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The Clock in the Brain: Anatomy of the Mammalian Circadian Timing System

Introduction

The circadian timing system in mammals is built from a neural network centered by the endogeneous clock located in the suprachiasmatic nucleus (SCN). Input pathways carrying photic and other informations are provided by the retina, intergeniculate leaflet and raphe nuclei, connecting the clock to the environment and provide entrainment to various stimuli. Output pathways are diverse and related to function. A major SCN-efferent trace via the paraventricular nucleus, spinal cord, the autonomic chain and the pineal gland couple the brain and the body to the clock mechanism. The present review presents some aspects of the history of circadian research and tries to summarize recent advances in the field. Viral transsynaptic tracings, the discovery of additional photopigments that may specificially entrain the clock, retinal circadian oscillations and central and peripheral clock gene transcription are among the exciting findings that may shed new light on the anatomical and functional properties of the circadian timing system.

The circadian timing system consists of the clock and of input and output pathways.

Circadian clocks are present in plants, fungi, bacteria and animals up to mammals including humans. Mammalian physiological processes such as sleep-wake cycles, locomotory activity, body temperature, oxygen utilization, water and food intake, oestrous, cardiovascular and digestive tract function are under circadian control, providing biological adaptation to the environment. However, students and scientists are disappointed when they try to look up respective knowledge in neuroscience textbooks.

On the other hand, in the scientific bibliographic system MedLine covering the last twenty-five years, more than thirty-five thousand citations were found containing the word "circadian". More than three thousand papers were listed when "suprachiasmatic nucleus" was searched for as keyword revealing that a large amount of knowledge on the structure and function of the circadian timing system is available to date. This discrepancy may be partly due to the fact that the system apparently consists of so many components connected by so many paths that it does not seem easily accessable—although the crucial component (suprachiasmatic nucleus) is known by many to be somehow responsible for timekeeping.

A general feature of this system is that it consists of three major functional components, i.e., (1) input pathways connecting the clock to the environment and synchronizing it to the internal milieu, (2) the clock oscillator that produces rhythmicity in the absence of external stimuli, and (3) output pathways connecting the brain's and the body's physiological parameters to the pacemaker (a schematic drawing is given in fig. 1). These outputs may be seen as "hands" of the clock, however, as is the case with melatonin, the hands may also take part in the "balance" mechanism. In fact, melatonin may be seen as a humoral input factor.

In search for the mechanisms of the clock's ins and outs and of clock-pineal interactions, many studies using neuronal tracing, lesioning of com-

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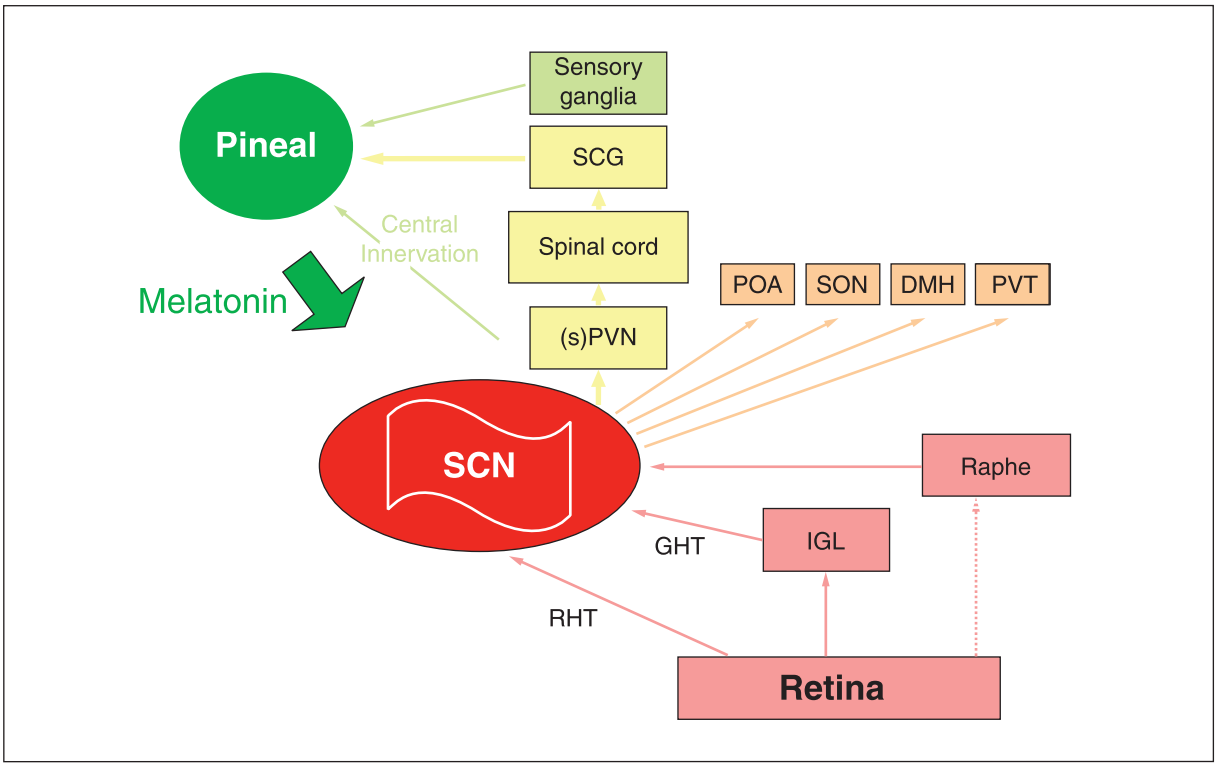

Fig. 1: Composition of selected structures and pathways of the circadian timing system in mammals. Only the structures that appear most important in that context are shown, and some of the connections are reciprocal (SCN inputs in light red, pineal regulating outputs in yellow, other outputs in light brown). *Abbreviations: DMH* dorsomedial hypothalamic nucleus, *GHT* geniculohypothalamic tract; *IGL* intergeniculate leaflet; *POA* preoptic area, *(s)PVN* paraventricular hypothalamic nucleus and its subparaventricular zone; *PVT* paraventricular thalamic nucleus; *RHT* retinohypothalamic tract; *SCG* superior cervical ganglia, *SON* supraoptic nucleus.

ponents and the investigation of functional parameters were conducted during the last three decades. They revealed a network consisting of the retina, hypothalamic suprachiasmatic and paraventricular nuclei, preganglionic sympathetic regions of the spinal cord, superior cervical ganglion and pineal gland. It became evident, however, that various other brain sites (e.g., intergeniculate leaflet, raphe nuclei) provide additional dominant input to the system and that diverse, partially reciprocal connections between several of these regions exist. In fact, the clockwork consists of multifarious feedforward and feedback pathways making a complete appreciation impossible. The present compilation therefore concentrates on the neuroanatomical features of the clock (the SCN) and its afferent and efferent connections, featuring an SCN-efferent string that regulates the pineal gland. To emphasize SCN-pineal interactions may be justified, since the pineal's hormonal output signal, melatonin, possesses dense binding sites in the SCN and thus influences its own regulator.

The article extends and updates reviews published previously [Reuss, 1993; Reuss, 1996] to which the reader is referred to for many previous data and references. Many overviews dealing with special aspects of SCN function give further information (e.g., [Gillette, 1997; Moore, 1997; Pévet et al., 1997; Ikonomov et al., 1998; Brown and Schibler, 1999; Dunlap, 1999; Golombek et al., 2000; Shearman et al., 2000; van Esseveldt et al., 2000; Wayne, 2000]).

The clock is located in the suprachiasmatic nucleus of the hypothalamus.

The suprachiasmatic nucleus (SCN) is a paired structure located bilateral to the third ventricle immediately dorsal to the optic chiasm (fig. 2A). In the rat, each nucleus consists of approximately 8,000 small neurons (7–11 µm soma diameter). Since there is general agreement that the SCN is the master circadian pacemaker, the present compilation will start with a description of the nucleus' intrinsic anatomy, then present input and output pathways and later deal with other components that are part of or closely related to the circadian timing system.

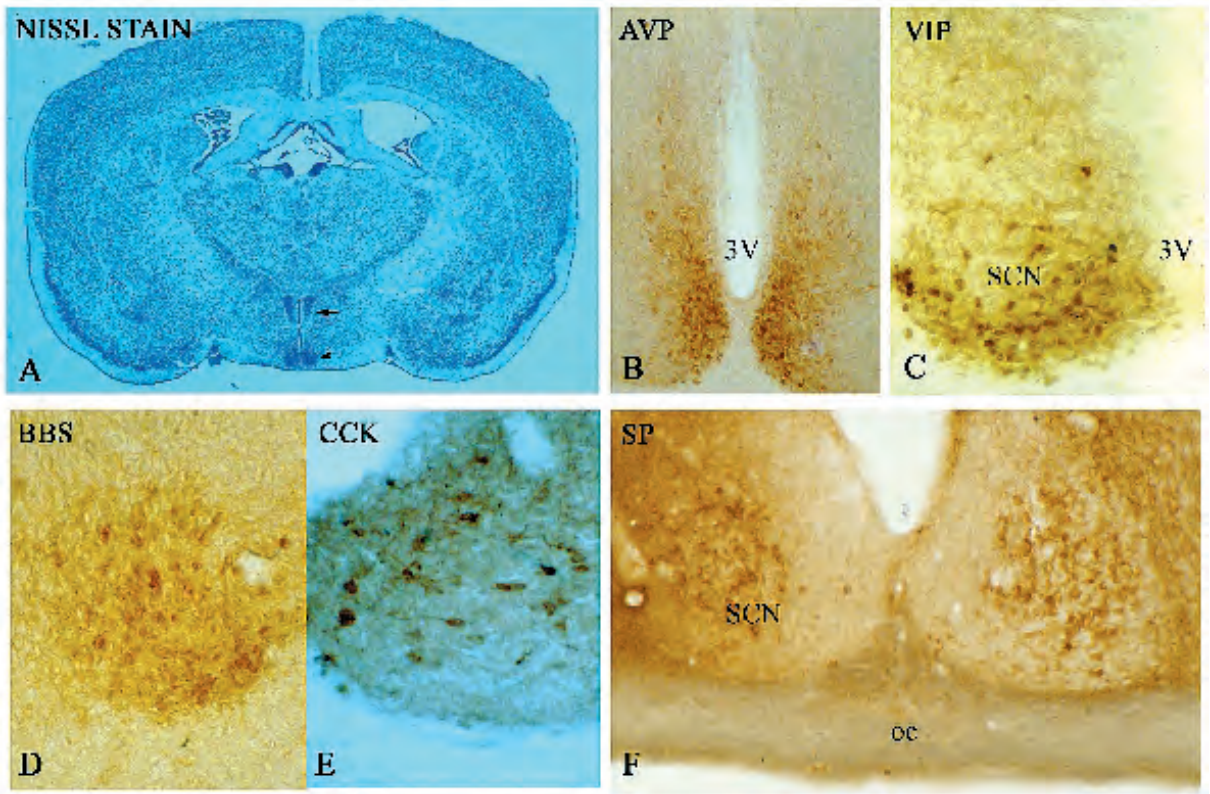

Fig. 2 A: Nissl-stained section of the rodent hypothalamus showing the locations of suprachiasmatic nucleus *(arrowhead)* and paraventricular nucleus *(arrow)*. The typical distribution of some neuroactive substances is shown for B: Arginine-Vasopressin (AVP), C: Vasoactive intestinal polypeptide (VIP), D: Bombesin (BBS), E: Cholecystokinin (CCK), and F: Substance P (SP). Abbreviations: *3V* third ventricle, *oc* optic chiasm.

Neuronal subpopulations of the SCN are characterized by their neuroactive substances.

Immunohistochemical and functional studies revealed that the SCN consists of several parts that may be characterized by their neurotransmitters and/or by their functional properties.

A considerable number of neuroactive substances are produced by neurons in the rodent SCN. Most extensively studied were the subgroups producing arginine-vasopressin (AVP, fig. 2B) and vasoactive intestinal polypeptide/peptide histidine-isoleucin (VIP/PHI, fig. 2C). Additional substances synthesized in the SCN include angiotensin II, bombesin/gastrin-releasing peptide (BBS/GRP, fig. 2D), calbindin, calcitonin gene-related peptide (CGRP), cholecystokinin (CCK, fig. 2E), enkephalin (ENK), galanin, γ-amino butyric acid (GABA), neurotensin (NT), neuronal nitric oxide-synthase (nNOS, fig. 4A–C), somatostatin (SS), substance P (SP, fig. 2F), thyrotropin-releasing hormone (TRH), tyrosine hydroxylase (TH), ubiquitin and VGF (a protein induced by

nerve growth factor; [van den Pol et al., 1989]). These cell bodies, in contrast to AVP- and VIP/PHI-positive perikarya, rather lack a distinct topographical localization in the SCN. Oxytocin (OT), gonadotropin- or corticotropin-releasing hormones were not observed or in small numbers of neurons only.

