CONTROL OF THE DAILY MELATONIN RHYTHM

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A model of time distribution by the biological clock mediated through the autonomic nervous system

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par ***Stéphanie Perreau-Lenz***

Contrôle du rythme journalier de Mélatonine

un modèle de distribution du temps par l'horloge biologique via le système nerveux autonome

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À ma famille

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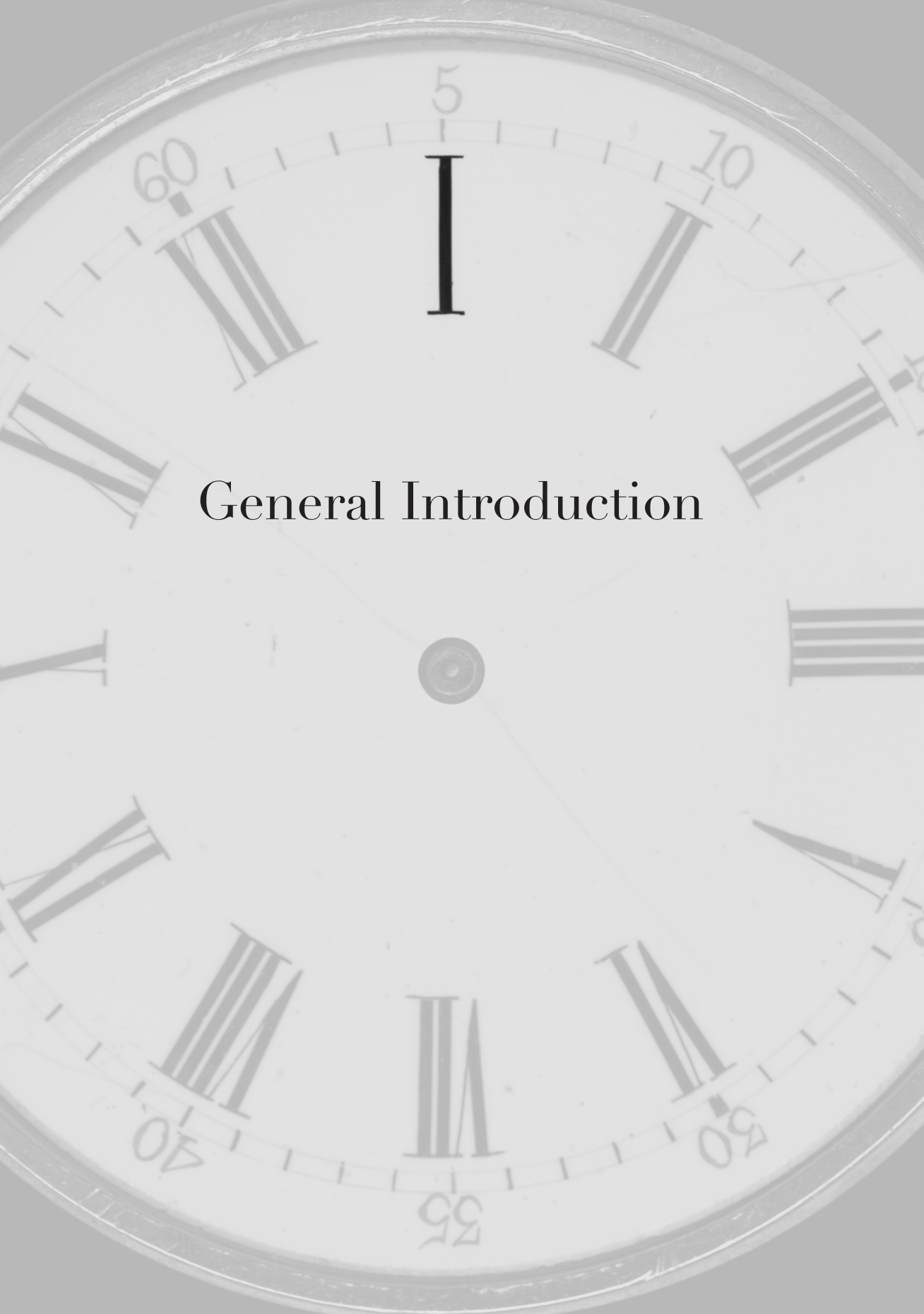

