Circadian Rhythms

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INTRODUCTION

Most organisms, including humans, exhibit daily rhythms in their physiology and behavior. Nearly all functions of the body show significant daily variations including activity, arousal, psychophysical performance, consumption of food and water, liver metabolism, urine volume and pH, blood pressure, heart rate, acid secretion in the gastro-intestinal tract, cortisol secretion (11,46,55). It is more surprising to find a variable which is static through time than one that shows a daily rhythm. This temporal variation obviously plays an important role in the body's homeostatic mechanisms and has a significant impact on the function of specific physiological systems, including the nervous system. In many cases, these rhythms are generated by endogenous processes referred to as circadian oscillators. These oscillators presumably function to synchronize biological processes with temporal changes in the environmental and to allow the temporal coordination of physiological systems.

The term circadian comes from Latin roots: *circa*, about and *dies*, day. A circadian rhythm is an endogenous rhythm which repeats with a period of close to 24 hours. Because these rhythms are generated from inside an organism, a fundamental feature of circadian oscillations is that they will persist when isolated from environmental time cues. However, because the period of these rhythms is not equal to exactly 24 hours, the oscillations will drift with respect to any 24 hour based time and are referred to as "free-running." The free-running period of an organism is under genetic control and will persist for generations in the absence of environmental time cues. One manifestation of this is that mutations can be isolated which alter the rhythms period. In Drosophila, investigators have been successfully using a molecular genetic approach to explore several aspects of the fly circadian system (32,80). Two genes have been identified, *period* and *timeless*, which are integral to the generation of circadian rhythms in Drosophila. For example, mutations in the *period* gene produce phenotypes which are arrhythmic or express altered periods. Recently, two single gene mutations which alter period have also been isolated in mammals (74,100). Hopefully, in the future, such mutations can be used to explore the genetic basis of mammalian circadian systems.

In order to function adaptively as time keeping systems, circadian oscillators need to be precise and stable in the face of environmental perturbations and, in general, they appear to satisfy these conditions. For example, in the mouse Mus musculus, it has been estimated that the circadian oscillator driving wheel-running activity exhibits a standard deviation of about 0.6% or just under 9 minutes per 24 hour cycle (72). Changes in temperature must be one of the major challenges to this homeostasis of period as the rates of most biochemical processes change 2 to 3X for every 10^{O}C change in temperature (Q_{10} = 2-3). Since even homeothermic mammals undergo daily rhythms in body temperature in the range of 2^{o}C, a circadian oscillator with a Q_{10} = 2-3 could exhibit transient period changes of several hours. This is obviously unacceptable for a timing system and the period of circadian oscillators typically show a Q_{10} of close to 1. This temperature compensation of period appears to be a universal feature of circadian systems, although little is known about the mechanisms responsible. This is just one example of the types of homeostatic mechanisms which had to evolve in order to enable biological oscillators to keep accurate time. Nevertheless, the period of a circadian oscillator is not a constant and can be modulated by both external and internal influences such as changes in photic environment, hormonal milieu, and even the age of an organism. In fact, some of these changes are essential if the oscillator is to be synchronized to the environment.

Circadian oscillators generate a rhythm which repeats with a frequency of close to but not equal to 24 hours. In order to function adaptively, these oscillators must be synchronized to the exact 24 hour cycle of the physical world. After all, there is limited utility in having a timing system which cannot be reset to local time. This process of synchronization is referred to as entrainment. Empirically, the phase (Φ) of the oscillator must be adjusted so that the endogenous period of the oscillator (tau, τ) equals the 24 hours period (T) of the physical world. Mathematically, this can be expressed at τ-T = $\Delta\Phi$. So for a primate with a τ of

25 hours to entrain to a T of 24 hours, the oscillator needs to be phase advanced by 1 hour per day. The formal properties of entrainment and circadian oscillators in general has been the subject of elegant analysis by Pittendrigh and colleagues (71). The daily cycle of light and dark, acting through light-induced phase advances and delays of the endogenous rhythm, is the dominate cue used by organisms, including humans, to synchronize their circadian oscillators to the environment. This is probably because dawn and dusk are the most reliable and noise-free indicators of time available to organisms in most habitats. But organisms have the ability to use many other cues for entrainment including social factors, temperature cycles, food availability. A major goal of circadian rhythms research is to understand the mechanisms by which light and other cues act to synchronize circadian oscillators.

In short, circadian rhythms show several fundamental features: 1) they are endogenously generated oscillations which repeat with a frequency of close to 24 hours, 2) they exhibit homeostasis of period including temperature compensation, 3) they are synchronized by periodic environmental signals, with the daily light-dark cycle being the dominant cue used. Obviously, not all daily rhythms are circadian and finding a day/night difference in a physiological or behavioral parameter is not sufficient to claim that the parameter shows a circadian rhythm. Daily rhythms that are driven exogenously will disappear when an organism is placed in constant conditions and the exogenous daily variable is removed. In contrast, a circadian rhythm will continue with a near 24 hour period for at least several cycles in constant external conditions. The phase of a circadian rhythm can also be synchronized to the phase of the light-dark cycle to which it is exposed.

Mammals have evolved a set of anatomically discrete cell populations which function as a physiological system to provide temporal organization on a circadian time scale. These structures are commonly referred to as a circadian system (Fig 1). In the simplest case, a circadian system can be modelled as having three components: 1) an oscillator or clock responsible for the generation of the daily rhythm, 2) input pathways by which the environment and other components of the nervous system provide information to the oscillator, and 3) output pathways by which the oscillator provides temporal information to a wide range of physiological and behavioral control centers. Although the presence of feedback loops between these components may make these separations somewhat arbitrary, they nevertheless represent a good starting point for discussion. In the rest of this chapter, we will consider each of these components in turn, as well as briefly discuss some of the medical implications of humans having a circadian timing system. Given the aim of this book, the discussion will focus on mammals and neurochemical mechanisms. Given space restrictions, many excellent and relevant references could not be included.

