells influences the period expressed.

5.6. Output signals of the suprachiasmatic nucleus

To explore the impact of extra oscillators on the expression of circadian rhythms, we implanted SCN's from two fetal donor animals into intact animals[54]. The most impressive aspect of the results was the absence of any measurable effect on the period or phase of the free-running circadian locomotor rhythm or on amount of activity, associated with the presence of 3-fold the usual complement of SCN oscillators. Furthermore, lesions of the host SCN subsequent to implantation of grafts had no detectable effect on the free-running locomotor behavior of the hamster. It is noteworthy that less than usual complement of SCN tissue is also compatible with free-running rhythmicity in that ablation of less than 75% of both SCN does not alter circadian function[75].

To examine the impact (if any) of SCN grafts in intact animals, on the metabolic activity of the host and the grafted SCN, we used radiolabelled 2-deoxyglucose uptake to index phase[66]. First, we established that we could measure a circadian rhythm in 2-deoxyglucose uptake in postnatal day 1 pups. Next, we housed host and donor animals in opposite LD cycles. On the first day after grafting, the donor clock retained its phase indicating that surgical isolation of the SCN from the rest of the fetal brain and implantation into the adult host animal, did not disrupt circadian rhythmicity in the donor clock. On the 14th day after grafting and thereafter, host and donor SCN were in synchrony, invariably with the phase of the host animal. Thus, the host SCN sends a signal which resets the phase of the grafted SCN and not vice versa.

5.7. What do transplants really do?

The broader question of what *transplants* do has been amply addressed[2,19] and the following possibilities are offered: (a) trophic effects of the graft; (b) grafted cells reinnervate host; (c) graft promotes regrowth in host; (d) paracrine signal reaches host by diffusion; (e) endocrine signal reaches host via circulatory system (Fig. 9). Each of these mechanisms have been implicated in the restoration of function by either neural and/or adrenal grafts and could mediate some of the effects of SCN grafts. The present results, using SCN grafts in lesioned and intact hosts suggest novel possibilities: (f) axonal growth from host brain to graft; (g) dendritic growth from graft to host; or (h) a diffusible signal from the host brain reaches the graft.

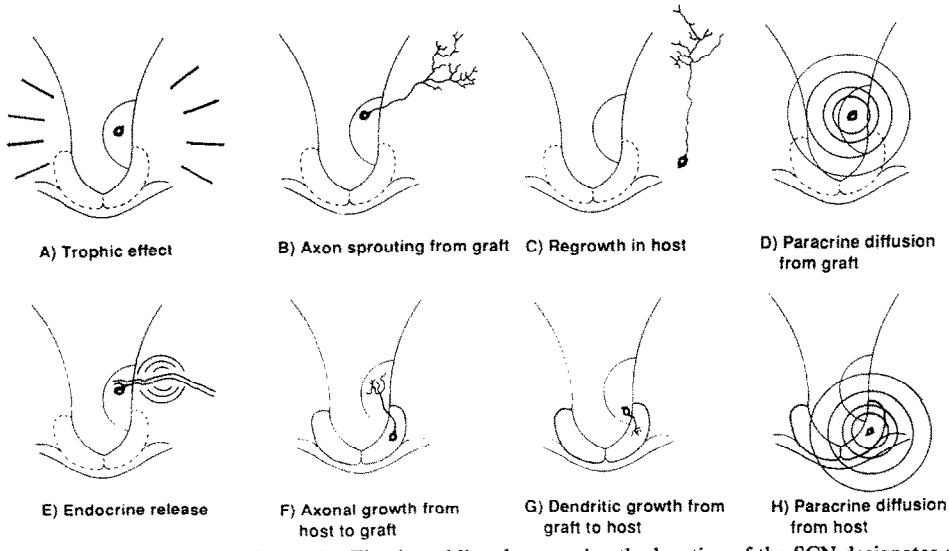

Fig. 9. Possible mechanisms of action of ventricular grafts. The dotted line demarcating the location of the SCN designates an SCN-lesioned host animal. A solid line indicates that the host SCN is intact.