In general, complex synaptic networks between different neuronal subpopulations appear to be present in the rat (and probably in the mammalian) SCN, where multiple modes of interactions were revealed by the dense reciprocal contacts between neurons containing AVP, SS, VIP/PHI and GRP [Romijn et al., 1997].

The transmitter architecture of the SCN is similar among mammals.

From data of several species, the impression is gained that the presence of most of these substances is a general feature of the mammalian SCN [Card and Moore, 1984; van den Pol and Tsujimoto, 1985; van den Pol and Dudek, 1993; Reuss, 1996]. For example, VIP is one of the peptides found in the SCN of all mammalian species investigated. Neuronal nitric oxide synthase (nNOS; see below) is present in similar amounts and somata distribution in the SCN of rats, hamsters, guinea pigs, rabbits, mice and men (see figs. 4A–C), and no mammalian species has been discovered that would lack nNOS in the SCN.

Most of the substances mentioned above were till now identified in the *human* SCN as well. For example, AVP, VIP and TRH mRNA were detected in neurons [Guldenaar et al., 1996; Zhou et al., 1996; Hofman, 1997]. Differences are small and concern neuropeptide Y (NPY) and galanin which were found in SCN neurons in man [Moore, 1989; Gai et al., 1990; Mai et al., 1991], while in the rodent SCN they were observed only in fibers and terminals (NPY see fig. 3B).

To what extent, for example, the presence of NPY- or galanin-mRNA in the mammalian SCN was studied, is presently unknown. In parentheses, it should be noted that a number of methodical parameters render it difficult to draw final conclusions on certain aspects of SCN morphology. For example, day-night differences in the expression of neuroactive substances may not or only inadequately be detected when respective changes were out of phase. A general problem could further be that many studies were conducted using albino rats which are known to exhibit neural defects of the visual system.

In addition to the apparent similarity of SCN structure in mammals, there is no evidence for any clear differences in the anatomical organization of the circadian system between diurnal and nocturnal animals. In a study of the diurnal rodent *Octodon degus*, no differences in the distribution of NPY, VIP, Met-ENK, AVP, SP and serotonin in the SCN were found in comparison to data stemming from nocturnal animals such as rat [Goel et al., 1999]. Even in the same species of grass rats *(Arvicanthis niloticus)*, no features of the SCN were found that would distinguish nocturnal and diurnal animals [Smale et al., 2001].

AVP neuron function is rhythmic.

Some transmitters should now be given some more detailed attention. The view that AVP-immunoreactive (-IR) neurons (fig. 2B) are somehow involved in rhythm generation or mediation was previously supported by several lines of evidence. The number of AVP-IR neurons in the SCN shows a clear day/night rhythm, and AVP-mRNA is distinctly augmented during the day in mice housed under a light/dark (LD) cycle or under continous dark (DD) conditions in the SCN, but not in hypothalamic paraventricular (PVN) or supraoptic nuclei [Jac et al., 2000]. Vasopressin release from dissociated rat SCN cells maintains in a circadian pattern even after several weeks of culture with an increase during the early subjective light-phase [Murakami et al., 1991; Watanabe and Yamaoka, 1997]. The AVP-containing neuronal subpopulation, in particular, shows a distinct circadian rhythmicity in spontaneous firing rate and membrane potential [Schaap et al., 1999]. Despite the apparent importance of AVP in the circadian timing system, the substance was not observed in the

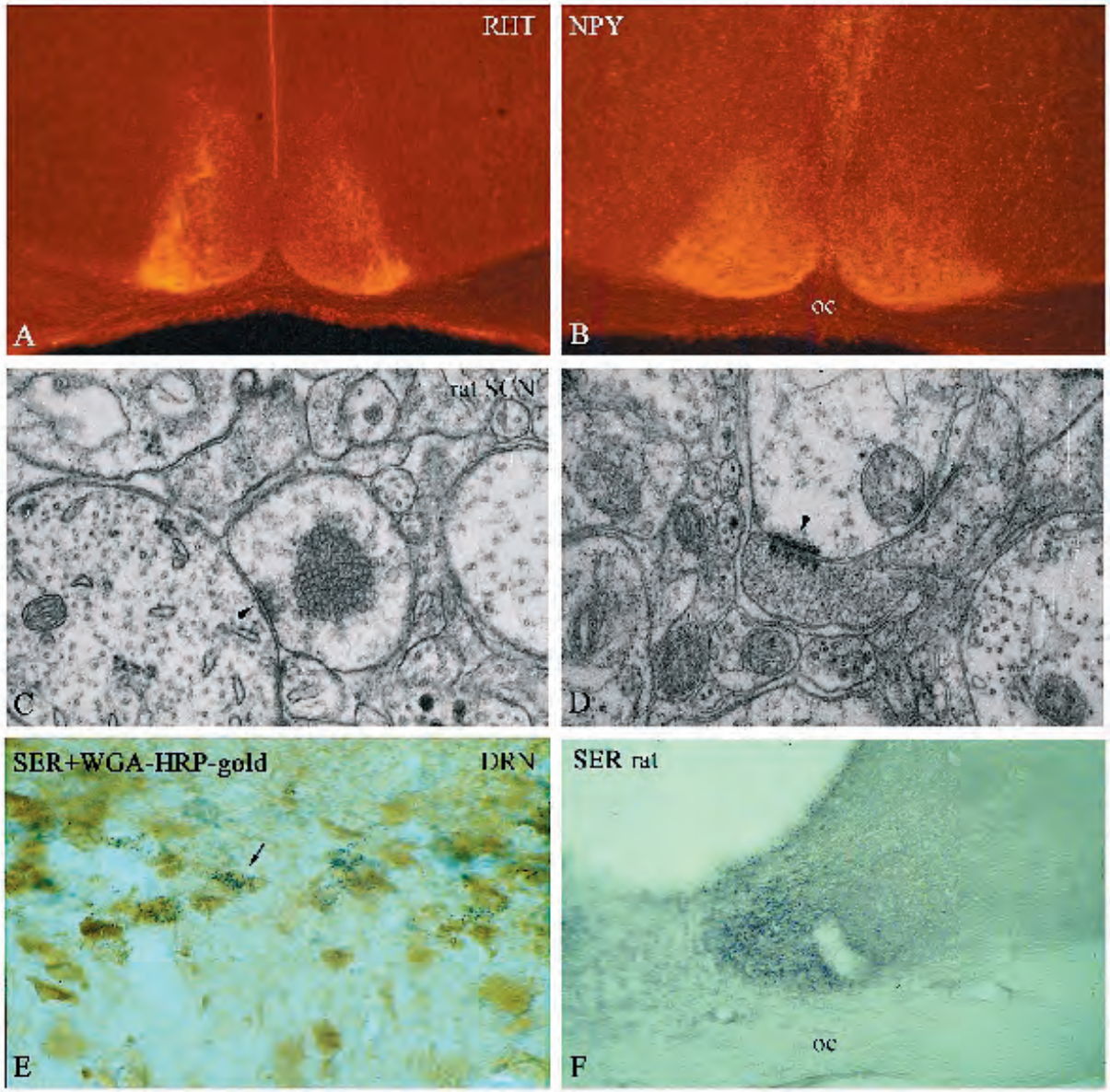

Fig. 3: Topography of three afferent systems in the rodent SCN, i.e., A: the retinohypothalamic tract *(RHT)*, demonstrated by anterograde transport of cholera-toxin B (CTB) injected into the eye, B: of the geniculohypothalamic tract *(GHT)*, demonstrated by NPY-immunoreactivity in adjacent sections of the dwarf hamster brain. C, D: Unidentified synapses (arrowheads) in the rat SCN. E: Retrograde neuronal tracing using WGA-HRP-gold complex injected into the rat SCN resulted in labelled neurons (black dots) in the dorsal raphe nucleus (DRN) that are also serotonin (SER)-immunoreactive (brown staining). The serotonergic projection to the SCN is shown in F. (Panels E, F stem from a cooperation with Hitoshi Kawano, Saga Medical School, Japan.) Abbreviation: *oc* optic chiasm.

SCN of tree shrews *(Tupaia belangeri)* killed in winter [Luo et al., 1995]. Our own studies of animals of the same species showed that the SCN contains some lightly stained AVP-IR neurons when animals were held under summer conditions suggesting that the substance is produced (or stored) to a low degree in the SCN of tree shrews (unpublished observations). However, a crucial role for AVP in rhythm *generation* was not shown yet, instead it was suggested that AVP release from the SCN rather represents the *output* of the circadian pacemaker [Kalsbeek et al., 1998].

Similarly to AVP, other neuroactive substances have recently been shown to exhibit circadian fluctuations in content or in release from the SCN. For example, SS-mRNA in the rat SCN is

augmented during subjective day even in DD [Nishiwaki et al., 1995] and neurons of the SCN release glutamate, aspartate and glycine in a circadian manner, as shown in slice cultures from the rat SCN [Shinohara et al., 1998]. It is unknown so far whether the latter represents the output or may rather be part of internal synchronizing mechanisms.

VIP neurons integrate afferent signals.

VIP neurons (fig. 2C), in contrast, exhibit a diurnal rhythm of mRNA and protein in the rat SCN with nocturnal peaks under LD but not in DD [Kalsbeek et al., 1998]. Their terminals were found to target AVP neurons in particular [Jacomy et al., 1999], and VIP may act directly on AVP neurons in the rat SCN to shift the phase of their rhythmic release [Watanabe et al., 2000]. Interestingly, VIP evoked a small phase-delay of the electrical activity rhythm when applied to the SCN slice in the early subjective night and a large advance at late night [Reed et al., 2001]. Thus the role of VIP neurons probably is to integrate and convey afferent entraining signals such as the photic signal (see below) to pacemaker neurons. Again, VIP cell function was found to be similar in nocturnal and diurnal rodents [Smale et al., 2001].

GABA is a major transmitter of the SCN.

Furthermore, there is multiple evidence that amino acid transmitters are of major importance for SCN function. GABA, thought to be the principal neurotransmitter in the SCN [Moore and Speh, 1993], is found in most if not all neurons, and the mRNA for its synthesizing enzyme GAD is coexpressed with AVP-, VIP- or SS-mRNA in many SCN neurons [Tanaka et al., 1997]. In addition, boutons in the rat and mouse SCN contain both GABA and VIP or AVP [Castel and Morris, 2000]. It was shown that GABA can promote AVP release from rat SCN slices [Isobe and Nishino, 1997]. Its functions further include the phase-shift and synchronization of electrical activity of dispersed SCN neurons [Liu and Reppert, 2000; Shirakawa et al., 2000] which otherwise exhibit a relatively wide range of spontaneous firing periods in vitro (i.e., 20–28 hs; see [Honma et al., 1998]). Evidence was found that GABA acts as excitatory transmitter during subjective day but as inhibitory transmitter during the night [Wagner et al., 1997], and a $GABA_B$ receptor agonist advanced the phase of SCN electrical activity at daytime and delayed it at night [Biggs and Prosser, 1998]. However, recent electrophysiological recordings from the rat SCN in vitro revealed that GABA may function as inhibitory transmitter during both subjective day and night [Gribkoff et al., 1999]. In the SCN, GABA is not synthesized in a circadian but in a diurnal pattern, since GAD mRNA in the rat SCN is augmented at daytime under LD but not in DD [Huhman et al., 1996; Huhman et al., 1999]. Rather, it appears possible that a circadian fluctuation in GABA release is present within the SCN (although not yet shown). This may synchronize rhythmically active neurons, as was suggested by [Liu and Reppert, 2000], and increase the circadian change in firing frequency [Strecker et al., 1997]. In the discussion of GABA actions in the SCN, however, the possible impact of extrinsic GABA sources such as the IGL (see below) should also be considered. There is, finally, also evidence that the release of GABA from SCN terminals is implicated in further transmission of light information [Kalsbeek et al., 1999].

Nitric oxide serves as a gaseous transmitter in the SCN.