General Introduction

CHAPTER 1

Introduction and Aims

A. BIOLOGICAL DAILY RHYTHMS

Even though they are encoded in our genes, most of us are oblivious to natural environmental rhythms. Almost every day, as the sun rises, we awaken to an internal or external alarm, dress, eat, and then head off to work. However, each morning, our serum cortisol, blood glucose, heart rate and blood pressure already start to rise before we wake up. In the artificial light of the workplace, we lose sight of the sun. We eat a second meal at midday and then work until sunset. After returning home, we eat a final meal. In the next hours our pineal gland starts to release melatonin, our thyroid gland starts to be stimulated by the increased release of thyroid stimulating hormone (TSH), and our plasma cortisol levels reach a trough. Our internal temperature drops, and we finally fall asleep, a state of unconsciousness that consumes a third of our lives. In the first hours of sleep our pituitary starts to release growth hormone and prolactin. While we sleep, the mice, rats and hamsters in our fields, houses and animal facilities wake up, become active and feed. And if you are a woman, although you must have noticed the recurrent and regular expression of your reproductive functions, most likely you are unaware of its time-window specificity in the early morning. Every day the gonadotropin-releasing hormone (GnRH) containing neurons become active in the early morning, and once every 28 days their activity, together with the rising plasma estrogen levels, results in the induction of a luteinizing hormone (LH) surge, thereby inducing ovulation. Although rodents have a totally different reproductive system, every 4 to 5 days the LH surge inducing ovulation occurs just before the beginning of their activity period. In addition, although to a lesser extent than seasonal rodents, we are also sensitive to seasonal variations, without noticing it- but testosterone secretion, semen quality and birth weight vary with winter or summer.

As this first paragraph illustrates, almost all functions of living organisms show a rhythmic expression. The rhythms of life can range from milliseconds to centuries. One of the oldest and best-known rhythms is the day-night rhythm – a clear and stable rhythm. Indeed, since, on earth, a day is almost always made up of a cycle of a light and a dark period, with a constant duration of 24-h, many biological functions obey the same 24-h rhythm. For a long time it was thought that the astronomic environment induced these rhythms. The first experiments that were carried out to study this

phenomenon were done by a French botanist, who showed that, although the daily rhythms could be influenced by the environment, they were mainly controlled by an intern mechanism, suggesting the presence of an endogenous biological clock (De Mairan, 1729). Later on, it was suggested that this phenomenon could also exist in rats (Richter, 1922), but it took 40 more years to show the endogenous nature of daily rhythms in human (Aschoff & Wever, 1962).

Following these first experiments on biological clock mechanisms, successive studies, realised in mammals, showed that many physiological and endocrine functions obey this rule of endogenous rhythms with a period close to 24-h, subsequently called circadian rhythms, "circa" meaning "close to" and "dies" meaning "day" in Latin. Main behaviours, such as locomotor activity, food intake, or sleep, present a daily rhythm that persists in constant conditions with a period close to 24-h. Next to the behavioural functions, several endocrine functions, such as LH, melatonin, corticosterone, or leptin release, also present circadian rhythmicity (Fig. 1).

The daily melatonin and corticosterone rhythms are the two most studied endocrine circadian rhythms, due to their pronounced amplitude, and due to the reproducible timing of their acrophase. Melatonin synthesis has the additional advantage of not being influenced by stress. Melatonin synthesis and release precisely follow the environmental photoperiod, giving a precise perception of daily and annual time to the rest of the body (Reiter, 1993). Also the anticipatory rise in corticosterone release preceding each activity period supports the biological clock in the distribution of its message of time. However, the distribution of this daily message by the biological clock does not seem to be uniform. As you can notice in Fig.1, all endocrine rhythms are clearly heterogeneous. Indeed, although hormones are released in a circadian manner, the time of their peak-release varies widely throughout the light/dark cycle. Moreover, some hormones, such as LH and corticosterone, always peak before the beginning of the activity period whatever the animal species, diurnal or nocturnal (i.e. in the morning for diurnal animals, but in the evening for nocturnal animals). But other hormones, such as melatonin and leptin, always peak during the dark period of the day, independent of the nocturnal or diurnal nature of the animal. The wide diversity in the expression of endocrine circadian rhythms suggests a complex mechanism behind the distribution of time by the biological clock.