5.8. Summary

The present results indicate that the status of the host animal and of cells within the graft influence the recovered response. The period of the free-run differs in animals bearing dissociated dispersed cell suspension grafts from those bearing whole tissue grafts. In host animals lacking an SCN, grafts restore free-running activity rhythms and drugs that act directly on the SCN influence the expression of restored rhythms. In intact host animals bearing SCN grafts, responses attributable to the transplanted SCN tissue have been hard to detect and the phase of metabolic activity of grafted SCN is set by the host SCN. If the condition of the graft and of the host brain influence responses of neural tissue grafts in other systems, as occurs with SCN grafts, this may account for variability of outcomes following grafting and has important clinical implications.

6. Cellular studies of a retinal biological clock

Gene D. Block, Michael E. Geusz, Sat Bir S. Khalsa and Stephan H. Michel

6.1. Introduction

The eyes of several mollusks contain circadian pacemakers. Eyes in isolation express circadian rhythms in the frequency of spontaneously occurring optic nerve impulses. In *Bulla gouldiana*, the cloudy bubble snail, the pacemaker is located among a group of approximately 100 electrically coupled neurons (basal retinal neurons, BRNs). The hypothesis that each BRN is a competent circadian pacemaker was based, until recently, on evidence that surgically reduced retinas containing only a few BRN somata continued to generate circadian periodicities[3]. The single cell-pacemaker hypothesis has now received experimental verification. BRNs dispersed in cell culture reveal circadian changes in membrane conductance that persist for at least two cycles in culture[47]. These conductance changes are not due to any form of communication between dispersed cells as individual cells isolated in microwell dishes continue to exhibit rhythmic changes in conductance[48]. These data provide the first direct evidence that individual neurons are capable of generating and expressing a circadian rhythm.

Our laboratory employs three strategies to study circadian pacemaker mechanisms: (1) we identify processes involved in pacemaker entrainment, as the entrainment pathway must ultimately terminate on a pacemaker element; (2) we analyze the mechanisms underlying pacemaker expression, as the origin of the expression pathway is a pacemaker component; and (3) we attempt to directly identify processes involved in rhythm generation.

6.2. Entrainment

We find that light leads to membrane depolarization that results in an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels[43,26]. Hyperpolarization or a reduction in Ca^{2+} flux also generates phase shifts but the PRC is shifted 180° on the time axis with respect to depolarization[27]. Using the fluorescent calcium indicator dye fura-2, we have recently confirmed that depolarization leads to a sustained increase in intracellular Ca^{2+} concentration (Geusz et al., unpublished results).

6.3. Expression

The circadian rhythm in optic nerve impulse frequency is driven by a circadian rhythm in membrane potential[44]. Membrane potential is relatively hyperpolarized during the subjective night and depolarizes by approximately 13 mV during the subjective day. This rhythm is due to the modulation of membrane conductance[58]. Membrane conductance decreases at subjective dawn and increases again at subjective dusk. These changes appear to be due to modulation of a TEA-sensitive K^+ channel[46,48].

6.4. Rhythm generation

While membrane conductances are involved in both pacemaker entrainment and expression, they do not appear to play a critical role in rhythm generation. Removal of Ca^{2+} from the bathing medium does not prevent the pacemaker from completing its cycle[28] nor does the removal of extracellular Na^+ (Khalsa, in preparation). Removal of extracellular Cl^- shortens the free-running period by up to 2.5 h but does not prevent rhythmicity[30]. Unlike transmembrane fluxes, transcription and translation appear to be critical for rhythm generation. Inhibiting protein synthesis or transcription leads to profound period lengthening and at higher concentrations the pacemaker is held motionless near circadian time 0 (ref. 29).