Another neuroactive substance that appears crucial for SCN function is nitric oxide (NO). Examples of NO-producing neurons in the SCN of rat, guinea pig and man are given in figs. 4A–C. Our initial demonstration of neurons exhibiting neuronal nitric oxide synthase (nNOS)-immunoreactivity in

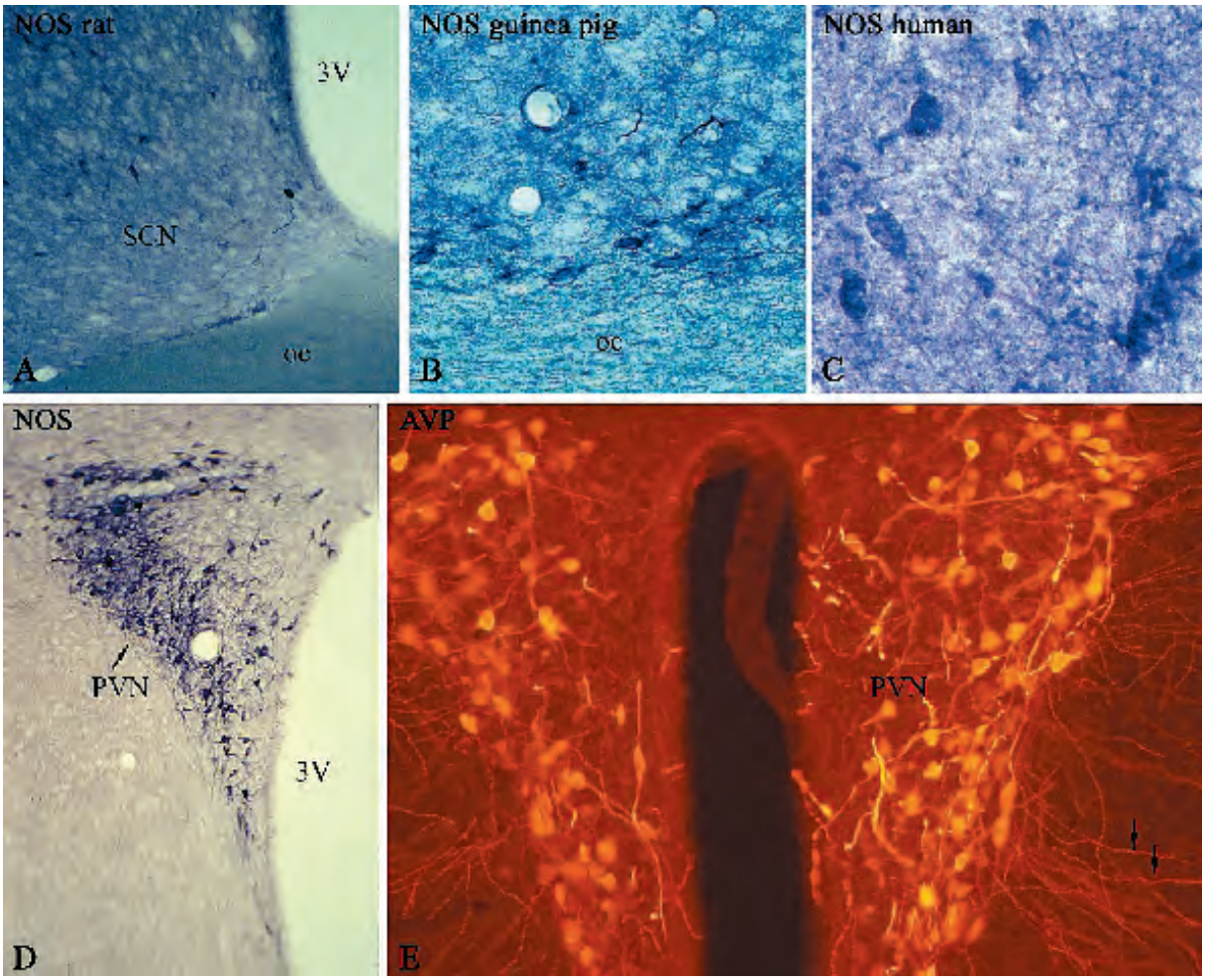

Fig. 4: Subpopulation of SCN neurons containing nitric oxide-synthase (NOS; demonstrated here by the NADPH-diaphorase method) as seen in the A: rat, B: guinea pig, and C: human brain. D: Distinct NADPH-staining of neurons is also present in the paraventricular nucleus of the rodent hypothalamus (PVN), which is also characterized by arginine-vasopressin (AVP)-immunoreactive neurons (E). Note immunoreactive fiber systems (arrows in E) that project to the posterior pituitary and the spinal cord autonomic regions. Abbreviations: *3V* third ventricle, *oc* optic chiasm.

the SCN of dwarf hamster and rat [Decker and Reuss, 1994; Reuss et al., 1995; Spessert et al., 1995] was followed by studies confirming the presence of nNOS-containing cell bodies and fibers in the rat and golden hamster SCN [Chen et al., 1997; Caillol et al., 2000]. The nNOS neurons belong to the photically activated cells in the SCN as revealed by anterograde RHT tracing [Decker and Reuss, 1994] and by the light-induced expression of the immediate-early gene protein c-Fos in these cells [Castel et al., 1997]. In addition, superoxide dismutase, thought to prevent superoxide-mediated inactivation of NO, was found in distinct regions of the mouse brain including the SCN, in particular in the ventrolateral subdivision [Oury et al., 1999] where most nNOS-neurons were observed. An additional source of NO in the SCN region may be astrocytes since a respective group of cells positive for endothelial NOS (eNOS) was found in rat and hamster [Caillol et al., 2000].

The functional impact of NO in the circadian timing system was demonstrated previously by findings that blocking NO production disrupts light transmission to the SCN [Amir, 1992], that NO synthesis is required for phase changes of SCN electrical activity [Watanabe et al., 1994] and that the enzymatic activity of nNOS, as determined by L-citrulline synthesis, is present in the rat SCN [Ding et al., 1994]. Furthermore, light-induced phase-advances of activity rhythms were attenuated upon intracerebroventricular application of L-NAME (a drug that blocks all three isoforms

of NOS in hamsters [Weber et al., 1995]. On the other hand, nNOS or eNOS knockout mice did not differ from wild-type animals in their ability to entrain to light/dark-cycles, leading to the assumption that neuronal and endothelial NO may not be necessary for photic entrainment [Kriegsfeld et al., 1999]. It is, however, probable that developmental mechanisms compensate the lack of NOS. For example, it was suggested that other guanylate cyclase-targeting substances such as carbon monoxide may compensate deleted NO-formation [Good, 1996]. Since compensational mechanisms seem not unusual in knock-out animals, the study of NO knock-outs may bear only limited validity. Blocking NO-action in intact animals, in contrast, interrupt the light-triggered cascade of glutamate release from retinal terminals in the SCN, subsequent N-methyl-D-aspartate (NMDA) receptor activation, intracellular increase of calcium, activation of nNOS, augmented production of cyclic guanosine monophosphate (cGMP), activation of protein kinase C and phosphorylation of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and the expression of immediate early genes [Ding et al., 1997] cf. [Golombek et al., 2000].

The compartments of the suprachiasmatic nucleus are functionally diverse.

There is evidence that the SCN is composed of several compartments which are morphologically and functionally heterogeneous and may work as independent oscillators. Previous studies had already demonstrated the functional diversity of the ventrolateral and dorsomedial regions when the use of retrograde transsynaptic viral tracing in rats recently revealed the separation into a “core”, i.e., the ventral part close to the optic chiasm, and a “shell”-region (the dorsal region surrounding the core; see [Leak et al., 1999]). Their functional diversity was suggested by the findings that these regions exhibit separate projection patterns as the core projects mainly to the shell and to intrahypothalamic targets, while the shell innervates intra- and extrahypothalamic regions to other hypothalamic sites [Leak and Moore, 2001]. In addition, the afferents terminate differently in the SCN, as direct and indirect visual inputs project predominantly to the core and non-visual mainly to the shell. Concomitantly, the circadian and the light-induced expression of *fos* family genes is different between these subdivisions of the rat SCN [Sumova et al., 1998; Schwartz et al., 2000], and the circadian oscillation of *rPer1* and *rPer2* clock genes (see below) was observed predominantly in the dorsomedial part, while nocturnal light exposure reduced expression of their mRNA mainly in ventrolateral neurons [Yan et al., 1999].

Functional studies as well demonstrated that locomotory activity and body temperature of rats are controlled by separate oscillators [Wideman et al., 2000]. Although their identity is largely unknown, studies utilizing SCN lesion and transplantation showed that the light-regulated (and RHT-targeted, see below) calbindin-containing subpopulation of the medial SCN is both necessary and sufficient for the control of circadian locomotory activity of hamsters [Silver et al., 1996b; LeSauter and Silver, 1999]. This region, however, did not exhibit rhythmic clock gene expression suggesting a functional compartimentation of the SCN into rhythmic and nonrhythmic parts [Hamada et al., 2001].

An interesting observation was recently described by [Jagota et al., 2000]. Electrophysiological recordings showed that the daytime peak of spontaneous activity found in frontal slices of the hamster SCN was separated into a morning- and an evening-peak when the SCN was sliced horizontally. Considering the functional separation of the nucleus into core and shell (see above) and their different afferent and efferent projection patterns, it appears likely that the preparations by [Jagota et al., 2000] may have disrupted intranuclear connections or have missed parts of the dorsomedial extent of the SCN or of the subparaventricular zone. The results, in any case, underline the presence of functionally different SCN subdivisions and reveal the importance of their afferent and efferent connections for clock function.

Finally, both SCN may even work as independent components of the clock mechanism since, in hamsters exhibiting a split of locomotory activity when housed in constant light, the left and the right SCN oscillate in antiphase, as demonstrated by expression of AVP, *c-fos* and clock genes *per1–3* [de la Iglesia et al., 2000].

The balance of the clock lies in its genes.

At least at this point the questions arise as to where and how the SCN-endogeneous rhythms are produced, i.e., which molecular mechanisms underly the rhythmic properties of SCN function. What actually is the "balance" of the clock? A number of clock genes have now been identified in mammals that are partly identical to those found in lower animals and plants, e.g., *Clock, period, Bmal1* and *cryptochrome*. The wealth of studies investigating these genes, their proteins and the various interactions between them are not among the subjects of the present review. Instead, the reader is referred to competent reviews (e.g., [Bunney and Bunney, 2000; Hastings and Maywood, 2000; Sancar, 2000; Whitmore et al., 2000]). A functional connection of the clock genes to a metabolic rhythm is provided by the finding that heterodimers of two clock gene proteins (CLOCK-BMAL1) activate vasopressin gene transcription in the SCN [Jin et al., 1999].

The present view is that clock genes are rhythmically transcribed and translated into proteins, and that these are involved in transcriptional feedback loops with positive and negative limbs, but it is unclear so far how molecular rhythms are translated into, for example, oscillating electrical activity. However, the pivotal role of clock genes also for the human circadian behavior was recently demonstrated when it was found that a mutation of the *hPer2* phosphorylation site is present in patient suffering from the familial advanced sleep phase syndrome [Toh et al., 2001].

Afferent pathways to the SCN provide photic and non-photic inputs.

In the absence of external time cues, endogeneous rhythms free-run, i.e., they switch from a 24-h period to a circadian rhythm of approximately 24.3-h period. There is general agreement that light is the most powerful factor that resets the clock in mammals. From a large number of structures providing input pathways to the SCN, three seem to be of major importance. Neuronal tracing, lesions and physiological studies revealed that major inputs stem from the retina, intergeniculate leaflet (IGL) and raphe nuclei. They all provide photic input, although the impact of the IGL and raphe includes or may serve primarily non-photic input. These afferents are topographically organized within the SCN but are anatomically overlapping, and functional interactions between these inputs exist. Some other sites are the sources of additional paths to the SCN but their functional implications are not as well investigated.

Retinal input to the SCN times the clock.

Retinal afferents to the SCN form the major part of the *retinohypothalamic tract* (RHT; see fig. 3A) which is known for more than one hundred years (cf. [Mai, 1978]). In 1888, Obersteiner published his observation of an "opticus root not to overlook" that enters the gray substance surrounding the third ventricle [Obersteiner, 1888]. On the basis of fiber tract studies in intact brains and experimental material, the RHT was later characterized by some authors, for example Frey [1937], Knoche [1956] and Blümcke [1958]. However, it was only in the early seventies of the last century that the existence of retinal projections to the hypothalamus was confirmed by means of modern neuroanatomical methods such as autoradiography and electronmicroscopy [Hendrickson et al., 1972; Moore and Lenn, 1972]. These studies demonstrated that the RHT leaves the optic nerve mainly in the anterior part of the optic chiasm, then turns dorsally to terminate in the suprachiasmatic nucleus where it predominantly innervates the ventrolateral part. This projection provides the morphological substrate for the regulatory effects of light on the SCN and thus on the circadian timing system; its primary function is to connect the SCN to the photic environment and thus to reset and synchronize the circadian clock.