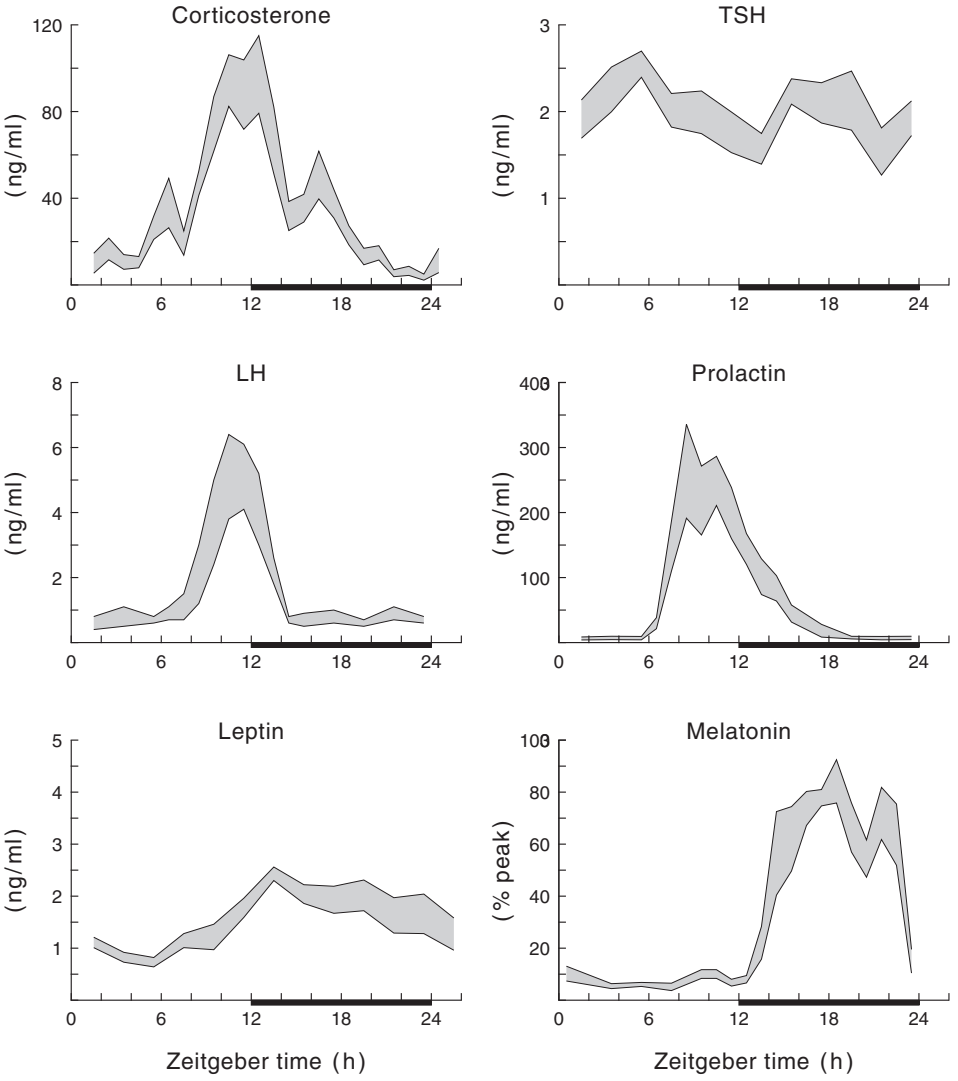

Figure 1 Diurnal rhythms of release of various hormones measured in rats. However, note the diversity in the release pattern along the light/dark cycle.

B. BIOLOGICAL CLOCK CHARACTERISTICS

based upon

Biological Clock: A Bodyguard of Temporal Homeostasis

Stéphanie Perreau-Lenz, Andries Kalsbeek, Paul Pévet & Ruud M. Buijs
Chronobiol Int (in press)

Endogenously set

Descartes already placed the biological clock in the brain but it is only since the early 1970s that the location of the master biological clock in the mammalian brain has been confirmed. The first indications for this location were provided by isolation and lesion studies, indicating a small bilateral brain structure (less than $1mm^3$) containing more than 10.000 neurons (16.000 in rats) and located in the anterior hypothalamus, the suprachiasmatic nucleus of the hypothalamus (SCN). Indeed, isolation or lesioning of this nucleus induced a complete loss of behavioural and endocrine daily rhythmicity (Moore & Eichler, 1972; Stephan & Zucker, 1972; Moore & Klein, 1974; Raisman & Brown-Grant, 1977; Reppert *et al.*, 1981; Watanabe & Hiroshige, 1981; Stoynev *et al.*, 1982; van Esseveldt *et al.*, 2000). *In vivo* glucose utilisation studies revealed that the SCN neurons are metabolically the most active in the middle of the day and the least active in the middle of the night (Schwartz & Gainer, 1977; Schwartz *et al.*, 1980). In addition, two early studies using multiple-unit activity recording, revealed that isolated either *in vivo* or *in vitro*, the SCN sustained its circadian rhythm in neuronal activity (Inouye & Kawamura, 1979; Shibata *et al.*, 1982), confirming the idea of the SCN being a "master clock". Indeed, although also other brain areas may show daily rhythmicity, the SCN is the only structure that can sustain its rhythmic activity with a period close to 24-h (Abe *et al.*, 2002) once it is isolated. The sustained circadian rhythm in neuronal activity in the SCN has even been confirmed at the level of the single neuron. Isolated SCN neurons maintained in culture *in vitro* are, indeed, able to sustain their neuronal activity rhythmicity on a long-term (months) basis (Green & Gillette, 1982; Groos & Hendriks, 1982; Mirmiran *et al.*, 1995; Watanabe *et al.*, 1995; Welsh *et al.*, 1995; Honma *et al.*, 1998), i.e. each SCN neuron seems to have a clock property which is set endogenously. The final proof that the SCN contains the "master clock" for the whole body was provided by transplantation studies. Transplants of intact SCN tissue from a donor restored circadian rhythms in SCN lesioned rats, hamsters and mice with the rhythmicity period characteristic of the donor tissue, i.e. ~20-h, ~24-h or ~28-h, depending on whether the tissue was derived from tau-mutant hamsters, intact animals or Clock-knock-out mice, respectively (Ralph *et al.*, 1990; Ralph *et al.*, 1993; Sujino *et al.*, 2003).