II. THE MAMMALIAN CIRCADIAN OSCILLATOR: THE SUPRACHIASMATIC NUCLEUS (SCN).

A. Localization of an oscillator: the SCN

One of the major goals of neuroscience research is to identify the anatomical substrates of specific behavioral control systems. This has proven frustratingly difficult in many cases. One of the success stories in this area has been the localization of most of circadian timing function to a bilaterally paired nucleus in the mammalian hypothalamus - the suprachiasmatic nucleus or SCN (Fig 2). For this reason, it is worth describing in some detail the evidence that this heterogenous population of cells is the locus of the mammalian biological clock. Initial studies utilized lesions to address the hypothesis that discrete parts of nervous system can function as a biological clock in mammals and concluded that a circadian oscillator was located somewhere in the ventral hypothalamus. Not much more progress was made until the early 1970s when new neuroanatomical tools were applied to the problem. Since lesion studies suggested that a circadian oscillator is located in the hypothalamus and light information from the retina must reach this oscillator, a logical approach was to look for anatomical pathways by which light information could be carried from the retina to the hypothalamus. This strategy lead to the demonstration of a direct retinohypothalamic tract (RHT). The hypothalamic sites in which these fibers terminated were the SCN and ablation of the SCN was quickly

shown to result in the loss of circadian rhythms (61,92). Of course, among other interpretational problems common to lesion studies, is the possibility that the SCN lesions could just be blocking the expression but not the generation of the rhythmic behavior. Nevertheless, these studies were the first to implicate the SCN as circadian oscillators.

If the SCN are the site of a circadian oscillator, then these structures should show oscillations in vivo. One of the first pieces of evidence of endogenous rhythmicity came from studies looking at cellular metabolism with 2-deoxy-D-glucose (2DG, 84). The cells of the SCN showed prominent circadian variation in their metabolic activity with glucose utilization peaking during the day when nocturnal rodents were at rest and fell to minimum during the night when the animals were active. One reason for this rhythm in metabolism may be that SCN cells also show a similar rhythm of spontaneous neural activity. But in vivo this rhythm is not restricted to the SCN and can be recorded in other regions of the central nervous system. In order to clarify the role of the SCN in the generation of these rhythms, Inouye and Kawamura (37) made a series of knife cuts around the SCN in order to isolate these structures from neural but not hormonal communication. When this was done, the rhythm in electrical activity continued inside the "island" containing the SCN but not in other regions of the nervous system. So, the SCN do indeed appear to be capable of generating circadian oscillators in an organism.

SCN tissue is also capable of generating circadian oscillations in vitro when isolated from the rest of the organism. Initial studies showed that the circadian rhythm in neural activity could also be recorded from a SCN brain slice preparation (30). Since that time, many studies have made use of this neural activity rhythm to explore SCN function in vitro. SCN tissue can also be shown to secrete the peptide vasopressin rhythmically (23). Currently, these in vitro preparations are being utilized to explore basic questions about how circadian oscillations are generated. Clearly, cells in the SCN have the ability to generate circadian oscillations both in vivo and in vitro.

The results of these experiments show that the SCN by themselves are oscillators and that, if the SCN are destroyed or functionally removed from an organism, many of the behavioral rhythms disappear. Ideally, one would want to show that if the SCN tissue is restored, the rhythms would reappear. Due to the advances in brain transplantation techniques, this experiment has turned out to be feasible. It is now well established that it is possible to restore rhythms in animals made arrhythmic due to SCN lesions by transplanting the SCN of another animal (45,76). Furthermore, it is possible to establish that the rhythm is being generated by the transplanted SCN tissue and not some trophic interaction with the host tissue. For these experiments, a mutant hamster was used which shows a circadian rhythm with a period much shorter than normal - the so-called *tau* mutant (74). When SCN tissue from *tau* mutant animals were transplanted into SCN-lesioned wild type animals, locomotor activity rhythms were restored with the shorter period of the donor tissue. Conversely, if SCN tissue from wild-type hamsters were transplanted into SCN-lesioned *tau* mutant animals, the restored rhythms exhibited the normal period of the donor animals (76). These experiments provide compelling evidence that the SCN are circadian oscillators in rodents.

Is circadian function localized exclusively to the SCN or do other regions also have this capacity? A few studies have raised the possibility of other circadian oscillators. For example, food entrainable and methamphetamine-induced circadian oscillations can be observed in SCN-lesioned animals (57). In many ways the presence of multiple circadian oscillators would not be surprising. In most vertebrate species, endogenous circadian oscillators have been localized to three structures: the SCN, the pineal gland and the retina. Until recently, mammals seemed to have been an exception to this rule, having such a major component of circadian function localized in the SCN. But recent work by Tosini and Menaker (94) provides a particularly clear demonstration that circadian oscillators exist outside of the SCN region even in mammals. In this study, a hamster's retina was cultured at room temperature and the secretion of melatonin measured. Non-mammalian retinas are known to exhibit daily rhythms in many variables including melatonin, so it was not unreasonable to look for rhythms in melatonin production. Indeed, the hamster retina was found to

produce melatonin rhythmically with levels high at night and low during day. A light-dark cycle can synchronize this rhythm in culture so the excised retina even has an intact light-input pathway. These observations prove that another circadian oscillator exists independently from the SCN and introduces the retina as another model system in which to explore circadian function in mammals.

B. Are circadian oscillations a network or intracellular property?

The SCN are made up of a dense accumulation of small neurons lying dorsal to the optic chiasm and lateral to the third ventricle (Fig 3). As previously described, these cells exhibit circadian rhythms in electrical activity, glucose utilization, and secretion. One of the first questions which needs to be addressed is whether these cells generate the rhythm through an intracellular or a network process. The answer is not yet known, but at least three lines of evidence suggest that synaptic interactions are not important for the generation of the daily rhythm. First, local injection of tetrodotoxin (TTX) into the SCN region in vivo blocked expression and photic regulation of a circadian rhythm in drinking behavior (86). TTX blocks voltage sensitive sodium channels and synaptic communication in neural tissue. However, when these treatments ended, the rhythms continued from a phase which suggested that the SCN oscillator was undisturbed by the experimental manipulation. Similar results were obtained with in vitro SCN preparations in which the expression of circadian rhythms of secretion and spontaneous neural activity were blocked by TTX but the oscillator itself appeared to be unaffected (22,103). In addition, the cells of the SCN generate rhythms in glucose utilization early in the development of the nucleus (84), prior to the majority of synapse formation. Finally, disassociated SCN cells in culture retain the ability to generate circadian oscillations (68,90,103). Under these condition, single SCN cells showed circadian oscillations in spontaneous neural activity which drift out of phase with one another as if each cell contains an independent oscillator (103). The simplest interpretation of this collective data is that synaptic interactions are important for the inputs to and outputs from the SCN oscillator but are not responsible for the generation of the circadian rhythm itself. However, resolution of this issue will have to await the demonstration that single, isolated SCN cells retain the ability to generate circadian oscillations.