Several neuronal cell groups of the SCN are targeted by the RHT.

Combined tracing and immunocytochemistry in rodents revealed that several neuronal subpopulations of the SCN are targets of the RHT. By electron microscopy, the termination of retinal afferents on VIP cells was shown in rats [Tanaka et al., 1993]. We previously reported light-microscopical evidence for the innervation of substance P- or nNOS-containing cells in the hamster SCN [Decker and Reuss, 1994; Reuss et al., 1994]. Light application resulting in c-*Fos* induction also showed that photically activated regions of the SCN accomodate neurons immunoreactive for AVP, VIP and NOS [Castel et al., 1997]. Further neurons contacted by the RHT were characterized by GRP- or calbindin-immunoreactivity (for further details and references, see [Reuss, 1996]).

In man, retinal afferents were characterized by comparing brains from cadavers with a lesion of one optic nerve with intact brains [Sadun et al., 1984] or by using the carbocyanin DiI as tracer [Friedman et al., 1991]. A recent postmortem tracing study of the human RHT revealed that retinal fibers innervate the ventral SCN with decreasing density from anterior to posterior, that only few projections reach the dorsal SCN, and that fibers synapse with NT-, VIP- and AVP-IR neurons [Dai et al., 1998a].

The present view concerning the optic system is that the RHT is functionally and anatomically separated from the primary optic tract. This is deduced from a number of major differences between the two pathways, starting with the finding that retinal ganglion cells projecting to the SCN are few in number. Functionally, the visual projections to lateral geniculate and optic tectum process *spatial* aspects of image formation and detection of motion, whereas the *temporal* analysis of light and the synchronization of endogenous rhythms with the environmental cycles of light and dark is provided by the RHT. Anatomically, the visual system exerts a strong contralateral predominance, while the RHT displays a bilateral projection pattern. However, differences with regard to laterality between species exist. For example, while RHT projections are rather symmetrical in hamsters [Johnson et al., 1988; Reuss et al., 1994], they show a greater contralateral projection in rat [Johnson et al., 1988; Levine et al., 1991] and ipsilateral predominance in primates including man [Sadun et al., 1984; Friedman et al., 1991]. However, the retina-SCN projection does not seem to accord with the basic principle of mammalian primary visual organisation, i.e., the extent of optic fiber decussation in the chiasm is inversely related to the degree of frontal orientation of the optical axes of the eyes ("law of Newton-Müller-Gudden"; [Magnin et al., 1989]). Contrary to the primary optic tract, the RHT lacks retinotopy: the injection of a retrograde tracer into the SCN labelled cell bodies in the retina without a topographical order in rat, hamster and mouse [Pickard, 1985; Youngstrom et al., 1991; Moore et al., 1995; Provencio et al., 1998]. However, nocturnal light (500 lux) exposure of the nasal part of the human retina is superior to the lateral part in reducing salivary melatonin concentration [Visser et al., 1999], and the comparison of the upper and lower visual fields showed that illumination of the upper visual field exerted a greater suppression [Lasko et al., 1999]. One possible explanation for these findings would be a relative concentration of retinohypothalamic neurons in the lower medial part of the human retina.

The major differences, however, between primary optic tract and RHT probably are location and type of photopigments.

Which retinal pigment mediates circadian photoreception?

There was little reason to doubt that RHT neurons receive their information primarily from photoreceptors containing the known group of vitamin A-based opsins (rhodopsin and three color opsins), and that these were responsible for all photosensory responses in the eye. This view, however, was questioned some years ago by the finding that bright light suppressed melatonin secretion in blind patients [Czeisler et al., 1995]. It was then found that transgenic mice in which rods and cones were selectively destroyed still exhibited phase-shifts of locomotor activity and suppression of melatonin synthesis by light of 509 nm wavelength [Freedman et al., 1999; Lucas et al.,

1999]. Very recently, the most potent wavelengths to suppress human melatonin synthesis were found to be in the region of 440–480 nm [Brainard et al., 2001; Thapan et al., 2001]. Quite surprising, these findings suggested the presence of additional retinal photoreceptors and/or pigment(s)—different from the scotopic and photopic visual system—that regulate the circadian clock, perhaps in concert with rods and cones (cf. [Lucas et al., 2001; Young, 2002]).

From a number of vertebrate photopigments, melanopsin stands out as a potential "circadian" photoreceptor (cf. [von Schantz et al., 2000]). In monkeys and mice, melanopsin is not expressed in rods and cones but is present in amacrines and few retinal ganglion cells. These cells exhibit direct intrinsic photosensitivity that is independent from photoreceptor input and peaks at about 480 nm [Berson et al., 2002; Hattar et al., 2002]. Interestingly, combined retrograde tracing and in situ hybridization in rats had shown that the majority of RHT neurons produce melanopsin [Gooley et al., 2001]. All these melanopsin-containing RHT ganglion cells also use PACAP, a well-known transmitter of the RHT (see below) [Hannibal et al., 2002].

Some melanopsin-containing ganglion cells additionally project to brainstem regions mediating the pupillary response [Hattar et al., 2002]. Interestingly in melanopsin-knockout mice, the pupillary light reflex was unaltered at low irradiances but was incomplete at high irradiances [Lucas et al., 2003]. The authors suggested that the melanopsin-associated and the rod/cone-based systems may be of complementary function. There are also hints against the view that melanopsin is the only circadian photoreceptor, since melanopsin knockout mice showed typical, although reduced, light responses such as entrainment and phase-shifting [Panda et al., 2002; Ruby et al., 2002]. A candidate substance for such an additional inner retinal photopigment is the clock gene product cryptochrome, since also mice lacking cryptochromes showed reduced pupillary light responses [Van Gelder et al., 2003]. Taken together, these findings reveal that photosensitive substance classes such as the opsins (including melanopsin) and others (including cryptochromes)—located in different retinal cell types—work together to compile non-visual light responses.

Glutamate and PACAP are primary transmitters of the retinohypothalamic tract.

With regard to the transmitter substances of the RHT, the current view is that two primary transmitters mediate retinal input to the SCN, i.e., glutamate (cf. [Ebling, 1996]) and pituitary adenylate cyclase-activating peptide (PACAP).

Glutamate. Recent data confirm that glutamate is the primary transducer of photic information to the SCN. The substance increases the electrical activity of SCN neurons and thus exerts the same effects as stimulation of the optic nerve or as photic stimulation of the eyes. Microinjections of the glutamate receptor agonist NMDA directly into the SCN phase-delayed hamster wheel-running activity [Mintz and Albers, 1997], and glutamate bath application phase-delayed the spontaneous single-unit activity peak-time [Franken et al., 1999]. NMDA microinjection into the SCN resulted in time-of-day-dependent phase-shifts also suggesting that glutamate mimics the actions of light on the clock [Mintz et al., 1999].

Glutamate receptors of types GluR1–7 and NMDAR1 were found in high density in the rodent SCN [Stamp et al., 1997]. They appear to be under circadian control since SCN neurons bearing the AMPA subtype of the glutamate receptor are augmented in number during the subjective day in animals held in constant dark [Chambille, 1999]. In addition, electrophysiological recordings showed that during night, more cells are responsive to light than at daytime in the hamster SCN in vivo and that the activation of the SCN by retinal afferents involve both NMDA and non-NMDA receptors [Cui and Dyball, 1996]. Likewise, bath application of glutamate or its agonists NMDA and AMPA showed excitatory effects on the electrical activity of rat SCN neurons in vitro [Schmahl and Böhmer, 1997]. Glutamate receptors responding to AMPA are colocalized with VIP, PHI and GRP suggesting that these peptidergic neurons are under the influence of glutamatergic afferents from retina or other sites [Peytevin et al., 2000].

PACAP. The second putative transmitter of the RHT, PACAP, is present in several retinal cell

types including the ganglion cells [Seki et al., 2000], and PACAP and glutamate are co-stored in a subset of retinal ganglion cells and in the retinorecipient regions of the SCN [Hannibal et al., 2000]. In the SCN of colchicine-treated rats, however, large amounts of PACAP-IR cell bodies were found [Piggins et al., 1996] suggesting that the substance is produced also in the SCN which may account for the circadian changes in its SCN content in constant darkness [Cagampang et al., 1998] and for the observation that its levels in the rat SCN were low during the light period and high during dark [Fukuhara et al., 1997].

PACAP was found to phase-shift the circadian rhythm during the subjective day in vitro [Hannibal et al., 1997]. In a recent study, a dose-dependent effect was found, i.e., PACAP resets the clock during the subjective night when applied at small doses (< 1 nmol/l), and during the subjective day at doses > 10 nmol/l [Harrington et al., 1999]. There is also evidence for a daytime-specific effect of PACAP on the phase of hamster wheel-running rhythm [Piggins et al., 2001a]. The functions of the peptide further include the activation of phosphorylation of CREB in the SCN during late subjective day, and that may be inhibited by melatonin [Kopp et al., 1997]. The interaction between glutamatergic and GABA-ergic afferents in the SCN seem to imply that glutamate activation is graded by PACAP so that the relative strengths of both signals encode the amplitude of response [Chen et al., 1999]. It was, however, also suggested that during light/dark transition the SCN changes dominant sensitivity from PACAP to glutamate [von Gall et al., 1998].

Some other substances may serve as transmitters of the RHT.

In addition to glutamate and PACAP, further RHT transmitter candidates are imaginable. The possible involvement of N-acetylaspartylglutamate (NAAG; a glutamate precursor) and of NPY has been discussed previously (cf. [Reuss, 1996]). NAAG, a neuron-specific dipeptide found in retinal ganglion cells, appears to act excitatory. Its amount in the SCN was drastically reduced upon optic nerve lesion, but there is also evidence for an additional non-retinal, unknown source of NAAG in the SCN [Moffett et al., 1990]. The present knowledge about NAAG function is sparse; it may just be reduced to glutamate [Castel et al., 1993].

Although it is known that gamma-type retinal ganglion cells are NPY-IR [Hutsler et al., 1993] and provide the RHT in cats [Murakami et al., 1989], the combination of both results provides at best indirect evidence that also NPY acts as an RHT transmitter. The question still must be addressed by combined retrograde tracing and immunohistochemistry.

Another substance found in retinal ganglion cells and in the SCN is GRP [McKillop et al., 1988]. It is unknown whether GRP neurons project to the SCN or whether the substance rather stems from intrinsic neurons. However, GRP phase-shifts the electrical activity of the rat SCN in vitro in a phase-dependent manner [McArthur et al., 2000]. In addition, lesion experiments have revealed that SP-containing ganglion cells in the rat retina project to the ventrolateral part of the SCN [Takatsuji et al., 1991] and electrophysiological investigations demonstrated that SP is an excitatory neuromodulator contributing to the expression of both NMDA- and non-NMDA-receptor-mediated components of RHT transmission, and that SP and glutamate work as agonist in series, SP upstream of glutamate [Kim et al., 1999; Kim et al., 2001]. SP, however, is produced not only by retinal ganglion cells but also by SCN neurons [Reuss and Bürger, 1994]. Moreover, recent data indicate that SP is a neurotransmitter of the RHT in rat, but not in hamster where SP is intrinsic to the SCN [Piggins et al., 2001b]. It appears also possible that GRP, SP and other substances discussed (e.g., acetylcholine) may act presynaptically in the SCN to modulate transmitter release from retino-afferent terminals (cf. [Ebling, 1996]).

Minor projections of the RHT reach beyond the SCN.

Many studies revealed that, apart from projections to the SCN, the RHT also innervates other hypothalamic sites. A retinal projection to the paraventricular nucleus (PVN) has long been supposed. The presence of a (sparse) paraventricular projection had previously been suggested by experiments on dog and rabbit in each of which a respective lesion was experimentally induced [Knoche, 1956]. In man, innervation of magno- and parvocellular parts of the PVN were described [Schaechter and Sadun, 1985] and have recently been observed upon anterograde neuronal traing in golden hamsters, but not in Djungarian hamsters [Youngstrom et al., 1987; Reuss et al., 1994]. Weak projections of the RHT have been found also to the lateral hypothalamus of rat [Mai, 1979; Riley et al., 1981], and to anterior, ventro- and dorsomedial hypothalamus of golden hamsters [Youngstrom et al., 1991]. Innervation of the supraoptic hypothalamic region, described as "tractus supraoptico-hypothalamicus" by Greving as early as 1928 [Greving, 1928], was later substantiated when some lateral projections to the human ventromedial supraoptic nucleus were found [Dai et al., 1998a]. Experimental studies using anterograde neuronal tracing in hamsters yielded similar results [Youngstrom et al., 1991; Reuss et al., 1994]. A tracing study in rats [Levine et al., 1994] has demonstrated that the supraoptic nucleus receives both direct and indirect innervation from the RHT, the latter via the peri-supraoptic terminal field, and that strong projections reach its perinuclear zone [Cui et al., 1997a]. This is an impression that was also gained by tract-tracing in the diurnal rodent *Arvicanthis niloticus* [Smale and Boverhof, 1999], in the common marmoset *Callithrix jacchus* [Costa et al., 1999] and in the tree-shrew *Tupaia belangeri* [Reuss and Fuchs, 2000].