Cell-autonomous Clockwork

Due to the fact that a circadian rhythm of neuronal activity persists in isolated SCN cells it was suggested that each SCN cell contains its own endogenous clock mechanism (Welsh *et al.*, 1995). The synchronisation of all these SCN cells would then give a unique clock output periodicity that can be transmitted to the rest of the body. However, Welsh et al. (1995) also revealed that in *in vitro* conditions, despite abundant functional synapses, circadian rhythms expressed by neurons in the same culture are not synchronized, suggesting the existence of yet another way of communication in the clock mechanism. Nevertheless, the understanding of the molecular clock mechanisms has grown extremely fast these last 20 years. Indeed, in 1984 two teams identified and localised in Drosophila a gene closely involved in circadian rhythms, which they called the period gene or *Per* gene (Bargiello *et al.*, 1984; Reddy *et al.*, 1984). Ten years later, the first so called "clock gene" was identified in mammals (Vitaterna *et al.*, 1994). And since the mid-90s, the number of (mammalian) "clock genes" has increased rapidly. The functioning of these clock genes in building the circadian clockwork has been widely studied these past years and huge progress has been realised. The present concept is that the molecular clockwork contained in SCN cells involves interacting positive and negative transcriptional-/translational-feedback loops built by the interaction of different clock genes, (see for review, Reppert & Weaver, 2002; Albrecht & Eichele, 2003). In rodents, the rhythmic expression of the clock genes involves 3 Period genes (*mPer1*, *mPer2* and *mPer3*), 2 Cryptochrome genes (*mCry1* and *mCry2*) and one Bmal gene (*mBmal1*). The rhythmic transcription of the Period genes and *Cry* genes involves self-sustained negative and positive feedback loops (Fig 2). However, further studies will be necessary to link the circadian expression of these loops with the various outputs of the biological clock. Recent studies were conducted in that direction by revealing a close link between the electrical activity of SCN cells and internal molecular clock mechanisms (Albus *et al.*, 2002; Kuhlman *et al.*, 2003).

Regionally Organised

Due to its high activity during the (subjective) light period and its low activity during the dark period (Schwartz & Gainer, 1977; Inouye & Kawamura, 1979; Schwartz *et al.*, 1980; Shibata *et al.*, 1982), the SCN is described as a group of autonomous clockwork cells functioning in a unimodal fashion and synchronized to give one uniform circadian signal to the rest of the body. However, from the earliest immunocytochemical studies it became clear that the SCN is far from being cell type homogenous (Swaab & Pool, 1975; van Leeuwen *et al.*, 1978; Sofroniew & Weindl, 1980). Indeed, in most of the mammalian species studied so far, the SCN can be divided in two main regions.