The mammalian circadian system did not evolve independently and comparative evidence is also consistent with the possibility that generation of circadian oscillations is an intracellular rather than a network process. Certainly, single celled organisms e.g. the dinoflagullate Gonyaulax polyedra and cyanobacteria exhibit circadian oscillations, so clearly these rhythms can be generated intracellularly. Further support comes from studies of the marine mollusk Bulla gouldiana. The eyes of this organism contain an oscillator that drives a circadian rhythm of spontaneous compound action potentials in the optic nerve. A population of electrically coupled cells known as basal retinal neurons (BRNs) are responsible for the generation of this rhythm through a daily cycle in their membrane potential. Isolated BRNs in culture continue to show a circadian rhythm in membrane conductance (54), thus, demonstrating that single cells in culture retain the ability to generate a circadian oscillation. Regardless of whether individual cells in the SCN are competent circadian oscillators, it is obviously important to understand how these cells communicate and remain synchronized with each other. Current knowledge about cell-to-cell communication in the SCN is the subject of a recent review (98) and will only be briefly discussed here.

C. Intrinsic transmitters: GABA and peptides

A variety of evidence suggests that the amino acid γ-aminobutyric acid (GABA) is the major transmitter used by SCN neurons. GABA is found in at least half of all presynaptic terminals in the SCN (18) and GABA and its synthetic enzyme are found in most SCN cell bodies (63). Furthermore, electrophysiological studies have recorded spontaneous and electrically evoked inhibitory postsynaptic potentials in the SCN which are GABA-mediated (43). Administration of GABA agonists cause phase shifts of behavioral rhythms during the day and alter photic regulation of the circadian system during the night

(75,95). In adult tissue, GABA is typically an inhibitory transmitter and most SCN neurons send projections to other cells in the SCN; thus, the typical SCN neuron may be best thought of as an inhibitory interneuron.

Ultrastructural studies support the subdivision of the rat SCN into three populations; a small rostral area, and caudally a dorsomedial and ventrolateral subdivisions. Many of the cells in the SCN express peptides and differences in peptide expression can serve as a basis to segregate SCN cells (58,97). In particular, a distinction is commonly made between cells expressing vasopressin (VA) which are found in the rostral and dorsomedial regions and those expressing vasoactive intestinal peptide (VIP) which are found in the ventrolateral region of the SCN (Fig 4). Retinal and other afferent innervation is largely confined to the ventrolateral regions. So, it is in these cells that integration of the majority of synaptic inputs is most likely taking place. There has been some speculation that the VIP-immunoreactive cells may play a critical role in processing photic input while the VA-immunoreactive cells may be responsible for the generation of daily rhythms. However, there is evidence that circadian rhythms can be expressed in both the ventrolateral and dorsomedial populations of SCN neurons (38) and, at this point, the functional roles played by these different cell-types are unclear. Moreover, the question of whether these anatomically-defined subdivisions of cells are also electophysiologically distinct remains to be answered.

In addition to VA and VIP, a number of other peptides and growth factors are expressed in SCN neurons including gastrin releasing peptide (GRP), the peptide histidine isoleucine, somatostatin, substance P, neurotensin and nerve growth factor. In many cases, these peptides appear to be co-localized with amino acid transmitters and presumably function as signalling molecules. One peptide which has received some recent attention is GRP which is expressed in neurons in the ventrolateral subdivision of the SCN. Application of GRP excited many SCN neurons in vitro and can cause phase shifts of the circadian system in vivo (69). But questions of how this or any other peptide functions in the local SCN circuits and their role in circadian function has not yet been resolved. The role of peptides in the SCN has been recently reviewed (38). The SCN, with its clearly defined circadian function and behavioral outputs, is an excellent location to start to explore the function of peptide signaling molecules.

4. INPUT TO THE SCN: HOW DOES THE ENVIRONMENT REGULATE THE CIRCADIAN OSCILLATOR

One approach to understanding circadian systems is to examine the neurochemical circuitry by which the SCN receives information from the environment. Besides addressing issues related to the sensory physiology of circadian systems, this approach could lead to the identification of components of the circadian oscillator. By systematically following the signal transduction cascade by means of which photic information reaches and regulates SCN neurons, it should be possible to identify mechanisms that generate circadian oscillations. One strategy that has been widely used to address these issues is a systems-level analysis of the effects of photic, pharmacological, and genetic manipulations on rhythms driven by the circadian system. Another strategy is to examine the effects of such manipulations on the cellular/molecular activities of SCN neurons. Both strategies are being successfully used to explore SCN function (See recent reviews; 38,66,95).

A. RETINOHYPOTHALAMIC TRACT (RHT)

The anatomy of the light input pathway to the SCN has been recently reviewed (Fig 5; 66). Briefly, in mammals, the effects of light on the SCN are mediated by unknown photoreceptors located in the retina. The primary pathway for transmission of photic information from the retina to the pacemaker for entrainment is the RHT. The RHT comprises a distinct subset of retinal ganglion cell axons that separate from the other optic axons at the optic chiasm to innervate the SCN. The photic information transmitted neurally to the SCN appears to be both necessary and sufficient for entrainment.