These projections may provide the morphological basis for the photic effects on mammalian physiology that are not mediated by the circadian timing system. Recent studies then showed retinal input to the median preoptic region of *Phodopus sungorus* [Reuss and Decker, 1997] and to the sleep-active, galaninergic ventrolateral preoptic nucleus of the rat [Lu et al., 1999].

The SCN receives non-retinal input from the intergeniculate leaflet, raphe nuclei and other sites.

A number of brain sites are now known to provide afferent fibers to the SCN. These include hypothalamic and thalamic regions, parts of the limbic system and of the brain stem. In addition, many neuroactive substances of unknown origin have been found to influence the SCN. Some of these sites and substances are addressed to in the following.

The intergeniculate leaflet integrates photic and non-photic information.

A major visual pathway connects the retina to the lateral geniculate nucleus. Between its dorsal and ventral parts lies the intergeniculate leaflet (IGL) which receives strong retinal input. There is general agreement that the IGL projects to the SCN by the geniculohypothalamic tract (GHT) providing a secondary, indirect photic input that terminates mainly in the ventral SCN. Indeed, the RHT and GHT display a partial overlapping in the SCN. Since there is evidence that the IGL mediates photoperiodic responses as well as non-photic entrainment of circadian rhythms [Menet et al., 2001]; cf. [Harrington, 1997], the functions of the IGL may cover the integration of photic and non-photic information. The primary transmitter of the GHT projection seems to be NPY (see fig. 3B). It is believed that NPY acts directly on pacemaker neurons as revealed by electrophysiological recordings from the hamster SCN in vitro [Biello et al., 1997]. Since NPY seems necessary for the adjustment of the endogenous circadian rhythm to changing light/dark cycles [Albers and Ferris,

1984], this secondary visual input to the SCN may enable it to adapt temporally to phase changes such as jet-lag.

Some authors considered that in higher primates including man the GHT projection is differently organized than in rodents (cf. [Moore, 1989; Chevassus-au-Louis and Cooper, 1998]). Although NPY neurons are present in the pregeniculate nucleus, the homologue of the rodent IGL, NPY fibers in the SCN are sparse. It is therefore suggested that the GHT may use different neurotransmitters. Candidates are GABA and ENK that were demonstrated in the rat and hamster GHT [Moore and Speh, 1993; Morin and Blanchard, 1995]. The presence and role of GABA in the SCN was discussed above. ENK was found in cell bodies in the IGL and in fibers in the SCN of many mammalian species. Recently, a high density of δ-opioid receptors which have the highest affinity for ENK was detected in the hamster SCN [Byku et al., 2000], and a respective agonist phase-advanced hamster wheel-running activity late in the subjective day [Byku and Gannon, 2000]. IGL neurons projecting to the SCN are characterized by GABA/NPY-immunoreactivity and those projecting to the contralateral IGL by GABA/ENK-immunoreactivity [Moore and Speh, 1993; Moore and Card, 1994]. The GABA-ergic fibers of the rat SCN were found to innervate VIP cells and to converge with serotonergic fibers [Francois-Bellan and Bosler, 1992].

The re-investigation of the IGL-SCN projection recently showed that additional projections exist to hypothalamic paraventricular, subparaventricular, periventricular and medial preoptic regions including those to neurons that produce dopamine [Horvath, 1998], thus providing a pathway by which visual signals may influence the neuroendocrine system independently from the SCN.

The raphe nuclei provide serotonergic input to the clock.

The second important non-retinal SCN afferent is the serotonergic projection stemming from the raphe nuclei (see fig. 3F). Its major function most probably is the modulation of pacemaker responses to light. Since the raphe nuclei receive retinal afferents in rats, gerbils, tree shrews and Chilean degus [Fite et al., 1999; Reuss and Fuchs, 2000; Fite and Janusonis, 2001; Su and Liu, 2001], the raphe-retina projection may be regarded as another indirect photic input to the clock. Previous retrograde tracing studies showed that the midbrain dorsal and median raphe nuclei (DRN, MRN) project to the SCN. According to retrograde tracing experiments in rats [Moga and Moore, 1997], additional input stems from the pontine median raphe. The major input was found to originate in the MRN, while minor input stems from the lateral group of the DRN [Moga and Moore, 1997]. Other studies revealed that also the medial part of the DRN innervates the SCN by a serotonergic projection ([Kawano et al., 1996], see fig. 3E) and that the MRN only lightly innervates the SCN [Vertes et al., 1999]. However, electrical stimulation of either the DRN or MRN attenuated light-induced Fos protein staining in the SCN and advanced the phase of running wheel activity in golden hamsters [Meyer-Bernstein and Morin, 1999]. As shown in vitro, serotonin advances the phase of the pacemaker during the day and delays it at night, similar to GABA (cf. [Prosser, 2000]). In addition, there is evidence that serotonin regulates SCN neurons by both pre- and postsynaptic inhibitory mechanisms [Jiang et al., 2000]. The serotonergic fiber system densely innervates mainly the ventral aspects of the caudal SCN, synapses on VIP neurons and also interacts with fibers forming the GHT [Kiss et al., 1984; Bosler and Beaudet, 1985; Guy et al., 1987].

Input from other, partly unidentified sources contribute to clock regulation.

Recent reports demonstrating binding sites pointed to some further substances (i.e., histamine, acetylcholine, leptin, sexual steroids) and sites that may exert influence on the SCN.

Combined retrograde tracing and immunohistochemistry conducted in female Syrian hamsters revealed input from preoptic area and amygdala neurons bearing oestrogen receptors suggesting

that sexual steroids influence the SCN via afferent neurons [de la Iglesia et al., 1999].

Retrograde and anterograde neuronal tracings showed that also various hypothalamic nuclei as well as other sites such as the infralimbic cortex, lateral septal nucleus, and thalamic PVN provide afferents to the rat SCN [Moga and Moore, 1997] and that some of these may use excitatory amino acids as transmitting substance [Moga and Moore, 1996]. As mentioned earlier, glutamate or its agonists showed direct excitatory effects on the electrical activity of rat SCN neurons in vitro [Schmahl and Böhmer, 1997].

The data regarding the biogenic amine histamine are somewhat controversial. Data from one group suggested that histamine, present only in scarce fibers in the SCN, does not play a major role in the regulation of circadian rhythms [Scott et al., 1998]. On the other hand, histamine affected the spontaneous electrical activity of medial SCN neurons [Stehle, 1991], prominent histamine H_2 receptor expression was recently detected in the rat SCN [Karlstedt et al., 2001], and it was found that the substance delayed the time of peak-firing neurons in a slice preparation of the hamster SCN by acting on NMDA receptors [Meyer et al., 1998]. A recent review [Jacobs et al., 2000] brought the substance back into discussion suggesting that histamine is the final neurotransmitter mediating entrainment to the LD cycle, that the substance is released in the SCN upon NMDA receptor activation, and that this is under the control of GHT-dependent inhibition of glutamate release.

While containing no intrinsic cholinergic neurons, the SCN receives synaptic input from cholinergic afferents that may stem from intrahypothalamic sources and/or from the basal forebrain and mesopontine tegmentum [Kiss and Halász, 1996]. Cholinergic receptors of nicotinic and muscarinic types have been demonstrated previously (reviewed in [Reuss, 1996]). Nicotinic receptor mRNA of the $\alpha 7$ subtype was recently found to be the most abundant type in the rat SCN, where nicotine may phase-shift the circadian system (cf. [O'Hara et al., 1998]).

In addition, receptors for leptin were found in the human SCN [Couce et al., 1997]. This substance, secreted by adipocytes as the protein product of the *obese* gene, has a number of functions including the attenuation of food intake and augmentation of energy expenditure [Rayner and Trayhurn, 2001]. Leptin application in rats activated hypothalamic neurons that project to the preganglionic sympathetic column of the spinal cord [Elias et al., 1998; Elmquist et al., 1998]. The sympathetic system itself inhibits the leptin system [Rayner and Trayhurn, 2001]. In rats and humans, leptin levels peak at night, and a circadian pattern was still found in humans undergoing continuous enteral feeding [Saladin et al., 1995; Simon et al., 1998]. These findings and the observation that dwarf hamsters were more responsive to leptin application under short-day than under long-day conditions [Atcha et al., 2000; Klingenspor et al., 2000] revealed that leptin-associated mechanisms are under the influence of the circadian system. Recent data suggested that the SCN, via sympathetic input to adipocytes, generates the diurnal leptin signal [Kalsbeek et al., 2001] which may provide a means by which the circadian system regulates food intake and energy expenditure.

Although the general pattern of afferents reveals that the sites receiving photic input (i.e., retina, IGL, raphe) project to the ventral SCN while others (e.g., hypothalamic, limbic) to its dorsal part, the situation is further complicated by considering the various interactions of different afferent systems in the SCN. This was revealed by the finding that serotonin ($5\text{-}HT_{1B}$) receptors are present on structures of two other afferent systems (i.e., RHT and GHT) in the rat SCN [Manrique et al., 1999; Pickard et al., 1999]. Furthermore, application of a respective agonist reduced the amplitude of glutamatergic potentials in the SCN evoked by optic nerve stimulation [Pickard et al., 1999] suggesting that these receptors may reduce retinal input to the SCN. Finally, NPY, which itself may phase-shift the clock, blocks GABA-induced phase-shifts [Biggs and Prosser, 1999], while NPY and melatonin, respectively, may block serotonergic phase-shifts of the rat SCN in vitro (cf. [Prosser, 2000]).

It is reasonable to conclude that all these pathways, viz. the RHT, GHT, and raphe-SCN projection as well as other unexplored or unidentified tracts, work together in adjusting the intrinsic neuronal rhythm of the SCN.

Do diencephalic or brain stem sites provide feedback to the retina?

An interesting question is whether retinal function is regulated by its target structures. In contrast to non-mammalian vertebrates in which a retinopetal projection is well-established, only few data were available that point to efferent fibers innervating the mammalian retina. Of particular interest would be a feedback from hypothalamic sites directly involved in the circadian timing system.

Some early studies using tracer techniques described the existence of a retinopetal projection stemming from the ventral hypothalamus in dog [Terubayashi et al., 1983], from the premamillary and posterior hypothalamic area in monkeys, cats, guinea pigs and rabbits [Labandeira-Garcia et al., 1990] or from the medio-lateral preoptic area in rat [Schütte, 1995]. In addition, we recently showed that retrogradely labelled neurons were occasionally found in basal hypothalamic regions outside the SCN upon Fluorogold injection into the vitreous body of the *Phodopus* eye [Reuss and Decker, 1997]. Another study, using tracer and lesioning methods, demonstrated VIP-ergic fibers in the optic nerve arising from the hypothalamus [Fogel et al., 1997].

With regard to the circadian timing system, it should be noted that there is multiple evidence for neurons of the raphe nuclei providing a serotonergic innervation of the retina as demonstrated by various methods including retrograde tracing and immunohistochemistry [Villar et al., 1987; Labandeira-Garcia et al., 1990; Reperant et al., 2000]. Finally, other visual and non-visual sites not directly involved in the circadian circuitry project back to retina.

Is there extraocular light perception?

In 1998, a surprising report described that intense light exposure of the cutaneous area behind the knee can shift the circadian rhythms of human body temperature and melatonin secretion [Campbell and Murphy, 1998]. This was discussed with devotion by the public press but was not verified by the scientific community. For example, light-exposure of the popliteal region did not suppress plasma melatonin in humans [Lockley et al., 1998; Hebert et al., 1999], and no phase-shifting effects of light on the rhythms of melatonin, cortisol and thyrotropin were observed when the human abdomen and chest was light-exposed [Lindblom et al., 2000]. With one exception [Jagota et al., 1999], rodent data demonstrated that the eyes are necessary for circadian and visual photoreception. Most researchers of the circadian field therefore suppose—as pointed out by [Yamazaki et al., 1999]—that "humans can be phase-shifted by some uncontrolled aspects of the paradigm used by Campbell and Murphy". However, a recent report by these authors described that photic stimulation of the popliteal region during sleep increased the duration of human REM sleep phase [Murphy and Campbell, 2001].

More circadian clocks are located outside the suprachiasmatic nucleus.