6.5. Pacemaker model

Our best guess for circadian pacemaker organization is depicted in Fig. 10. Briefly, the circadian pace-

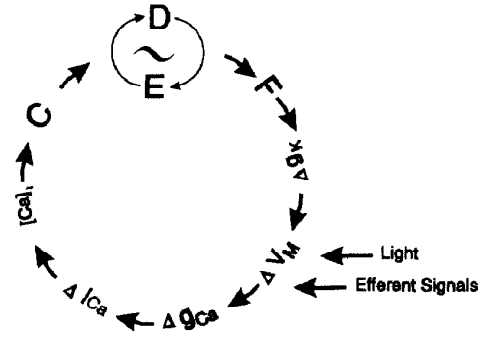

Fig. 10. Model for circadian pacemaker system within *Bulla* retinal neurons. D and E represent the state variables of an intracellular feedback loop generating the 24-h periodicity. F depicts the initial steps of the output pathway that are as yet unidentified. C represents the final steps of the entrainment pathway. See text for additional details.

maker within the basal retinal neuron consists of at least two feedback loops. An intracellular loop most likely involves transcription and translation as rate determining steps and is responsible for generating the long circadian time constants. In addition to the intracellular feedback loop, a transmembrane loop is formed by virtue of the fact that membrane potential is both an input and output to the neuronal pacemaker. Environmental synchronizing signals act through membrane depolarization, voltage gated Ca^{2+} channels, a transmembrane Ca^{2+} flux and a sustained increase in intracellular Ca^{2+}. On the output side, rhythm expression is mediated by pacemaker modulation of a TEA-sensitive K^+ channel, driving a rhythm in membrane potential that results in a circadian rhythm in impulse frequency. We propose that depolarization-induced phase shifts during the subjective night are caused by an increase in the level of intracellular Ca^{2+} and hyperpolarization-induced phase shifts during the subjective day are due to a reduction in a persistent diurnal Ca^{2+} flux[27]. This diurnal flux is caused by voltage-gated Ca^{2+} channels opening during the depolarized phase of the circadian membrane potential cycle. Some support for this proposal is provided by experiments in which pulses of lowered bath Ca^{2+} levels lead to phase shifts similar to those generated by membrane hyperpolarization[27]. Future experiments will be directed towards further characterizing the role of intracellular Ca^{2+} in rhythm entrainment and generation.

7. Convergence and divergence in chick pineal regulation

Martin Zatz

The circadian rhythm of melatonin production by the pineal gland is the most robust and reliable overt hormonal rhythm in vertebrates. In all classes, synthesis (and release) of melatonin (derived from tryptophan via serotonin) is low during the day and goes up many-fold during the night. In mammals, the rhythm of melatonin production is unique to the pineal gland and entirely dependent on the sympathetic innervation which conveys photic and circadian regulation to the gland. The rat pineal is itself neither rhythmic nor photosensitive, but responds to its neurotransmitter norepinephrine (NE) by *increasing* the synthesis of melatonin from serotonin[31]. In contrast, the chick pineal is itself both rhythmic and photosensitive, even in dispersed cell culture[11] and responds to its neurotransmitter NE by *decreasing* the synthesis of melatonin.

We have been using chick pineal cells to identify and interrelate perturbations that act on the melatonin-synthesizing apparatus and to distinguish them from those acting on the pacemaker underlying rhythm generation[86]. Generally, agents that can acutely raise or lower melatonin output without ever inducing phase shifts in the free-running rhythm are considered to act solely on the melatonin-synthesizing apparatus, whereas agents that can induce phase shifts are considered to act (however indirectly) on the pacemaker mechanism. Further experiments are then aimed at dissecting the relationships between the pathways converging on melatonin regulation. Results and interpretations concerning some of these effects and relationships are summarized in Fig. 11.

Cyclic AMP is a key regulator of melatonin production in the chick pineal, as it is in the rat pineal. Agents that raise cyclic AMP levels, such as forskolin (FSK) or vasoactive intestinal peptide (VIP) and cyclic AMP analogs, such as 8-bromocyclic AMP (8BrcAMP), acutely increase melatonin output. Agents that lower cyclic AMP levels, such as NE or light (L), acutely decrease melatonin output. In contrast to their effects in certain neuronal systems, none of these agents (except L) demonstrably affected the temporal pattern in subsequent cycles of the melatonin rhythm; they do not perturb the underlying circadian pacemaker[84]. For