1. Glutamate

There is a variety of evidence that the amino acid glutamate is a transmitter at the RHT/SCN synaptic connection and that this transmitter plays a critical role in mediating photic regulation of the circadian system (14). Anatomical studies report that identified RHT terminals innervating the SCN show glutamate-immunoreactivity associated with synaptic vesicles (8,19). A variety of glutamate receptors have been localized to the SCN by both in situ hybridization and immunocytochemistry (28). There is electrophysiological evidence that exogenous application of GluR agonists excite SCN neurons (6, 87) and that GluRs mediate the excitatory post-synaptic potentials recorded in the SCN (42). Application of GluR agonists cause phase shifts of a rhythm of neural activity recorded from the SCN in vitro (20,89). Finally, GluR antagonists block light-induced phase shifts and Fos-induction in the SCN in vivo (2,13).

Despite this strong evidence that glutamate is a transmitter released by the RHT, there are many unanswered questions as to how the circadian oscillators in the SCN respond to this glutamatergic stimulation. In the simplest case, light causes the release of glutamate which initiates a signal-transduction cascade in SCN neurons which ultimately results in a phase shift of the circadian system. This model is strengthened by the findings that GluR antagonists prevent light-induced phase shifts in vivo and that exogenous glutamate can cause phase shifts in vitro. More problematic is the finding that GluR agonists injected into the SCN region do not cause light-like phase shifts (53). Of course there are many possible pitfalls in the interpretation of this type of experiment. For example, It seems unlikely that the injection of glutamate into the SCN region would stimulate the same cells in the same manner as synaptic activation of the RHT. Nevertheless, the currently available data clearly challenge any simple interpretation of the behavioral experiments with GluR antagonists. At present, behavioral evidence suggests that GluR activation is necessary but not sufficient to generate "light-like" phase shifts. More work is clearly needed to delineate the signal-transduction cascades by which glutamate is acting in the SCN and to understand how these cascades influence the phase of the circadian system.

2 Nitric Oxide (NO)

One possible consequence of NMDA GluR activation is the stimulation of nitric oxide synthase (NOS) and several pieces of evidence raise the possibility of a role for NO in the light-input pathway to the SCN. First, anatomical studies have described the presence of NOS in the SCN (3). Second, NO production has generally been linked to NMDA-induced cGMP production and administration of cGMP produces phase shifts of the circadian rhythm of neuronal activity recorded from the SCN in vitro. (73). Finally, NOS inhibitors prevent NMDA-induced phase shifts of circadian rhythms both in vitro and in vivo (20).

3. Changes in gene expression

Although the signal transduction events occurring downstream from GluR activation in the SCN are not well understood, one important consequence of photic stimulation is the regulation of gene expression in the SCN (44). In many neurons, one consequence of GluR stimulation is activation of immediate-early genes including *c-fos*. The proteins coded for by these genes, including Fos, appear to be generally involved in the transduction of extracellular signals to changes in gene expression and, in some cases, changes in immediate-early gene expression can be used as a cellular marker of neuronal activation. Photic regulation of *c-fos* mRNA and Fos-like immunoreactivity (Fos-LI) in the SCN of rodents has been extensively demonstrated. These studies have shown that photic regulation of Fos in SCN neurons is correlated with light-induced phase shifts of the circadian system. For example, induction of *c-fos* mRNA by light in the hamster SCN shows the same phase dependence and intensity threshold as does phase shifting. The functional significance of light-induced Fos expression is still unclear. Light can still entrain the circadian system of mice lacking the *c-fos* gene although a reduction was seen in the magnitude of light-induced phase shifts (35). In another study, the

intraventricular administration of *c-fos* and *jun-B* antisense oligonucleotides was reported to decrease expression of these transcription factors and inhibit light-induced phase shifts (104). These results suggest that while *c-fos* activation may contribute to the normal entrainment process, it is not absolutely required for photic regulation or generation of circadian rhythms.

Fos-induction has also been widely used as a cellular marker for light-responsive cells to address questions about how experimental manipulations alter photic input to the SCN. A number of studies have reported that both NMDA and AMPA/KA GluR antagonists inhibit light induction of Fos expression in the SCN (2,25). At least one study has also found that the intraventricular injection of NMDA induces Fos expression in the SCN (25). The effects of AMPA/KA GluR agonists have not been examined. These pharmacological studies generally suggest a role for both NMDA and AMPA/KA GluRs in mediating photic regulation of SCN neurons in vivo.

4. Peptide co-transmitters

The possibility of peptide co-transmitters within RHT terminals is specifically suggested by the presence of dense-core vesicles among the glutamate-containing synaptic vesicles (8). Two likely candidate co-transmitters are N-acetylaspartylglutamate (NAAG) and substance P (SP). Immunocytochemical localization of NAAG to many retinal ganglion cells and the SCN has been reported (58). Optic nerve transection decreased NAAG immunoreactivity in the SCN - a finding consistent with the suggestion that NAAG is contained in terminal fields of the RHT. Although the functional role of NAAG is unclear, it can both directly activate GluRs and is also hydrolyzed extracellularly to form glutamate. One physiological study found that the iontophoretic application of NAAG increased the firing rate and potentiated glutamate-induced responses in SCN neurons in culture (6).

There is also some evidence that SP may play a role as a retinal co-transmitter. Anatomical evidence for the presence of SP in the RHT has been found for a number of species including humans (64). Application of SP in vitro has been reported to increase 2DG uptake, excite a population of SCN neurons, induce Fos expression, and cause phase shifts of the circadian rhythm of electrical activity (88). More recently, a SP antagonist has been found to block light induced Fos expression in vivo (1). It will be important to examine the effects of this inhibitor on light-induced phase shifts of the circadian system and to see if SP alters glutamate release from the RHT. To date, the results are all consistent with the possibility that this peptide is a co-transmitter at the RHT/SCN synaptic connection.

B. GENICULOHYPOTHALAMIC TRACT (GHT)

The retinal ganglion cells which innervate the SCN also project to a subdivision of the lateral geniculate - the intergeniculate leaflet (IGL; Fig 6; 66). The IGL, in turn, has a population of neurons that project to the SCN through a geniculohypothalamic tract (GHT). The projection neurons that make up the GHT appear to contain neuropeptide Y (NPY) and GABA and these transmitters may be co-localized. Both of these molecules exert effects on the circadian system. This pathway appears to be involved both in the processing of photic information but also in mediating the effects of some non-photic entraining stimuli.