Although the SCN in mammals is considered as the central "master" circadian pacemaker which drives most, if not all, rhythmic physiological processes, it was discussed previously that oscillators outside the SCN may exist (cf. [Reuss, 1996 p. 557]). For example, the comparison of clock gene expression and locomotory activity rhythms under chronic metamphetamine treatment revealed that the rhythm of locomotion in rats is directly driven by a pacemaker outside the SCN in which caudate-putamen and parietal cortex are involved [Masubuchi et al., 2000].

Structures in which SCN-independent rhythms were detected include retina and pancreas (see below). Recent progress, moreover, has raised the possibility that every cell may be able to express circadian oscillations, as was shown for rat fibroblasts upon a serum shock in culture [Balsalobre et al., 1998]. It has been speculated that these oscillations are under the control of the central pacemaker. Indeed, it has been made clear that fibroblasts—in contrast to SCN cells—are *not* able to generate selfsustained circadian rhythms [Allen et al., 2001]. Parenthetically, it should be emphasized that the term "oscillator" does not imply a "pacemaker" function.

Circadian rhythms were found in the rodent retina.

A presumed site for the extra-suprachiasmatic generation of circadian rhythms was the mammalian retina. Evidence came from the observation that a rhythm of visual psychophysical sensitivity persisted after SCN lesions [Terman and Terman, 1985]. Removal of the pineal or of other glands or pre-chiasmatic optic nerve section did not abolish rod outer segment disk shedding [LaVail and Ward, 1978; Teirstein et al., 1980]. Further, upon SCN lesions that resulted in complete behavioral arrhythmicity, the disk shedding pattern was similar to controls [Terman et al., 1993]. These studies, however, have been conducted in vivo so that an endogenous circadian rhythm in the mammalian retina remained to be shown, until the crucial experiments were reported by [Tosini and Menaker, 1996]. The authors demonstrated that in the hamster retina a circadian rhythm of melatonin synthesis is present *in vitro*, and that such a rhythm persists in the mouse retina under culture conditions at 27 °C, but not at 32 °C or 37 °C [Tosini and Menaker, 1998]. The interesting question arises whether a slave oscillator exists in the retina that is inhibited by physiological body temperature? The melatonin-forming enzyme, N-acetyltransferase (NAT) was localized to retinal photoreceptors in rats [Niki et al., 1998] while in mice photoreceptors appear to be necessary for the expression of the melatonin rhythm but not for synthesis itself [Tosini and Menaker, 1998], thus leaving the question open which retinal cells produce melatonin. Recently, a circadian rhythm of the pH was found, possibly generated by photoreceptors [Dmitriev and Mangel, 2001].

A metabolic circadian rhythm is present in the pancreas.

At the same time, a series of in vitro experiments demonstrated that a circadian pattern of insulin secretion from perifused pancreatic islets with periods of 22–26 hours exists, and that the rhythm of secretion may be phase-shifted by melatonin [Peschke and Peschke, 1998]. Further studies revealed that melatonin may have a direct, inhibitory influence on pancreatic insulin synthesis and secretion while serotonin rather augmented these parameters [Peschke et al., 1997]. More recently, a study employing autoradiography and reverse transcription polymerase chain reaction revealed that a melatonin receptor of the Mel_{1a} subtype is located on pancreatic island cells [Peschke et al., 2000b].

SCN-dependent oscillations are found throughout the body.

One of the clock genes mentioned above, Cry1, is transcribed in a circadian pattern in liver and skeletal muscle [Miyamoto and Sancar, 1999]. The same is true for rat period homologue rPer2 mRNA in various tissues including brain, heart, lung, liver and kidney [Sakamoto et al., 1998], and for mPer1 in cultured liver, lung and skeletal muscle [Yamazaki et al., 2000]. However, these rhythms were abolished by SCN lesion or dampened after few days in vitro, respectively, suggesting that the peripheral expression was governed by the SCN. Thus the question arises as to how the SCN as the master circadian pacemaker may synchronizes all peripheral “slave” oscillators to the central clock?

A clock gene rhythm was also detected in peripheral mononuclear leukocytes [Oishi et al., 1998] which are not morphologically connected to the SCN revealing that a humoral substance may mediate the circadian signal. The presence of a diffusible coupling signal by which an SCN transplanted into an SCN-lesioned animal may control circadian locomotory activity was previously suggested by [Silver et al., 1996a]. A recent paper [Stokkan et al., 2001] demonstrated that restricted feeding may entrain the rhythm of clock genes in the liver independently of the SCN. According to the authors, the results suggest a mechanism by which peripheral oscillators may be coupled to the SCN through rhythmic behaviour such as feeding. Buijs and Kalsbeek [2001] proposed a model in which peripheral clocks synchronize the activity of an organ thus supporting the central clock. Feedback signals then allow the

SCN to integrate both central and peripheral clock mechanisms. It is open, however, whether a humoral signal from the SCN is indeed necessary in vivo. As shown by viral tracings, polysynaptic neural efferents of the SCN reach many organs via sympathetic and parasympathetic motor pathways [Ueyama et al., 1999; Kalsbeek et al., 2000a; la Fleur et al., 2000]. More exciting news on that item are, to my opinion, warranted during the next years.

SCN output pathways influence the hypothalamic neighborhood.

Tracing or lesion studies and those using a physiological approach revealed that the SCN exerts its influence on several sites within and outside of the hypothalamus [Miller et al., 1996]. They demonstrated that projections exist to hypothalamic regions dorsal and posterior to the SCN. Recently, retrograde tracing experiments depicted that the SCN core (see above) projects to the peri-suprachiasmatic regions, lateral subparaventricular zone and ventral tuberal area. The shell innervates densely the medial preoptic area, medial subparaventricular and dorsomedial hypothalamic nuclei [Leak et al., 1999].

SCN-(sub)paraventricular projections have major functional impact.

Combined with immunohistochemistry, tracings revealed that VIP- or AVP-containing cell groups of the rat SCN provide these efferents [Teclemariam-Mesbah et al., 1999]. Similar results were obtained from the human SCN. Its main projections, as judged from AVP and VIP fibers arising from the nucleus, aim at the anteroventral and dorsomedial hypothalamic nuclei and the subparaventricular and ventral paraventricular hypothalamic regions [Dai et al., 1997]. A subsequent postmortem anterograde tracing study confirmed these projections and demonstrated labelled fibers near AVP and CRH neurons of the human PVN [Dai et al., 1998b]. With the exception of a dense innervation of the ventral PVN in humans, the results revealed very similar general projection patterns as observed in the rodent brain.

In the context discussed here, projections from the SCN to the subparaventricular zone (SPZ) and/or the paraventricular nucleus (PVN; figs. 4D, E) are of high importance. A recent paper demonstrated that the paths involving the SPZ are responsible for the circadian regulation of sleep and body temperature [Lu et al., 2001]. The PVN, which consists of several magnocelluar and parvocellular subdivisions, coordinates neuroendocrine and autonomic mechanisms by a complex series of neural connections to and from various hypothalamic regions [Swanson and Sawchenko, 1980; Sawchenko and Swanson, 1983].

Early autoradiographic investigations have revealed a suprachiasmatic projection to the PVN [Berk and Finkelstein, 1981], and there is evidence from recent studies that GABA-ergic, inhibitory as well as glutamatergic, excitatory influences of the SCN on the magnocellular PVN exist [Boudaba et al., 1996; Csaki et al., 2000; Cui et al., 2000; Cui et al., 2001]. Blocking of GABA-ergic transmission in the bilateral PVN during the day resulted in elevated pineal melatonin synthesis [Kalsbeek et al., 2000b]. Assuming that at daytime the GABA-ergic output of the SCN inhibits the path that is responsible for the increase of melatonin synthesis at night, these data would also explain our previous observation that electrical PVN stimulation during the daytime further diminished melatonin synthesis [Reuss et al., 1985; Olcese et al., 1987]. Very recently, the activation of the SCN-PVN projection system was found to be phase-dependent inasmuch as light induced c-Fos-immunoreactivity in paraventricular projection neurons of the SCN to a higher degree at late than at early night [Munch et al., 2002]. These output neurons were mainly VIP- or GRP-IR and contained AVP to a lesser degree.

Other details on the PVN's role in the SCN-pineal pathway and on the involvement of its hormone AVP have been discussed in detail previously (cf. [Reuss, 1996]). A recent re-investigation confirmed the hypothesis of reciprocal regulation of AVP and melatonin release [Isobe et

al., 2001a; Isobe et al., 2001b]. In conclusion, the impact of the PVN in the rhythm-regulating system is suggested by the findings that it is the target of retinal fibers and SCN efferents, that it projects to autonomic spinal regions thus integrating neuroendocrine and autonomic mechanisms and that lesions or stimulation of its neurons influence functional connections of the retino-pineal pathways. Finally, the SCN-PVN connection is a primary candidate site for the conversion of peak activity between SCN and pineal melatonin synthesis (see [Reuss, 1996] for a detailed discussion).

The SCN projects to the neuroendocrine hypothalamus.

Strong GABA-ergic inhibitory and glutamatergic excitatory effects of the SCN were also revealed by electrophysiological recordings in rats to be elicited on the supraoptic nucleus [Cui et al., 1997b] and it was suggested that these are involved in the regulation of daily changes of neurohypophysial hormone secretion. Electrophysiological studies further suggested extensive reciprocal connections of the SCN and its targets including the arcuate and peri-supraoptic nuclei [Saeb-Parsy et al., 2000]. In addition, neuroendocrine cells of the medial preoptic, periventricular and arcuate nuclei of the hypothalamus are targets of SCN efferents [Horvath, 1997]. Since some of these neurons produce dopamine, a pathway is conceivable by which the SCN may exert its (indirect) influence on the hypothalamo-pituitary-gonadal axis. A similar path may be responsible for the circadian regulation of the estrous cycle. The SCN, necessary for the preovulatory luteinizing hormone surge, directly innervates (probably via VIP-ergic fibers) GnRH neurons in the preoptic area of the female rat [Van der Beek et al., 1997]. Since sexual steroids influence the SCN via afferent neurons (see above), there is again evidence for reciprocal influences within the system.

Lesion studies showed that knife cuts dorsocaudal to the SCN did not block gonadal responses to short days in Siberian hamsters suggesting that these signals are not mediated by SCN efferents projecting in dorsomedial or dorsocaudal direction [Song and Bartness, 1998]. It appears that rather the ventromedial hypothalamic area is required for the gonadal regression induced by short days [Bae et al., 1999]. There is experimental evidence that the latter structure may amplify rhythmic output from the SCN, may contain a feeding-entrained oscillator, and that the subparaventricular zone may integrate information from several oscillators [Choi et al., 1998].

SCN output pathways also reach extrahypothalamic sites.

Mono- and polysynaptic pathways connect many neural and non-neural sites to the SCN. Moderate to sparse SCN projections have been found to the paraventricular and paratenial thalamic nuclei, and to the bed nucleus of the stria terminalis (BNST) and the zona incerta [Leak et al., 1999]. Combined tracing and immunohistochemistry showed that these projections stem from AVP neurons and, to a small extent, from GRP neurons of the rat SCN [Novak et al., 2000]. Electrophysiological recordings in hamsters suggest that the BNST is strongly coupled to the SCN since both circadian and ultradian rhythms were in phase with the SCN, while those in other hypothalamic and extrahypothalamic regions were not [Yamazaki et al., 1998].

Neuronal tracings using a transsynaptically transported virus and its immunohistochemical detection revealed polysynaptic efferents reaching as far as to liver and thyroid, adrenal and salivary glands in rats [Ueyama et al., 1999; Kalsbeek et al., 2000a; la Fleur et al., 2000]. This method also demonstrated that the SCN is connected to many sympathetic and parasympathetic motor pathways regulating systems under tonic control [Ueyama et al., 1999]. Autonomic efferents originate from the dorsomedial SCN and arise in part from AVP neurons but VIP-, GRP- and SS neurons of the ventrolateral part are also involved [Ueyama et al., 1999].