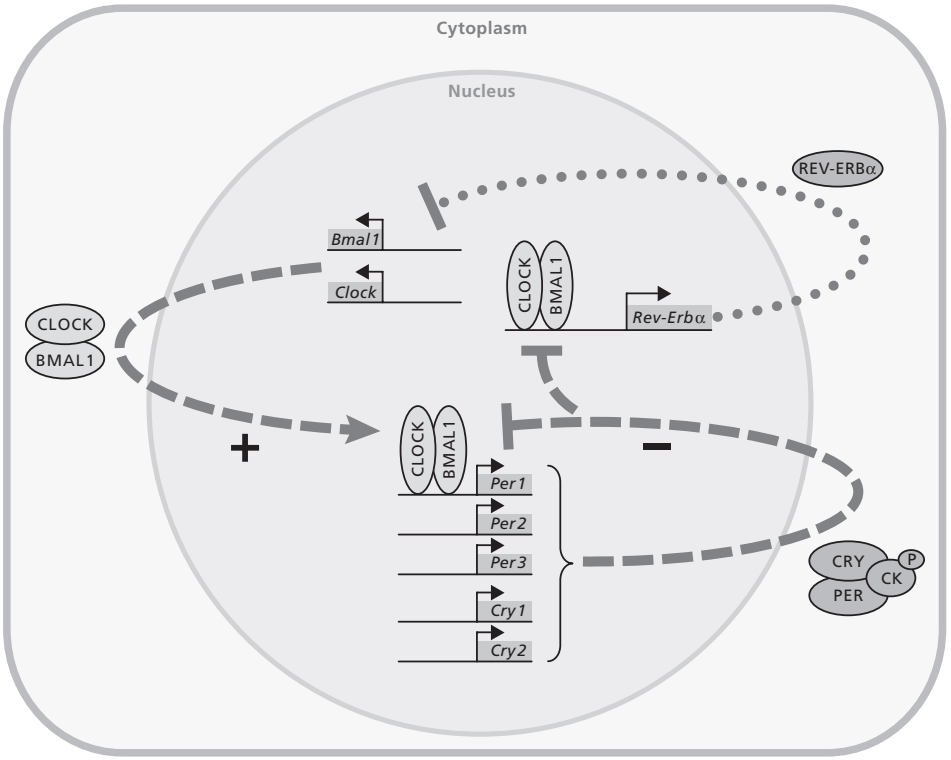

Figure 2 Cell autonomous clockwork model. This model consists of interacting positive and negative feedback molecular loops. *Bmal1* gene is rhythmically expressed (during the dark period), and BMAL1 proteins form heterodimers with CLOCK proteins (constitutively expressed throughough the day). CLOCK-BMAL1 heterodimers enter the nucleus and activate transcription of *Per* and *Cry* genes through E-box enhancers. This first step constitutes the positive feedback loop. Once translated, *PER* and *CRY* proteins form heterodimers and are phosphorylated by casein kinases (CK) (such as casein kinase Iε for PER2) before entering the nucleus and inhibiting their own transcription by associating with the CLOCK-BMAL1 complex, thereby constituting the inhibitive feedback loop. The daily molecular cycle is finally sustained by the transcriptional inhibition of the gene *Rev-Erbα*, with the protein REV-ERBα repressing *Bmal1* transcription during the day.

The dorsomedial area of the SCN specifically expresses the peptide vasopressin (VP), while the ventral area is characterized by vasoactive intestinal peptide (VIP) expressing neurons (Stopa *et al.*, 1984; Card *et al.*, 1988; Cassone *et al.*, 1988; Moore & Speh, 1993; Moore *et al.*, 2002). Besides, a large part of the SCN is made up of neurons containing the inhibitory transmitter γ-aminobutyric acid (GABA) (Okamura *et al.*, 1989; Moore & Speh, 1993; Moore *et al.*, 2002). Therefore, even when taking into con-

sideration only the transmitter content of SCN neurons, a clear subdivision becomes apparent, with partly overlapping populations of SCN neurons. Other peptidergic transmitters, such as gastrin releasing peptide (GRP) (van den Pol & Gorcs, 1986), peptide histidine isoleucine (PHI) (Ishikawa & Frohman, 1987), angiotensin II (Kilcoyne *et al.*, 1980), substance P (SP) (Mai *et al.*, 1991; Otori *et al.*, 1993), calcitonin gene-related peptide (CGRP) (Park *et al.*, 1993), or somatostatin (SS) (Card *et al.*, 1988), are expressed in SCN neurons only to a minor extent. But also these peptides present a location preference within the SCN. GRP-ir, PHI-ir, SP-ir and NT-ir cells are located in the ventrolateral part of the SCN, while enkephalin-ir, and calretinin-ir cells are located in the dorsomedial part of the SCN (Moore *et al.*, 2002). In addition, while VIP and PHI co-localise with GRP (Albers *et al.*, 1991), no co-localisation could be shown so far for VIP or VP and SS (Tanaka *et al.*, 1996). SS-containing cells are located at the interface of the dorsomedial and ventrolateral part of the rat SCN (Card *et al.*, 1988). Such a central subdivision can also be observed in hamsters and mice for cells that contain a calcium binding protein, calbindin D_{28K} (CalB) (Silver *et al.*, 1996b; Ikeda & Allen, 2003).

VP is a peptide for which a clear diurnal pattern of peptide content, intranuclear SCN release and cerebrospinal fluid levels has been shown (Schwartz & Reppert, 1985; Reppert & Uhl, 1987; Burbach *et al.*, 1988; Yamase *et al.*, 1991; Tominaga *et al.*, 1992; Kalsbeek *et al.*, 1995). In addition, a clear circadian gene expression pattern of this peptide has also been revealed (Uhl & Reppert, 1986; Cagampang *et al.*, 1994). The VP concentration in the cerebrospinal fluid peaks in the middle of the day for both nocturnal and diurnal animals, suggesting a close association with the circadian pacemaker system. Furthermore, the circadian pattern of VP release can be correlated to the circadian pattern of neuronal activity of the SCN (Gillette & Reppert, 1987). On the other hand, the fact that VP-deficient Brattleboro rats still present intact circadian rhythms is confusing regarding the involvement of VP in the generation and/or transmission of circadian rhythms. The second main SCN peptide, VIP, also shows a diurnal rhythm in its gene expression (Glazer & Gozes, 1994; Ban *et al.*, 1997), content (Isobe & Muramatsu, 1995) and *in vitro* release (Shinohara *et al.*, 1994). In contrast to VP, VIP mRNA expression, and content peak in the middle of the night. In addition, VIP expression is strongly influenced by environmental light conditions. Interestingly, VIP- and PHI-deficient mice present disrupted circadian rhythms as well as deficits in the response to light, suggesting that VIP/PHI peptides are critically involved in both the generation of circadian oscillations as well as in the normal synchronisation of these rhythms (Colwell *et al.*, 2003). Moreover, knock-out mice for the VIP receptor VPAC(2) show even more severe circadian deficits (Harmar *et al.*, 2002) reinforcing the idea of a crucial role for VIP signalling in circadian functioning.