Cells in the IGL are known to be light-sensitive (33) and the GHT pathway may contribute to the processing of photic input. In many sensory neurons, NPY acts presynaptically to modulate transmitter release and may play a similar role in the regulation of RHT input to the SCN. Likewise, $GABA_B$ receptors may modulate the release of glutamate by the RHT (40) and light-induced phase shifts (75). IGL lesions are associated with relatively subtle changes in the circadian system's response to light including changes in the magnitude of light-induced phase shifts, period changes, and slower adjustment to new light-dark cycles (66). However, many features of the circadian system's response to light are unaffected in IGL-lesioned animals, suggesting that this pathway is not essential for the major portion of photic regulation of the circadian system. But this pathway does appear to have an important role in mediating the effects of other non-photic stimuli.

A number of experimental treatments act to produce phase shifts during the day but not during the night including activity induced by novel stimuli (67) and benzodiazepines (95). These phase shifts are dependent upon an intact IGL as lesions abolish both benzodiazepine (41) and activity induced (67) phase shifts. The use of antibodies to reduce NPY binding also reduced activity-induced phase shifts (4). A similar pattern of phase shifts during the day is generated by NPY administration in vitro and in vivo through a mechanism which may be dependent on GABAergic transmission (36). Recent studies in cultured SCN cells suggest that NPY can act presynaptically to inhibit GABA-mediated synaptic transmission through an inhibition of calcium currents (9). This evidence suggests that the GHT plays a critical role in mediating the effects of some non-photic stimuli on the circadian system.

C. Other neurotransmitter systems

1. Serotonin (5HT)

The SCN receives a dense serotonergic projection from the midbrain raphe nuclei that terminates predominantly in the retinorecipient region of the nucleus (62). It is well established that 5HT receptor agonists cause phase shifts of the SCN circadian oscillator when administered at times in the circadian cycle during which light does not cause phase shifts both in vitro (52) and in vivo (26). In addition, a variety of evidence suggests that this projection modulates photic input to the SCN. Neurotoxic destruction of the serotonergic input to the SCN alters the relationship between the light-dark cycle and locomotor activity and increases in 5HT levels alter the effects of light on the circadian system (65). Finally, 5HT and 5HT agonists inhibit optic nerve induced field potentials in the SCN brain slice preparation, light-induced Fos expression and phase shifts of the circadian rhythm of wheel-running activity (78). Interestingly, 5HT antagonists have been reported to enhance light-induced increases in the firing rates of SCN neurons (105) and light-induced phase shifts (77). These results raise the possibility that 5HT may be involved in a tonic inhibition of the light-input pathway to the SCN. In addition, these studies are all consistent with the hypothesis that the serotonergic innervation of the SCN serves to modulate light-regulated glutaminergic input. Understanding this pathway is likely to be important in understanding the links between disruptions in circadian function and affective disorders (see later discussion).

2. Acetylcholine (ACh)

It has long been suggested that ACh plays a role in the light-input pathway. Fibers immunoreactive for choline acetyltransferase are known to innervate the SCN (99) apparently from the cholinergic regions of the basal forebrain and brain stem (5). Furthermore, electrophysiological studies indicate that some SCN neurons are excited by cholinergic agents. In addition, administration of the ACh receptor agonist carbachol caused large phase shifts in the SCN neuronal activity rhythm; this response is mediated by muscarinic receptors perhaps of the M1-subtype (50). The intraventricular administration of carbachol caused phases shifts in vivo which can be blocked by GluR antagonists (12). This result raises the possibility that some of the behavioral effects of carbachol may be due to the stimulation of glutamate release. So, while ACh does not appear to be a transmitter directly in the light input pathway, it may act to modulate photic information reaching the SCN.

3. Histamine (HA).

The SCN receives a prominent histaminergic innervation from the tuberomammillary nucleus. Based on anatomical considerations, the histaminergic transmitter system has been suggested to represent a regulatory center capable of altering arousal throughout the nervous system. Depending on the subtype of receptor activated, HA can have excitatory (H1) or inhibitory (H2) actions on SCN neurons (49). Administration of HA can cause phase shifts of the in vitro neural activity rhythm and the in vivo locomotor activity rhythm (15). These results suggest that HA may be involved in modulation of light-input to the

circadian system but, at present, the functional significance of this regulation is unknown.

4. Melatonin.

The mammalian pineal gland secretes melatonin rhythmically under the neural control of the SCN (see discussion below). The SCN are also a target of this hormonal output as there are a high density of melatonin receptors in the SCN (102). One consequence of activation of these receptors is the inhibition of neural activity perhaps through the activation of potassium currents in SCN cells (40). Melatonin also caused phase shifts of the circadian rhythm of neuronal activity of SCN neurons in vitro (51). Behaviorally, administration of melatonin caused phase shifts of the locomotor activity rhythm during the day and may modulate the effects of light during the night (7).

5. OUTPUT FROM THE SCN: HOW DOES THE OSCILLATOR IN THE SCN REGULATE OTHER PHYSIOLOGICAL AND BEHAVIORAL SYSTEMS

Most of an organism's physiological and behavioral parameters show a daily rhythm. In many cases, these rhythms are driven from a circadian oscillator located in the SCN. Physiological or behavioral parameters which exhibit daily rhythmicity due to the activity of cells in the SCN are known as "outputs" of the circadian system. Metaphorically, these outputs are sometimes referred to as "hands of the clock" to distinguish them from the mechanisms responsible for the generation of the rhythms. It is not clear if this intellectual distinction will hold up experimentally. In general, outputs of the circadian system are rhythmic but not temperature compensated. Theoretically, if an output is held constant, this should not alter other rhythms driven from the SCN. A major problem in circadian rhythms research is to understand the mechanisms by which the circadian oscillator located in the SCN regulates such a wide-range of physiological outputs. There is evidence for two types of signals originating from SCN and conveying phase information: hormonal and neural outputs.