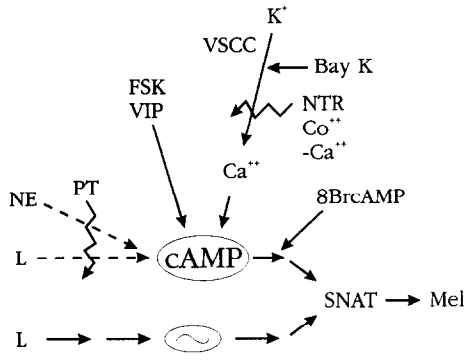

Fig. 11. A scheme summarizing the effects of agents on melatonin (Mel) production and their interactions. Abbreviations are explained in the text.

light, experiments with pertussis toxin (PT) distinguished the pathways by which L exerts its acute and entraining effects[85]. PT could block the acute inhibitory effect of L (or NE), but PT did not prevent the phase shifts induced by light pulses. Other experiments indicated that daily release of NE, which does not entrain the melatonin rhythm, does contribute (like daily L) to rhythm regulation by helping to prevent damping and sustain a robust rhythm.

Perturbations of calcium flux through the plasma membrane have also been shown to affect melatonin production. Agents that increase calcium influx through voltage sensitive calcium channels (VSCC), such as Bay K 8644 (Bay K) or depolarizing concentrations of extracellular potassium ions (K^+), increase melatonin production, whereas agents that decrease calcium influx, such as nitrendipine (NTR), cobalt ions or low extracellular calcium concentrations ($-Ca^{2+}$) acutely decrease melatonin output[83]. Competition experiments[88] and direct measurements[82] indicated that these agents act through cyclic AMP. Again, in contrast to results in neuronal systems, none of these agents demonstrably affected the underlying circadian pacemaker. The circadian system in chick pineal cells further differs from neuronal systems in that perturbations of membrane potential and cyclic GMP analogs have also failed to induce phase shifts in the melatonin rhythm. The circadian pacemaker in chick pineal cells is not, however, impervious to perturbation. In addition to pulses of light and darkness, pulses of ouabain, hypertonic or hypotonic media, protein synthesis inhibitors or temperature change have been shown to induce phase shifts in the melatonin rhythm. The mechanistic relationships among these perturbations remain unknown.

The importance of cyclic AMP regulation in the pineal encouraged the presumption that cyclic AMP mediates the actions of the pacemaker on melatonin production. Recent experiments, however, strongly indicate that the pacemaker acts *with*, rather than *through*, cyclic AMP to regulate melatonin production in chick pineal cells[87]. The key result was the demonstration of a persistent rhythm of melatonin output in the constant presence of maximally effective concentrations of FSK (and cyclic AMP) or 8BrcAMP. The synergy and convergence between the actions of cyclic AMP and the circadian pacemaker are implicit in the scheme shown in Fig. 11. At a given level of cyclic AMP, the different states (phases) of the pacemaker can determine different rates of melatonin synthesis and, at a given state (phase) of the pacemaker, different levels of cyclic AMP can determine different rates of melatonin synthesis.

The scheme shown in Fig. 11 completely separates and then converges the pathways for light's acute ('masking') and entraining (phase-shifting) effects. The nature and site of *convergence* between these pathways is unknown, but it is shown as occurring at the regulation of serotonin *N*-acetyltransferase (SNAT), the pivotal enzyme in melatonin synthesis, which is itself induced at night. There is precedent in both plants[49] and fungi[12] for dual pathways, 'direct' and 'indirect' (clock-mediated), for the regulation of specific gene expression by light. The nature and site of *divergence* between light's pathways is also unknown; it remains to be determined, for example, whether the acute and phase-shifting effects of L are mediated by the same photopigment. The acute effect of L can be partially and plausibly explained in terms of known mechanisms of cyclic AMP regulation and enzyme induction. The substances and processes mediating the entraining effects of light, however, as well as those mediating pacemaker functions, remain entirely mysterious.