Although the precise location of glutamate neurons within the SCN still needs to be demonstrated, it was recently proven that glutamate is indeed yet another transmitter present in the SCN (Hermes *et al.*, 1996; Sun *et al.*, 2000; Cui *et al.*, 2001; Sun *et al.*, 2001). Until now, its presence was only demonstrated in SCN neurons projecting to target areas, such as ventromedial preoptic area (VMPO), ventrolateral preoptic area (VLPO), or paraventricular nucleus of the hypothalamus (PVN). Although glutamate might not be necessary in the building or in the maintenance of the internal clockwork mechanism, it could play, as VP, an essential role as an output transmitter of the biological clock. This idea was recently strengthened by the demonstration of disrupted circadian output rhythms (locomotor and firing rate neuronal activities) but intact molecular circadian clockwork in mice lacking the enzyme phospholipase C β4, coupled to the metabotropic glutamate receptors in the SCN (Park *et al.*, 2003).

However, although these different transmitters have all been revealed in the SCN, their respective implication in the generation of circadian rhythms varies and still needs further investigation. Assuming that the rhythm of VP measured in the cerebrospinal fluid is reflecting the actual release of VP by the SCN in each of its targets, VP has, until now, been the only SCN transmitter for which the circadian release pattern has been determined *in vivo*. But so far, for none of the other cited neurotransmitters has the actual *in vivo* release pattern been demonstrated in their specific targets, and it cannot be deduced from the knowledge on rhythms in mRNA or peptide content. This lack of knowledge very much complicates the prediction about the role of specific SCN transmitters in the control of, for instance, endocrine rhythms. As long as we cannot measure the actual release of a certain transmitter, the only relevant technical approach is to apply antagonists in the SCN target areas at different times of the L/D-cycle and to measure whether a specific rhythm is affected by this procedure.

Integration Centre

The biological clock is set endogenously to generate a rhythm of approximately 24-h but can be set exactly to 24-h by its synchronization to the environment. In order to be synchronised to the environment the biological clock has to sense the environment. In chronobiology, the entraining or synchronising factors of the clock are named Zeitgebers. In most species the main Zeitgeber is the light/dark cycle. The time-scale used is expressed as Zeitgeber Time and ZT0 corresponds to the beginning of the light period when animals are maintained in light/dark conditions (LD). When animals are maintained in constant darkness (DD), the time scale is expressed as Circadian Time and CT0 corresponds to the onset of the subjective daytime period.

Different Zeitgebers are divided into two major groups, i.e. photic and non-photic.

Photic inputs

Light information, sensed at the level of the retina, is transmitted to the SCN, where it induces an immediate increase of the activity of most SCN neurons (Groos & Meijer, 1985; Meijer *et al.*, 1998). In addition to this immediate effect, light also has a long-term effect on the biological clock. It can, indeed, phase-shift the internal clockwork mechanisms in order to synchronise the period of biological rhythms with the environment. This effect of light on the circadian clock differs along the L/D cycle. Indeed, a similar light pulse applied at the beginning or at the end of the activity period of an animal induces either a delaying phase shift or an advancing phase shift, respectively.