A. HORMONAL OUTPUTS.

Evidence for hormonal or some other diffusible signals comes from transplantation experiments (76). These studies have shown that rhythmicity can be restored to SCN-lesioned animals following implantation of tissue containing the SCN into the third ventricle. As previously discussed, these experiments provided compelling evidence that the SCN are circadian oscillators in mammals but also provide an important tool to look at the mechanisms by which SCN output alters other physiological systems

There are several pieces of evidence which suggest that a hormonal/diffusible factor secreted from the SCN is an important output signal for the circadian system (91). First, some behavioral rhythms recover within 4 days after transplantation of the SCN before much axonal outgrowth from the transplant is noted (45). In addition, some successful transplants can be placed in locations distant from SCN/hypothalamus (45). Third, transplantation of disassociated SCN cells can restore rhythmicity (90). The interpretational problem common to all of these studies is the difficulty in ruling out all neural outgrowth. Resolution of this problem may come from studies which place SCN grafts into a polymer capsule or into the anterior chamber of the eye; conditions which allows humoral communication with the brain but prevent neurite outgrowth (76). Of course, interpretation of negative results from these type of experiments would be extremely difficult.

One of the best studied examples of hormonal output from the SCN is the rhythmic secretion of the peptide VA. Peripherally, this peptide is secreted by the pituitary and functions as an antidiuretic hormone. Centrally, VA also acts as a signaling molecule and is highly expressed in a population of cells in the SCN. These cells drive a prominent daily rhythm in the levels of VA in the cerebrospinal fluid in mammals. If the SCN are lesioned, then VA levels are dramatically reduced and no rhythmicity can be detected (81).

Furthermore, SCN cells express a circadian rhythm in VA mRNA in vivo (96) and SCN cells in vitro secrete VA rhythmically (23). Finally, if embryonic SCN tissue is transplanted from a normal rat into a VA-deficient Brattleboro rat, the rhythm in VA is restored (24). Since host animals can not make VA, the source must be donor tissue containing the SCN. The function of this rhythm in VA is currently unknown and is an interesting area for future work.

B. NEURONAL OUTPUT

Anatomical knowledge of the output pathways of the SCN have been reviewed by Watts (101). In short, axonal projections from SCN neurons terminate within the SCN, other hypothalamic regions, and a few regions outside the hypothalamus. The largest projection from the SCN goes to the subparaventricular region of the hypothalamus, a region with widespread connections throughout the limbic system. Most SCN neurons contain GABA (18,63,99) suggesting that the output is generally inhibitory. There is evidence that these neuronal pathways are functional and are involved in the communication of signals from the SCN to other parts of the nervous system. First, the local injection of TTX into the SCN region blocks the expression of a rhythm in drinking activity (86). Second, knife cuts around the SCN which cut efferent fibers block the expression of several rhythms (37,92). Third, some transplants appear to be healthy, are in an appropriate position, and have cells expressing normal peptides; yet do not restore rhythmicity. These data are all consistent with the idea that neural connections play an important role in driving overt rhythms. The interpretation problem common to all of these studies is that the experimental treatments might interrupt hormonal as well as neural output from the SCN.

Among the rhythms under neural control of the SCN is the circadian synthesis and secretion of the pineal hormone melatonin. In mammals, the pineal gland is not directly light sensitive but is photically regulated through a complicated neuronal pathway involving the SCN. The hormone secreted by the pineal is melatonin and the SCN drives a daily rhythm in its secretion. Control relies on a multisynaptic pathway via the sympathetic nervous system to maintain and entrain the rhythmic synthesis and secretion of this hormone (Fig 7; 60).

The neural pathway from the SCN to the pineal passes first to the paraventricular nuclei (PVN). Ablation of either the SCN or PVN results in the loss of the rhythm in pineal melatonin levels. Most SCN neurons contain GABA, an inhibitory neurotransmitter, and it is most likely that the excitation of SCN neurons inhibits neurons in the PVN. The PVN neurons project to the spinal cord and make synaptic connections with preganglionic cell bodies which, in turn, innervate the superior cervical ganglia of the sympathetic nervous system. Stimulation of the PVN increases sympathetic outflow through activation of cholinergic preganglionic sympathetic neurons. Sympathetic neurons release norepinephrine which drives a rhythm in pineal melatonin by increasing N-acetyltransferase activity. Disruption of the pathway from the SCN to the pineal gland at any level (destruction of the SCN itself, knife cuts of SCN afferents, or pharmacological blockade of the sympathetic innervation) interrupts the circadian pattern in the synthesis and secretion of the hormone (60). SCN transplants do not appear to restore the melatonin-mediated photoperiodic response in hamsters (45). Clearly, this output of the circadian system is under neural control.

There is evidence for both neuronal and hormonal outputs from the SCN. Signals could vary with the specific physiological system being regulated. For example, rhythmic secretion of melatonin could be under neural control while locomotor activity is under hormonal control. Alternatively, the signals could be redundant with a specific physiological system receiving both neural and hormonal signals from the SCN. But the issue of how cells in the SCN regulate other physiological systems is one in which little work has been done and it is clearly an important area for future research.

6. MEDICAL IMPLICATIONS OF CIRCADIAN SYSTEM

Although the experimental studies described in this review were mostly performed in rodents, the general principles developed by this research are likely to apply to humans. Humans, like other organisms, exhibit daily rhythms in many physiological and behavioral parameters (11,46,55). Because of experimental difficulties in isolating humans from environmental influences, in many cases, it is not yet clear whether these rhythms are really circadian or instead are diurnal, i.e. driven by external cues. Nevertheless, it is clear that humans have an endogenous circadian timing system including SCN and a RHT. There is every reason to think that the SCN function as circadian oscillators in humans. First, humans suffering from dementia have problems with the timing of their sleep/wake cycle which has been reported to be correlated with loss of neurons in the SCN (56). Furthermore, patients with tumors or other damage to the hypothalamic area including the SCN exhibit disruptions in their daily rhythms (10,85). Since the human SCN expresses many of the same neurochemical markers as have been described in rodents (59), it seems likely that many of the neural pathways described in rodents will also be relevant for humans.