8. Putative oscillator proteins in the *Aplysia* eye circadian system

Arnold Eskin and Costas Koumenis

A question in circadian biology whose time has come is: "What is the molecular mechanism of circadian pacemakers?". Elucidation of this mechanism will require the identification of the molecular components

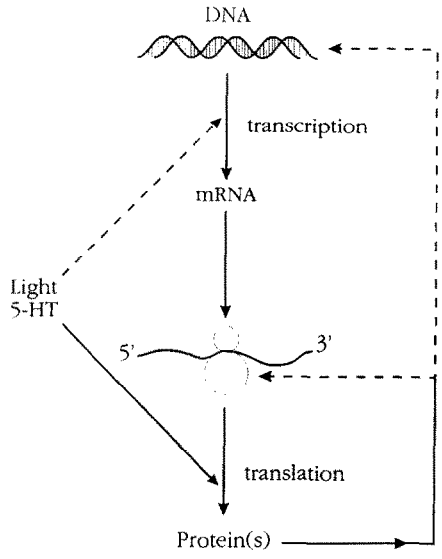

Fig. 12. Critical role of transcription and translation in the feedback loop of the oscillator (model).

of the circadian oscillator and the determination of how these molecules interact with one another to generate a circadian oscillation. Three lines of evidence suggest that macromolecular synthesis may be an important process in the circadian oscillator[34,73]. First, continuous alteration of the level of translation or transcription leads to changes in the free-running period of circadian rhythms and brief perturbations of translation or transcription lead to phase shifts in rhythms[17,80,55]. Second, inhibitors of translation block phase-shifting effects of entraining agents in several systems[57,23,4] and gene expression appears to be involved in entrainment pathways in still other systems[33]. Third single-gene mutations have been shown to affect the period of circadian rhythms in several organisms. Furthermore, a detailed study of the *per* mutation in *Drosophila* shows that *per* mRNA and protein levels oscillate and that expression of the protein appears to be essential for the oscillation of its mRNA[21]. These studies on the role of macromolecular synthesis in the circadian oscillation have led to a model of the oscillator (shown in Fig. 12) in which transcription and translation are critical processes in the feedback loop of the oscillator[34,21]. Furthermore, entrainment of this model oscillator is proposed to occur by environmental cycles regulating the synthesis of specific mRNA or protein components of the oscillator. Critical tests of this model require the identification of candidates for molecular components of the pacemaker, i.e., mRNAs and proteins whose synthesis is important for the normal function of the circadian oscillator.

We have used a biochemical approach to screen for proteins that may serve as components of the circadian system in the eye of *Aplysia*. Our screening strategy is based upon the model above, together with information we have previously obtained concerning the entrainment of the circadian oscillator. The rhythm of the eye of *Aplysia* is entrained over at least two pathways[34]. One rather direct pathway mediates the effect of light upon the eye while the other pathway mediates effects of efferent nerve activity on the eye. Effects of the efferent pathway on the rhythm appear to be mediated by release of the neurotransmitter, serotonin (5-HT) in the eye. Because light and 5-HT appear to act through opposite actions on some element of the oscillator, the model predicts that light and 5-HT will have opposite actions on the synthesis of some protein components of the oscillator. We have searched for such proteins by exposing eyes to radioactive amino acids in the presence of light or 5-HT and then studying individual proteins using two-dimensional polyacrylamide gel electrophoresis[57,79]. Also, because transcription and translation appear closely coupled in the eye circadian system, we searched for proteins whose synthesis was rapidly affected by a transcription inhibitor[35].

Using such criteria we have found over ten proteins that we consider putative oscillator proteins (POPs). The next stage in this research requires the identification of the function of these proteins. To do this, we have been obtaining partial amino acid sequences of POPs. Thus far, we have been able to identify four POPs and sequence information is available on another one. POP-1 belongs to the lipocortin/annexin family of proteins[56], POP-2 is PORIN, POP-3 and POP-5 appear to be related to the heat shock family of proteins (GRP78 and HSP70) and POP-4 may also be a stress-related protein, for it is similar to the stringent starvation protein of *Escherichia coli*[35]. These identifications of POPs have provided reagents such as antibodies and gene sequences that we are using to study additional properties of the proteins. Also, we are examining functions of these proteins in the circadian system using pharmacological agents to alter their functions and perturb the circadian rhythm. Finally, we are developing techniques that will allow us to perturb the synthesis of specific POPs or their mRNAs. A critical future issue will be to determine whether any of the POPs are functionally related to one another. It is through functional relationships between POPs that a model of the molecular structure of the circadian pacemaker will emerge.