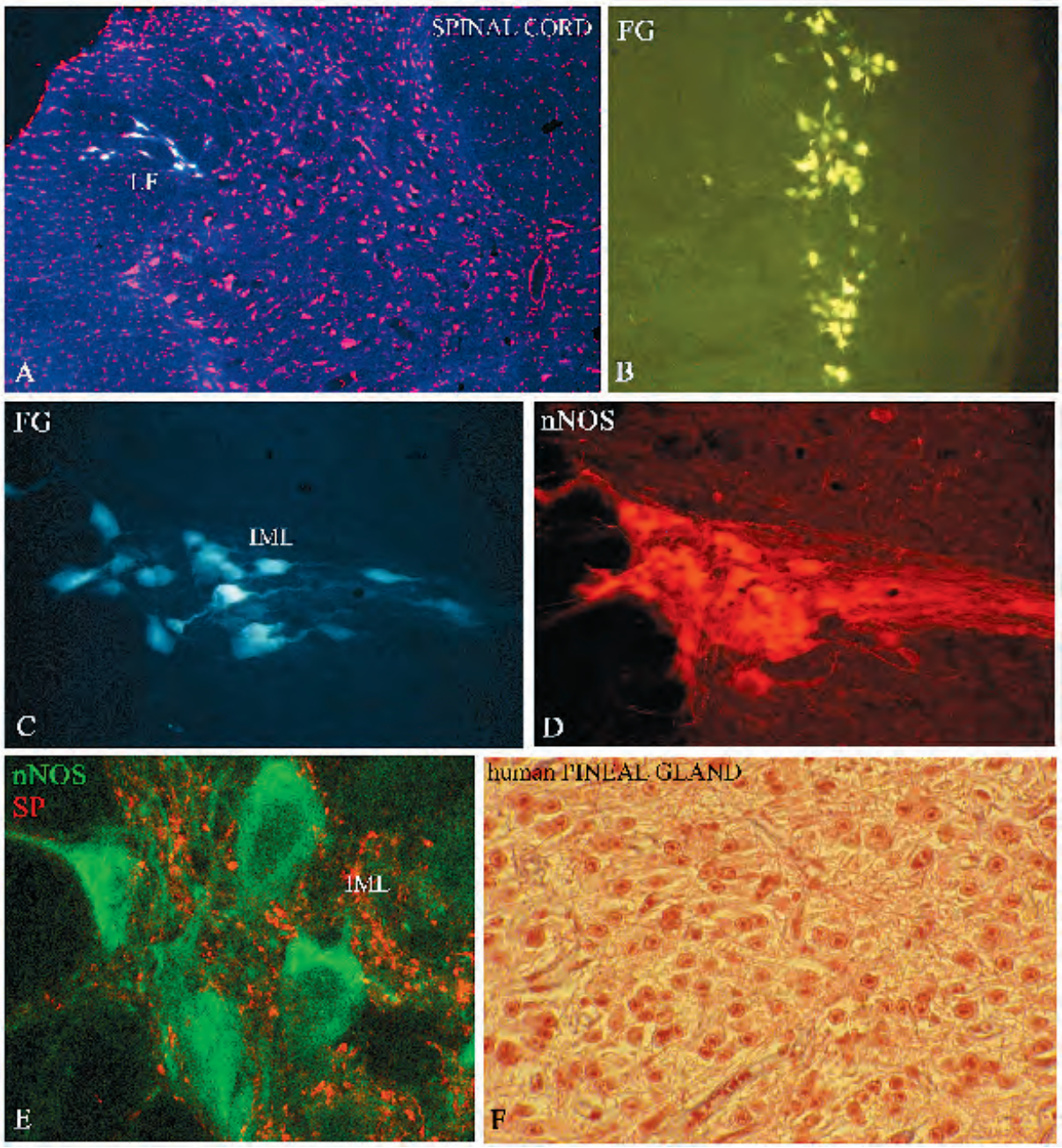

Fig. 5 A: Location of preganglionic sympathetic neurons (PSN) in the lateral funicle (LF) in a cross section of spinal segment Th1 of the rat spinal cord, seen after injection of Fluorogold (FG) into the superior cervical ganglion and retrograde axonal transport (ethidium bromide counterstain). B: FG-labelled PSN are located in clusters in the intermediolateral nucleus (IML) in a longitudinal section of the dwarf hamster spinal cord. The comparison of C and D, taken from the same cross section of the rat spinal cord, shows that most identified PSN contain neuronal nitric oxide synthase (nNOS)-immunoreactivity. E: Neuronal NOS-IR cell bodies and processes (green fluorescence) are contacted by substance P (SP)-IR terminals (red fluorescence) of putative supraspinal origin. (Panels C–E stem from a cooperation with Martina Reuss). F: Pinealocytes of the human pineal gland, characterized by light nuclei with distinct nucleoli.

The hypothalamic paraventricular nucleus projects to the spinal cord.

The hypothalamic PVN is seen as an interface between neuroendocrine and autonomic systems. The efferent projection pathways of the hypothalamic PVN include central fibers reaching the pineal gland directly (see below) and fibers to the spinal autonomic regions (cf. [Reuss, 1996]) via the rostral ventrolateral medulla or bypassing this structure [Pyner and Coote, 2000]. Paraventricular efferents terminate in autonomic nuclei where preganglionic sympathetic neurons (PSN) are located, i.e., the intermediolateral nucleus (IML), lateral funicle (LF) and central autonomic area (figs. 5A, B). These projections most probably use AVP and OT as (mainly inhibitory) transmitters as well as a cocktail of modulating neuropeptides (cf. [Benarroch, 1994; Reuss, 1996]). PVN-efferent fibers are shown in fig. 4E (arrows).

In the dorsal and medioventral parvocellular subdivisons of the PVN, projection neurons that innervate the rat spinal cord were identified by retrograde tracing [Teclemariam-Mesbah et al., 1997]. These neurons were contacted by VIPergic boutons that may stem from the SCN [Vrang et al., 1997]. Spinally projecting neurons in different subdivisions of the rat PVN were determined recently to express AVP, OT, dynorphin or enkephalin mRNA in specific pattern partly related to function [Hallbeck et al., 2001].

There is, furthermore, evidence for spinal projections from the lateral, dorsomedial and ventromedial hypothalamus (for details and references, see [Reuss, 1996]).

The brainstem raphe nuclei provide input to several components of the circadian timing system.

In the context discussed here, the most important raphe projection perhaps is the serotonergic innervation of the SCN (see above and figs. 3E, F). However, other sites within the circadian timing system also receive afferent projections from raphe nuclei the functional implications of which are presently unknown. For example, serotonergic neurons of the raphe nuclei provide a serotonergic innervation of the retina [Villar et al., 1987; Labandeira-Garcia et al., 1990; Reperant et al., 2000] which may be interpreted as the morphological correlate of a raphe-retina-feedback mechanism. A functionally parallel tract may be seen in the raphe serotonergic projections to the intergeniculate leaflet [Morin, 1994]. In addition, the nucleus raphe magnus provides non-serotonergic input to the hypothalamic PVN while the dorsal and median raphe nuclei provide only sparse fibers in rats [Larsen et al., 1996]. Finally, anterograde tracing studies in the same species demonstrated that direct neuronal projections from the dorsal raphe to the deep pineal and the pineal stalk are present [Møller and Hay-Schmidt, 1998]. By its projections to several sites of the system, the raphe complex probably is involved in many regulatory mechanisms that have a circadian component (cf. [Reuss, 1996]).

The pineal gland acts via hormonal output.

The pineal gland (fig. 5F) is located dorsally to the lamina tecti. Neural signals (dealed with in the following chapters) are changed in the gland into an endocrine signal. The best-investigated humoral output is melatonin but other indolamines such as serotonin, as well as other substances including amino acids and nitric oxide are also produced and secreted (cf. [Reuss, 1996]). Although it is clear that melatonin formation is under sympathetic influence, there are few data that would uncover the functional implications of other pineal substances. Transporters and receptors for some of these compounds were recently detected. For example, glutamate and GABA transporters are expressed by pinealocytes and glial cells [Redecker, 1999; Berger and Hediger, 2000]. Glutamate receptors have been found in the primate pineal gland on glial and neuronal-like cells [Mick, 1995]. Glutamate

and aspartate are secreted from rat pinealocytes in vitro by exocytosis and it was demonstrated that these amino acids may inhibit melatonin secretion [Yamada et al., 1996; Yatsushiro et al., 1997]. The presence of catecholaminergic, GABA-ergic and peptidergic neurons or pinealocytes (containing AVP, OT, MSH, ENK, SS, GnRH or SP) have been described previously (cf. [Reuss, 1996]). All these compounds may represent a humoral output or serve intrinsic function in regulating the synthesis of melatonin or of other substances. Melatonin, secreted in a diurnal pattern with high nocturnal levels, serves as a humoral signal to convey day length information, and thus functions as nature's contraceptive in seasonal breeders (cf. [Reiter, 1991]). Correspondingly, sites of actions were detected in many structures involved in the regulation of reproduction from the hypothalamus to the gonads (cf. [Pang et al., 1998]). In humans, as summarized comprehensively in a recent review [Vollrath, 2001], it functions as a feedback signal for the circadian oscillator, mediates maternal-fetal communication of daylength, depresses body temperature, and has sleep-promoting properties. Discussed are further a role in the pathophysiology of seasonal affective disorders, interactions with the immune system and possible oncostatic properties, as well as preventive action in shock, inflammation and ischemia [Cuzzocrea and Reiter, 2001].

The sympathetic system regulates pineal melatonin synthesis.

Preganglionic sympathetic neurons are cholinergic, contain many neuropeptides and predominantly are nNOS-IR (figs. 5C, D). They receive a dense SP-ergic innervation from supraspinal sites ([Reuss and Reuss, 2001], see fig. 5E). Axons of PSN of the upper thoracic segments, building the sympathetic trunk, convey information to sympathetic ganglia. In this regard, the superior cervical ganglion (SCG) as the source of the pineal gland's noradrenergic innervation is of major importance. Neurons in the cranial part of the SCG form the internal carotid nerve (nervus conarii) and thus provide the sympathetic innervation of the pineal gland [Kappers, 1960; Bowers et al., 1984; Reuss and Schröder, 1988; Reuss and Moore, 1989]. According to a wealth of knowledge, this input is responsible for the nocturnal increase of pineal melatonin synthesis. For example, removal of the ganglia blocked the nocturnal increase of the melatonin-forming enzyme in the rat pineal, and electrical stimulation of the cervical sympathetic trunk activated the system [Klein et al., 1971], cf. [Reuss, 1996]. It is however not clear yet whether increased amounts of pineal noradrenaline (NA) receptors or rather nocturnally increased NA release from postganglionic sympathetic fibers are the reason of augmented melatonin synthesis at night. The amount of pineal NA, in any case, exhibits only minor day/night-differences in rats and hamsters [Hermes et al., 1994; Míguez et al., 1998]. Recent studies demonstrated that the mRNA for β_1-adrenergic receptors in the rat pineal exhibits maximal levels at mid-dark [Møller et al., 1997; Pfeffer et al., 1998], while β-adrenergic receptor ligand binding sites are increased both in the afternoon and in the second half of the night [Pfeffer et al., 1998]. The molecular mechanisms underlying the regulation of pineal melatonin synthesis were comprehensively reviewed by [Stehle et al., 2001].

Co-localized to NA in SCG neurons are several neuropeptides including NPY. Combined tracing and immunohistochemistry showed that also the majority of pinealopetally projecting cells in the SCG contain NPY [Shiotani et al., 1986; Reuss and Moore, 1989]. The effects of NPY on the rat pineal were first demonstrated in vivo in our laboratory [Reuss and Schröder, 1987] to be either excitatory or inhibitory on melatonin synthesis depending on the state of the system (i.e., day or night conditions) and were later confirmed by in vitro-studies (cf. [Olcese, 1994]). In addition to its diurnal variation in the pineal gland of the dwarf hamster [Reuss and Olcese, 1995], there is also evidence for a seasonal rhythm since NPY-IR nerve fiber profiles in the pineal of the European hamster exhibited maximal density in mid-winter [Møller et al., 1998].

The nonsympathetic innervation of the pineal gland stems from diencephalic and other sites.

As mentioned above, pineal melatonin synthesis is under sympathetic control. However, several studies mainly using morphological methods demonstrated extrasympathetic input to the gland, the functional impact of which remains largely enigmatic (cf. [Korf and Møller, 1984; Reuss, 1996]). Upon ganglionectomy, for example, pineal NPY in rats did not totally decrease [Piszczkiewicz and Zigmond, 1992], some NPY-IR fibers remained in the pineal of mink and sheep [Møller et al., 1990; Cozzi et al., 1994], and CGRP- and SP-fibers persisted in the rat pineal [Matsushima et al., 1994; Matsuura et al., 1994]. According to the present knowledge, extrasympathetic influence on the pineal gland comes from cholinergic and peptidergic sources.

Peptidergic fibers in the pineal stem from central sites and peripheral ganglia and may influence the gland's metabolism.