In recent years, it has become clear that light is an important environmental signal responsible for the synchronization of the human circadian system (16). This has led several groups to investigate the use of light for therapeutic manipulations of the human circadian system (93). With these observations also comes the recognition that within the last 100 years dramatic changes have occurred in the temporal environment to which humans are exposed. With the widespread use of artificial lights and airplanes, many people experience rapid changes in their light-dark cycle. These changes can disrupt our endogenous timing system. Let us next briefly consider a few aspects of the human circadian system: desynchronization of the human circadian system caused by jet travel or shift work; circadian variation in the effects of pharmacological agents; the possible use of melatonin to manipulate the human circadian system; and, finally, the possible link between the circadian system and affective disorders. While there are many other interesting aspects of the human circadian system, these topics at least provide an introduction as to how the circadian system impacts human biology.

A. Desynchronization of the circadian system

In our modern world, increasing numbers of people have been exposed to effects of rapidly moving across time zones or working during the night. The result is a group of symptoms collectively known as "jet lag". While there is a lot of variation in individual symptoms, many people experience disruption of sleep, gastro-intestinal disturbances, decreased vigilance and attention span, and lack of energy. While most people have no difficulties tolerating an occasional case of jet lag, repeated shifts creates greater problems. One recent report even suggested that jet lag of players may be a factor affecting the outcome of baseball games (79). More seriously, consider people whose jobs require constant changes of schedule, e.g. health care professionals, pilots, and other shift workers. While it is difficult to directly link shift work with demonstrable physical illnesses, commonly reported health consequences include stomach diseases, sleep disturbances, and fatigue (55, 93). Besides physical problems, these workers are unlikely to be performing optimally. Humans undergo daily rhythms in many cognitive and motor functions with human performance normally at a minimum between 3 and 5AM (11,55). Persons working during these hours are likely to be sleepy, inefficient, and accident-prone.

There are many factors that contribute to jet lag including fatigue and stress which may be independent of the circadian system. But, other symptoms are undoubtedly a direct result of the desynchronization of the circadian system. In general, circadian systems can be thought of serving at least two different kinds of functions. One is to ensure that an organism is synchronized to the physical world. Another, less appreciated function, is to ensure that the various physiological systems inside the organism remain synchronized. Both of these functions are compromised under conditions of rapid travel between time zones or changes in the scheduling of work. For example, consider a person travelling from North America to Europe which involves a shift of at least 8 time zones and which may take up to a week to

resynchronize. In the meantime, the traveler's performance minimum (3:00 to 5:00AM old time) will now be occurring at 11:00-13:00. In order to maximize synchronization to a new time zone or schedule, the best strategy appears to be to maximize exposure to entraining signals especially light and social cues. So, the best advice in order to speed adjustment to a new schedule is to be immediately active in the new daytime and to sleep during the new night, eat meals at local times, and spend the day out in well-lit environments. But, even after exposure to all of these new environmental signals, it will still take a few days to readjust so it may just be best to simply recognize this and to allow some time for adjustment after shifting to a new schedule or time zone.

B. Circadian variation in drug effects

Since most physiological and behavioral parameters exhibit daily rhythms, it is not surprising that drug effects both desired and undesired (i.e. toxicity) will vary with the time of day. In one of the early observations of this phenomena, it was reported that the mortality of mice due to an injection of E.coli endotoxin was 80% during the middle of their inactivity/sleep time, but below 20% in the middle of the activity time (31). This is not an isolated or unusual result, and the effects of many drugs are now known to vary depending on the time of day (46). This daily variation is not due to some mysterious process but rather can be mostly explained by two observations. First, temporal variation has been documented in the rates of absorption, metabolism, and excretion. These factors will all impact the concentration of drug which actually reaches its intended target. Second, many tissues and cells show temporal variations in their response to the drugs which do reach them. These observations form the basis of the hope that by scheduling drug treatments for certain times of day, clinical outcomes can be improved.

Daily variation in the time of symptom onset may be common in a number of diseases and medical emergencies. One extensively studied example is asthma in which the majority of patients experience symptoms mainly at night (70). These patients have a daily rhythm of bronchial constriction with the greatest constriction seen between midnight and 8:00AM which, accordingly, is the time when most respiratory failures occur. Understanding this type of rhythm can, at the very least, lead to improved monitoring at certain times of day as a preventive measure. In addition, drugs used for treatment of asthma are apparently more effective when given before bed than during other times of day (46). So, in this case, the normal pharmacological goal of keeping drug concentrations constant through time appears to be less effective than allowing drug concentrations to fluctuate.

Even in diseases in which the symptoms are not so obviously temporally patterned, there is still evidence for diurnal variation in drug effects. For example, most of the drugs used in chemotherapy are toxic to both host and malignant cells so there is an unusually narrow window between therapeutic and toxic effects. There are good reasons to think that the timing of drug administration may be an important therapeutic variable. There are data which suggest that both the toxicity and therapeutic benefits of anticancer drugs vary with a daily cycle (27). If these rhythmic variations are out of phase with each other, then time of treatment may represent a variable which can be exploited to maximize the benefit/cost ratio for the use of anticancer drugs. In one early study, mice were injected with leukemia cells and treated with DNA synthesis inhibitors cyclophosphamide and 1-B-D-arabinofuranosylcytosine at different times of day (83). Without treatment, most of the mice died within the 75 day trial. The number of animals which survived the tumor inoculation varied depending on the time of cyclophosphamide administration. Treatment in the beginning of inactivity/sleep period for these animals led to a 40% survival rate while, the same treatment given at the beginning of the animals' active period, led to over 90% survival. Of course, there are many possible reasons for these data and the question of whether such scheduling strategies can actually improve clinical outcome for humans is one which still needs to be answered. But, it seems likely that varying drug doses over the course of the day could lead to much more effective drug treatment strategies.