9. Genetic and molecular analysis of the *Neurospora* circadian clock

Jay C. Dunlap, Jennifer J. Loros, Keith A. Johnson, Kristin M. Lindgren, Benjamin D. Aronson, Deborah Bell-Pedersen, Qiuyun Liu and Norman Y. Garceau

The biological clock is generally believed to be comprised of a feedback loop that operates, in all organisms displaying a circadian rhythm, at the level of the single cell. Hence, microbial systems, and systems amenable to genetic analysis, have long been appreciated for the insights they can provide into the mechanism of the clock and the means by which clocks act to regulate the metabolism and behavior of cells. The best molecularly studied microbial clock system is the ascomycete fungus *Neurospora crassa*, in which a circadian biological clock controls several aspects of growth and development. In particular, the timing of the initiation of conidiogenesis has been well studied and has allowed genetic and molecular techniques to be used to study the circadian clock itself from two converging angles[13,12,15].

One approach has been to examine the pathways whereby clocks act to control cellular metabolism and behavior. Initial efforts targeted the isolation of genes whose transcript levels are controlled by the clock. Two such genes, designated *c*lock *c*ontrolled *g*ene-1 (*ccg*-1) and *c*lock-*c*ontrolled *g*ene-2 (*ccg*-2), were identified by a subtractive hybridization procedure[40]; both are morning specific genes. Nuclear run-on assays[41] have pinpointed transcription as the primary point of clock regulation. DNA sequence and gel mobility shift analysis of these two clock regulated genes and their regulatory regions have suggested possible targets for clock mediated regulation and preliminary experimental data has identified a trans-acting protein factor(s), a putative clock-regulatory factors, that binds to the promoter region of *ccg*-2. The factor(s) appears to be present in morning protein extracts but not in evening extracts, its presence coincidental with the time of expression of *ccg*-2. *Ccg*-2 encodes one of the major fungal hydrophobin proteins required for the formation of the hydrophobic outer rodlet layer of conidia[1]. As spore surface hydrophobicity is a dominant determinant of pathogenicity in many fungal pathogens, a role for the clock in determining the immediate pathogenic potential of some fungal spores is suggested. Finally, as both *ccg*-1 and *ccg*-2 are known to be regulated by light as well as the clock, these genes provide excellent molecular correlates for studying the complex interface between genes, environmental factors and an organism's environmental niche.

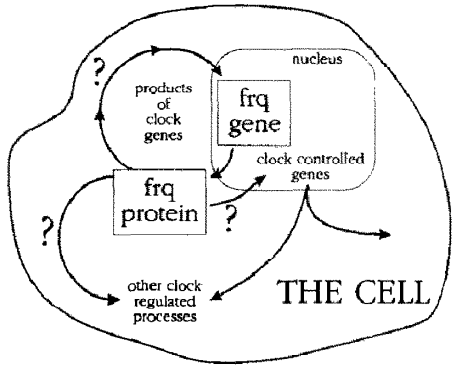

Fig. 13. Role of *frq* gene in the feedback loop of the oscillator.

A second approach has been to identify and characterize putative clock components through the identification, cloning and characterization of genes, that when mutated, alter the expression of wild-type rhythmicity. Towards this end, the *period*-4 and *frequency* loci have been cloned and the latter characterized in some detail[42]. The *frequency* locus encodes two non-overlapping transcripts; the smaller transcript has little capacity to code for a protein while the larger transcript has the potential to code for a 989 amino acid protein and appears to have an unusually long 5′ untranslated region. Sequence analysis of the previously isolated eight *frq* alleles has allowed tentative assignment of the single amino acid substitutions that can lenghten or shorten the period length and disrupt temperature compensation. In general, conservative substitutions have led to short period changes and more potentially disruptive amino acid substitutions have resulted in long-period mutants. Disruption of the gene has established that it is not essential. Strong preliminary data speak to the importance of *frq* in the actual assembly and operation of the clock. First of all, the amount of *frq* transcript is rhythmic, with a peak in the early morning. Thus the *frq* gene both regulates the clock (as exemplified by the phenotypes of the various *frq* alleles) and is regulated by the clock (as exemplified by the rhythm in *frq* transcript) and it appears that *frq* must be placed within rather than just affecting the feedback loop of the oscillator (Fig. 13).

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