An additional source of extrasympathetic influence on the pineal are peptidergic neurons. Central fibers are thought to innervate the gland, connecting the hypothalamic PVN and the habenular nuclei directly to the pineal, i.e., bypassing the sympathetic pathway. Vasopressinergic and oxytocinergic fibers were detected previously in the gland. Effects of AVP were found on rat melatonin synthesis in vivo and in vitro (see above), while this was not the case with oxytocin (OT) in vitro [Reuss et al., 1993]. A study using perifused rat pineal glands in vitro also revealed peptidergic influences on melatonin synthesis, i.e., VIP and PHI enhanced cAMP efflux and melatonin secretion, NPY blocked NA-induced melatonin secretion, while CGRP, OT, AVP and SP were ineffective with regard to melatonin secretion [Rekasi et al., 1998]. However, OT binding sites were found in high density in the ovine pineal gland [Rahmani et al., 1997] but it is still open to which aspects of pineal function they may relate. Interestingly, intrinsic OT-IR neuron-like cells were detected in the bovine pineal gland recently [Badiu et al., 2001].

Recent tracing studies demonstrated that several nonsympathetic ganglia are putative sources of pineal innervation. Retrograde tracing and combined immunohistochemistry revealed that the trigeminal ganglion sends SP- and CGRP-ergic fibers to the rat pineal (cf. [Reuss, 1999]). In addition, the rat pineal is innervated by PACAP fibers the majority of which are thought to stem from the trigeminal ganglion [Møller et al., 1999]. Additional evidence from tracing studies suggests that fibers originating in the sphenopalatine and otic ganglia innervate the rat pineal gland [Møller and Liu, 1999], and these ganglia also contain peptidergic neurons.

In the tree shrew *(Tupaia glis)* pineal gland, leu-ENK fibers were detected [Phansuwan-Pujito et al., 1998] as well as bundles of SP- or CGRP-fibers which were left intact following superior cervical ganglionectomy, while NPY-fibers disappeared almost completely [Kado et al., 1999]. In the same species, frequent GABA-ergic synapses but no immunoreactive cell bodies were detected in the pineal [Sakai et al., 2001]. In the tree shrew and in rat, mouse, hamster and guinea pig, these terminals were of putative nonsympathetic origin [Sakai et al., 2001]. In addition, secretoneurin is present in rodent pineal fibers of presumed sympathetic and central origin, and the substance was found to inhibit serotonin and melatonin release [Simonneaux et al., 1997].

Finally, gonadotropin and gonadal steroid receptors were observed in the human pineal gland [Luboshitzky et al., 1997b]. It was suggested that gonadal steroids may modulate melatonin secretion. Interestingly, gonadotropin receptors exhibit higher levels in the winter [Luboshitzky et al., 1997a].

It should be noted that effects on melatonin synthesis were in the focus of most physiological studies while other functions that are putatively under the influence of peptidergic and other substances were only poorly covered.

Acetylcholine dampens melatonin synthesis.

Acetylcholine (ACh) receptors of the nicotinic and the muscarinic types were demonstrated in the rodent pineal previously. Electrophysiological recordings from rat pinealocytes during the daytime recently showed that spontaneous multiunit activity was increased by ACh application [Schenda and Vollrath, 1998b]. In this study, nicotinic stimulation required a ten-fold higher dose than muscarinic stimulation. However, nicotinic receptors seem to mediate the depolarizing effect of ACh on pinealocytes [Letz et al., 1997]. They also mediate the ACh effect on glutamate exocytoses and on the inhibition of NAT activity in cultured rat pinealocytes [Yamada et al., 1998]. It is suggested that ACh negatively controls melatonin synthesis, possibly by prejunctional inhibition of NA release or by inhibition of the cAMP cascade [Drijfhout et al., 1996; Yamada et al., 1998].

Two sources are conceivable from which pineal ACh may originate. At first, the substance may stem from pinealocytes, and there is indeed evidence that pineal cells produce ACh. Rat pinealocytes exhibited immunoreactivity against the ACh-forming enzyme, choline acetyltransferase, even after 48 h in culture, and the gland's ACh content, as measured by high performance liquid chromatography, increased about ten-fold at night [Wessler et al., 1997]. A second possibility is that ACh stems from nerve fibers originating, for example, from parasympathetic neurons in the sphenopalatine ganglion (cf. [Møller and Liu, 1999]).

Pineal function is modulated by intrinsic factors.

Nitric oxide synthase, detected in the guinea pig pineal gland as early as in the late sixties [Vollrath and Schmidt, 1969], has since been investigated in several studies (cf. [Reuss et al., 1997]). In a number of species including rat and sheep, it is present in fibers that may also contain VIP or PHI and is probably of parasympathetic or intrinsic origin [Lopez-Figueroa and Møller, 1996; Spessert et al., 1998]. It was recently found that NO stimulates cGMP formation in the pineal gland [Maronde et al., 1995; Spessert et al., 1998]. NO may also have a negative influence on the gland since, in vitro, NO donors inhibited melatonin synthesis in rat and bovine pineal cells [Maronde et al., 1995] and decreased spontaneous electrical discharges recorded from the rat pineal gland [Schenda and Vollrath, 1997], the latter probably by mediating the depression induced by β-adrenoceptor activation [Schenda and Vollrath, 1998a]. It is likely that NO belongs to the intrinsic substances that are produced and active directly in the gland to modulate melatonin synthesis.

Another neuroactive substance that may increase pineal cGMP formation is C-type natriuretic peptide [Müller et al., 2000]. Built in the gland itself [Middendorff et al., 1996] or taken up from the cerebrospinal fluid, its receptor is found in highest levels in membranes of rat pinealocytes. Calcium channels were considered as principal targets for cGMP in the rat pineal [Sautter et al., 1997]. However, the functions of cGMP in the pineal gland are still unclear (see [Reuss, 1996], for discussion) while it is without doubt that activation of cAMP induces the nocturnal increase of melatonin synthesis.

Melatonin receptors are present in the SCN.

Melatonin receptors were identified in a number of neural and non-neural sites. Both types, Mel_{1a} (also known as MT_1) and Mel_{1b} (also known as MT_2), were detected in the rodent SCN. The Mel_{1a} type exhibits a diurnal augmentation of density as does its mRNA in the rat SCN [Neu and Niles, 1997]. It is believed that it may transduce the acute inhibitory actions of melatonin on the SCN, as was revealed by studies using a mouse model with targeted disruption of this receptor [Liu et al., 1997] or by the application of competitive antagonists in rats [Hunt et al., 2001]. In the *Phodopus* SCN, its mRNA is coexpressed with AVP mRNA in some neurons suggesting that

AVP neurons of the SCN may respond to melatonin and thus participate in photoperiodic signalling [Song et al., 2000]. This observation fits well with the finding that melatonin inhibits AVP-release from dispersed neurons of the rat SCN [Watanabe et al., 1998]. However, recent evidence suggests that the Mel_{1b} receptor type is responsible for the inhibition of AVP release from the SCN in vitro [Isobe et al., 2001b]. An inhibiting influence of melatonin on the rat SCN was also revealed by increases in the volume of cell nuclei upon inactivation of the pineal or melatonin synthesis [Peschke et al., 1996]. Interestingly, a stimulatory influence of the pineal or melatonin on the ventromedial hypothalamic nucleus has been found suggesting a positive feedback of the gland on this hypothalamic nucleus [Peschke et al., 2000a].

The actions of melatonin further include phase-advance of the circadian clock. It was shown that melatonin administration advanced the evening rise in light-induced c-fos in the rat SCN in vivo [Sumova and Illnerova, 1996], and advanced the time of peak electrical activity when applied to SCN slices at two windows of sensitivity corresponding to dusk and dawn [McArthur et al., 1997]. Since respective antagonists blocked the melatonin-mediated phase advances, it is suggested that the Mel_{1b} type may mediate melatonin-induced phase-shifts [Dubocovich et al., 1998].

Melatonin receptors in the pituitary pars tuberalis are important for gonadal regulation.

The pars tuberalis of the pituitary is thought to be the site at which melatonin exerts its gonad-regulating and neuroendocrine effects. This structure mediates seasonal effects of melatonin on secretion of prolactin and other hypophyseal substances. Several modes of melatonin action were considered, e.g., direct effects on endocrine anterior pituitary cells and/or an influence on the process of differentiation of precursor cells in the pars distalis (cf. [Hazlerigg, 2001]).

Melatonin binding sites were identified in several neural and non-neural sites.

Melatonin may also influence retinal ganglion, amacrine and horizontal cells via the Mel_{1a} receptor [Fujieda et al., 1999]. Ganglion cells exhibited the most intense receptor immunoreactivity which was, to a lesser degree, observed also in dopaminergic and GABA-ergic amacrine cells of the guinea pig retina [Fujieda et al., 2000].

In addition, melatonin receptors were found in the thalamic PVN of *Phodopus sungorus* [Song et al., 2000], in the human cerebellar cortex (Mel_{1b} in glial cells, Mel_{1a} in non-Purkinje neurons; see [Al-Ghoul et al., 1998]) and in the central grey matter of the rabbit spinal cord [Wan et al., 1996]. In non-neuronal sites, melatonin receptors are present on Leydig cells of the rat testis [Valenti et al., 1997], on rat pancreatic island cells (Mel_{1a} [Peschke et al., 2000b]), on villi of the small intestine [Lee and Pang, 1993] and in proximal tubules of the guinea pig kidney (Mel_{1a}; see [Song et al., 1997]). The functional roles of melatonin in the regulation of many neuronal and non-neuronal sites, however, are largely unknown.

Physiological and pathophysiological changes occur in the aging circadian system.

An interesting question is which, if any, morphological changes underlie the observed age-related functional decline of the circadian timing system (cf. [Hofman, 2000]). For example, hamsters aged 18–22 months were much less sensitive than young animals to the phase-shifting effects of light as measured by activity rhythm, fos protein induction and CREB phosphorylation in the SCN [Zhang et al., 1996]. However, the responsiveness of the mice circadian timing system to melatonin does not decrease with age [Benloucif et al., 1997].

While in rat no effects of age on SCN volume, cell number or size were observed [Madeira et al., 1995], aged rats under an LD-cycle did not exhibit day/night-differences in VIP mRNA that were ob-

served in young animals [Kawakami et al., 1997; Krajnak et al., 1998]. This age-related loss, however, was not observed for AVP mRNA [Krajnak et al., 1998]. The hypothesis of a causal relation between the age-related loss of AVP-neurons in the SCN and the decrease in circadian organization of behavior was also not supported by a study comparing the number of AVP neurons and locomotory activity of young and old voles [Van der Zee and Bult, 1995].

On the other hand, aged dwarf hamsters showed a drastic increase in the number of SP-IR neurons in the ventral SCN [Reuss and Bürger, 1994] and cAMP fluctuations in the SCN of the same species were clearly diminished in aged animals [Reuss and Rimoldi, 1998]. The SCN also exhibits changes in serotonergic functions in aged animals [Duncan et al., 2000]. Taken together, these results reveal that SCN components are differently affected by age.

In the human SCN, a loss of VIP neurons were observed in males, but not in females, and the circadian rhythm in AVP-IR neuron number is disrupted with age (cf. [Zhou and Swaab, 1999; Hofman, 1997]). A recent study, however, demonstrated the periods of body temperature, and of melatonin and cortisol production to be the same in young humans and in a group aged 64–74 years [Czeisler et al., 1999].

Morphological alterations of the circadian timing system with advanced age may not necessarily be restricted to the SCN since respective changes were previously observed also in the paraventricular and supraoptic nuclei of the human hypothalamus [Ishunina and Swaab, 1999] and in the rat pineal gland [Reuss et al., 1986; Reuss et al., 1990; Míguez et al., 1998].

With regard to pathophysiological changes, clinical studies correlated temporal disorganization such as sleep disturbances to many neurological diseases including epilepsy, dementia, multiple sclerosis and neuromuscular disorders (cf. [Turek et al., 2001]). Morphologically it was conspicuous that AVP and neurotensin neurons were lost in the SCN of patients suffering from senile dementia of the Alzheimer type (SDAT) which was suggested to be one of the morphological substrates underlying the clinically observed changes in circadian rhythmicity in these patients [Stopa et al., 1999]. In addition, AVP-mRNA was three times lower in SDAT patients compared to controls and the clear day/night-rhythm was absent [Liu et al., 2000].

Although a causal relationship remains to be shown, it is interesting that the nocturnal period of melatonin secretion exhibited seasonal differences in patients suffering from the seasonal affective disorder syndrome but not in healthy volunteers [Wehr et al., 2001].

It is held that probably all living cells and organisms are somehow under the influence of an internal system regulating behavior on a 24-hour basis as an adaptation to the earth's rotation. A better insight into anatomy and connections of the structures responsible for rhythmic regulation may therefore contribute to our understanding of mammalian body functions and their control in health and disease.

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