C. Melatonin

In humans, as in other mammals, melatonin is secreted by the pineal gland during the night but not during the day. This rhythm is due to both circadian regulation and acute light-induced suppression of melatonin secretion. Both mechanisms ensure that the secretion of melatonin fairly accurately follows the night and have led to the description of melatonin as a "dark" hormone. In many temperate-zone mammals, this nightly "dark" signal is intimately involved in the control of seasonal changes in physiology and behavior. Although there is little direct evidence that melatonin mediated seasonal changes in humans, the onset of melatonin secretion at night does appear to be a good marker for the phase of the human circadian system (47). The human SCN contains melatonin binding sites and administration of melatonin (0.5mg), by itself, caused phase shifts of the human circadian system: phase delays during late night and phase advances during the morning (47). In addition, there is some evidence that melatonin can act as a sleep-inducing agent. In one study, a group of healthy adults were administered melatonin (1-10mg) in the middle of the day. Melatonin was found to induce sleep, lower body temperature, and caused feelings of sleepiness and fatigue (21). On the basis of these results, it has been proposed that melatonin could be used to help workers or travelers adjust to new schedules.

There is some evidence that melatonin may be therapeutically useful in this regard. Several studies have reported that timed melatonin administration can help with re-adjusting the circadian system after jet-lag or shift work (17,47). Moreover, in some insomnia patients, administration of melatonin has been reported to improve the sleep quality or the phasing of sleep (29). But, overall, our understanding of melatonin's effects on human biology is still in its infancy and much more work will have to be done before the possible therapeutic value of melatonin administration can be determined.

D. Affective Disorders

Several observations suggest a link between disruptions in circadian function and affective disorders (34). Certainly, many patients suffering from depression exhibit disruptions in the timing of sleep/wake cycles, cortisol levels, melatonin secretion and body temperature. Similarly, many treatments which have anti-depressive effects like timed photic stimulation, sleep-depravation, serotonin-uptake inhibitors also alter circadian rhythms. Dysfunction in serotonergic pathways have been suggested to play a role in affective disorders and are frequently treated with agents that alter serotonergic neurotransmission. The serotonergic projection from the raphe to the SCN (see previous discussion) may very well be the anatomical substrate by which affective disorders alter the human circadian system. Of course, it has been extremely difficult to establish more then a correlative link. Perhaps the best evidence comes from studies of seasonal affective disorder or SAD. SAD is characterized by recurrent cycles of fall-winter depression and spring-summer remission (82). The seasonal nature of the symptoms immediately suggests a role for the circadian system which, in many mammals, plays a central role in mediating seasonal changes in behavior and physiology. Furthermore, SAD has been successfully treated by timed exposure to bright light (48,93). But, overall, the exact role of the circadian system in any affective disorder is still open to debate and remains an important area for future research.

General Summary

The finding that humans and other organisms have endogenous circadian timing systems raises a number of issues. Among them: What are the mechanisms by which light and other environmental stimuli synchronize these systems? What are the mechanisms by which cells generate these oscillations? How are these cells and cell populations integrated to form a coherent timing system? How are these oscillators coupled to the various outputs they control? What are the consequences for human biology as well as the natural history of other organisms? In this review, I have briefly described some of the current work in each

of these areas. In keeping with the topic of this book, the focus was on mammals and neuropharmacological issues.

Some of the knowledge gained from addressing these issues may very well be unique to this physiological system. For example, the biochemical processes involved in the generation and temperature compensation of rhythms with a 24-hour time base are likely to be novel. Other questions involving sensory input to the circadian system, coupling between oscillators, and output from the circadian system are all basic issues of communication central to neuroscience research. In closing, I would like to argue that some features of the circadian system make it an excellent model system to address many core issues in neuroscience research. Many of the behavioral and physiological outputs of the circadian system are precise, quantifiable, and functionally important. This allows the productive use of both neuropharmacological and genetic approaches. Anatomically discrete and well defined pathways control these behaviors. Finally, SCN neurons are amenable for detailed cellular and molecular analysis by all of the tools of modern neuroscience. For these reasons, I believe that the circadian system will be one of the first mammalian behavioral control systems which will be understood at a variety of levels from behavioral to molecular.

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FIGURE CAPTIONS

Fig.1. A circadian system can be modeled as having three components: (1) an input pathway by which environmental information synchronizes the oscillator, (2) a set of elements which feedback on one another to form the oscillator, (3) an output pathway by which the oscillator is coupled to the processes it controls.

Fig 2. Dark-field photomicrograph of a section of hamster brain which has been immunocytochemically stained for the peptide vasoactive intestinal peptide. Core questions for the field of circadian rhythms include: (1) what are the cellular/molecular mechanisms which allow these cells to form functional circadian oscillators? (2) how does environment regulate this oscillator? and (3) how are these cells coupled to the various processes under their control?

Fig 3. Live, unstained SCN cells viewed by infrared differential interference contrast videomicroscopy. Left: scale bar = 100μm; Right: scale bar = 10μm. 3V, third ventricle; SCN, suprachiasmatic nucleus; OC, optic chiasm.

Fig 4. Schematic representation of immunocytochemical distributions in a single SCN of the mouse. VIP-immunoreactive cell bodies are represented by the open circles while VA -immunoreactive cell bodies are represented by filled circles. The Nissl-defined SCN is shown with the hatched line. 3V, third ventricle; SCN, suprachiasmatic nucleus; OC, optic chiasm.

Fig 5. In mammals, photic regulation of the circadian system is mediated by unknown photoreceptors which are located in the retina. These photoreceptors project to the SCN directly through a mono-synaptic projection from retinal ganglion cells known as the retinohypothalamic tract or RHT. The RHT appear to be both necessary and sufficient for synchronization of the circadian system by light. Evidence suggests that the amino acid glutamate (Glu) and the peptides substance P (SP) and NAAG are signaling molecules at this synaptic connection.

Fig 6. Photic information can reach the SCN through an indirect pathway which parallels the RHT. The retinal ganglion cells which form the RHT also project to a subdivision of the lateral geniculate - the intergeniculate leaflet (IGL). Neurons in the IGL, in turn, project to the SCN through a geniculohypothalamic tract (GHT). Evidence suggests that the amino acid GABA and neuropeptide Y (NPY) act as signaling molecules at this synaptic connection.

Fig 7. Diagram illustrating the neural pathway by which the SCN controls the secretion of pineal melatonin. Melatonin feeds-back to influence neurons in the SCN. IML, intermediolateral cell column of the spinal cord; Mel, melatonin; PVN, paraventricular nucleus; RHT, retinohypothalamic tract; SCG, superior cervical ganglion; SCN, suprachiasmatic nucleus.