It has been shown, both in rodents and in humans, that the classical visual photoreceptors (rods and cones) are not required for the transmission of light to the circadian system (Lucas *et al.*, 1999; Brainard *et al.*, 2001; Thapan *et al.*, 2001). Recently, Berson *et al.* (2002) demonstrated that a small subset of retinal ganglion cells (RGCs) projecting to the SCN is specifically photosensitive. In parallel, Provencio *et al.* (1998) uncovered the existence of a new putative photopigment, a vitamin-A based molecule named melanopsin, in RGCs. Melanopsin was first discovered in the intrinsically photosensitive melanocyte pigment cells of the frog skin. Melanosomes, the melanin-containing organelles of amphibian melanophores, are dispersed throughout the cytoplasm when the skin is exposed to light, but in darkness the melanosomes aggregate around the nucleus (Rollag *et al.*, 2003). Soon after this discovery it was demonstrated that, in mammals, melanopsin was only present in the specific RGCs projecting to the SCN (Provencio *et al.*, 2000; Gooley *et al.*, 2001; Hannibal *et al.*, 2002; Hattar *et al.*, 2002; Provencio *et al.*, 2002). In addition, the melanopsin sensitivity peak precisely matches the action spectrum of the most potent wavelength for shifting the circadian system, i.e. 450-500 nm (blue-green light) (Takahashi *et al.*, 1984; Miyamoto & Sancar, 1998; Thapan *et al.*, 2001). However, the idea that the melanopsin system was the only means for light transmission to the circadian system had to be revisited when light responses were measured in melanopsin-photopigment deficient mice (Panda *et al.*, 2002; Ruby *et al.*, 2002; Lucas *et al.*, 2003). Indeed, although responses to light were attenuated by 40-45%, these different studies showed that melanopsin is not the "one and only" system of photosensing regarding the circadian system. Recently, Panda *et al.* (2003) have elegantly shown that, although mice lacking melanopsin still retain nonvisual photoreception, mice lacking both the outer-retinal rod and cone system as well as melanopsin, exhibited a complete loss of photosensitivity as well as their photo-entrainment capacity. These recent results clearly revealed the concomitant implication of both visual and nonvisual photoreceptor systems in the nonvisual photic responses in mammals. Consequently, many questions remain to be solved in order to understand fully the photosensitive system related to circadian rhythms.

However, once the light is sensed in the retina, the light information is transmitted to the SCN via both direct and indirect neuronal pathways.

RGCs project down the retino-hypothalamic tract (RHT) (Hattar *et al.*, 2002) and reach directly the ventrolateral part of the SCN. Light stimulation of the retina induces a direct secretion of the excitatory aminoacid neurotransmitter glutamate within the VIP-containing cell part of the SCN (de Vries *et al.*, 1994; Ding *et al.*, 1994; Mikkelsen *et al.*, 1995; Pennartz *et al.*, 2001). Although different glutamate receptors may be involved (Paul *et al.*, 2003), glutamate has been shown to be crucial in the direct effect of light (Vindlacheruvu *et al.*, 1992) as well as in the synchronizing effect of light on the circadian clock, (see for review, Ebling, 1996; Hannibal, 2002). Apart from glutamate, pituitary adenylate cyclase-activating polypeptide (PACAP) is also an important transmitter used to transmit light information. PACAP is co-expressed with glutamate (and melanopsin) in the RHT (Hannibal *et al.*, 2000) and it may potentiate the effect of glutamate in the SCN (Harrington *et al.*, 1999; Kopp *et al.*, 2001). However, recent studies revealed that PACAP is efficient in shifting the clock only during daytime, while glutamate shifts the clock when applied during the night (Gillette & Mitchell, 2002).

In addition to this direct RHT projection, light information is transmitted to the SCN by an indirect neuronal pathway. RGCs also project to neuropeptide Y (NPY) containing cells in the intergeniculate leaflet (IGL) which subsequently project to the ventral part of the SCN via the geniculo-hypothalamic tract (GHT) (Pickard *et al.*, 1987). The rhythm of NPY measured within the SCN during L/D-conditions, with two peaks corresponding respectively to the light/dark and dark/light transition, disappears totally in DD (Calza *et al.*, 1990; Shinohara *et al.*, 1993a; Shinohara *et al.*, 1993b). Since RHT and GHT projection areas seem to overlap within the SCN (Stopa *et al.*, 1995), and since the same set of RGCs innervates the SCN and the IGL (Pickard, 1985; Treep *et al.*, 1995), it seems that the indirect pathway plays a role as a modulator of the light information. On the other hand, ablation of the IGL does not prevent phase shifting to light, but it does prevent arousal-induced phase-shifts. It therefore seems that the IGL and GHT have a predominantly non-photic role (Hastings *et al.*, 1997).

Non-photic inputs

Although light is the main Zeitgeber in most species, including mammals, other cues, too, can have an important function as a Zeitgeber and can synchronise the biological clock. This property for non-photic synchronisation might find its relevance in early life (Reppert & Weaver, 1993; Challet & Pévet, 2003). At a time when the RHT is not mature yet, it may be necessary that the circadian clock of foetus or neonates

senses other cues in order to be in phase with the mother. Indeed, administration of drugs such as benzodiazepines (Turek & Van Reeth, 1989; Cutrera *et al.*, 1994b) or exogenous hormones such as melatonin (Armstrong & Redman, 1985; Pitrosky *et al.*, 1999), or corticosteroids (Pinto & Golombek, 1999) can synchronize the biological clock in the long term and can induce phase-shifting of the biological rhythms. Although (maternal) hormones might be the most important non-photic Zeitgebers, especially during foetal development, arousal and food-availability also have strong Zeitgeber properties in adult life. Indeed, if the light transmission system is disrupted, other cues like arousal (the use of an alarm clock), feeding schedule, forced locomotor activity, or certain drugs taken at a fixed time can then be useful to remain in phase with the environment, (see for review, Mrosovsky, 1996; Challet *et al.*, 2003). Even though non-photic inputs have not been studied as extensively as photic inputs, neuronal inputs seem to be clearly involved. Since lesioning of the IGL induces total disappearance of NPY and enkephalin (two main transmitters expressed in the IGL) within the SCN (Harrington & Rusak, 1986), and since it prevents phase shifting induced by arousal (Hastings *et al.*, 1997), drug injections (i.e. triazolam or chlordiazepoxide) (Biello *et al.*, 1991; Meyer *et al.*, 1993), or temporal and caloric feeding restriction (Challet *et al.*, 1996), the GHT pathway seems to play an important role in the synchronization by non-photic stimuli (i.e. arousal) of the biological clock. Another pathway that might play an important role is the 5-HT-ergic system, originating in the median raphe nucleus, located in the rostral brainstem (Meyer-Bernstein & Morin, 1996; Leander *et al.*, 1998; Morin & Meyer-Bernstein, 1999) in most rodents, and also from the dorsal raphe nucleus in rat (Moga & Moore, 1997). Indeed, lesions of the serotoninergic fibers of the raphe nucleus also prevent phase-shifting induced by triazolam (Cutrera *et al.*, 1994a), 8-OH-DPAT (Schuhler *et al.*, 1998) or temporal and caloric restriction feeding (Challet *et al.*, 1997). However, the precise mechanism involved in the transmission of non-photic inputs to the SCN still needs to be clarified. Certain drugs might act by inducing arousal/activity (Van Reeth *et al.*, 1991; Antle & Mistlberger, 2000) or by activating afferent projections to the SCN, whereas hormones like melatonin might act directly on the SCN (van den Top *et al.*, 2001).

Time dealer

As presented in the previous paragraph, major progress has been made in the past 10 years in the understanding of the molecular clockwork of the biological clock. Indeed, it has been recently shown that SCN cells contain several so-called clock genes, interacting with each other in self-sustained gene-protein-gene feed-back loops (see for review, Reppert & Weaver, 2001; Albrecht & Eichele, 2003). Subsequently, the same clock genes were also found to be expressed rhythmically in other structures,

both in the central nervous system (Yamazaki *et al.*, 2000; Abe *et al.*, 2002) and in the periphery (Damiola *et al.*, 2000; Yamazaki *et al.*, 2000; Hara *et al.*, 2001). But once these structures are isolated, the oscillation of their clock genes is not sustained, suggesting a necessary entrainment or synchronization of "peripheral oscillators" by the master clock (Yamazaki *et al.*, 2000; Balsalobre, 2002). Despite the discovery of the clock genes, however, some important questions still remain. For example, how do we explain the large diversity in the peak activity of different behaviors and endocrine functions with the unimodal activity pattern of the SCN described so far with multi-unit recordings (Inouye & Kawamura, 1979; Shibata *et al.*, 1982), and the 2-deoxy-glucose technique (Schwartz & Gainer, 1977; Schwartz *et al.*, 1980)? Until recently the SCN was considered a "homogenous" structure, giving one global timing signal. However, it is becoming more and more difficult to explain how the SCN is able to control different functions with separately timed 24-h rhythms as diverse as shown in Fig.1, when distributing only one uniform message. Although several hormones are released in a circadian manner, the time of their peak-release varies greatly throughout the light/dark cycle. The picture becomes even more complicated when one realizes that in diurnal and nocturnal species the SCN activity functions in an identical way, i.e. despite the anti-phase of the sleep/wake patterns, the main activity period of the SCN is found during the light period in either type of species (Schwartz *et al.*, 1983; Shibata & Moore, 1988; Hofman & Swaab, 1993). Moreover, only some endocrine rhythms are associated with the timing of behavioural activity, whereas others remain phase-locked to their position within the light/dark-cycle. For example, LH and corticosterone plasma concentrations have their acrophase before the beginning of the activity period (i.e. in the morning for diurnal animals, but in the evening for nocturnal animals), but the acrophase of melatonin and leptin release is always found during the dark period, independent of the nocturnal or diurnal nature of an animal. Yet, the question that arises is how the SCN distributes its message of time, which is built within each of its cells, to the rest of the body? Is a specific population of SCN cells responsible for each specific output? Or, alternatively, are all SCN neurons involved in the creation of one uniform output signal of the biological clock that is differently interpreted by the various clock targets?

The question of how the message of time is distributed by the SCN was first addressed in transplantation studies. The restoration of the circadian rhythms of locomotor activity and drinking behaviour in SCN-lesioned animals, within a week after having received SCN grafts (Ralph *et al.*, 1990), even if encapsulated (Silver *et al.*, 1996a), proved that the SCN has the potential to distribute at least part of its message by simple diffusion of substances in the control of behavioural functions. Indeed, SCN-secreted molecules such as TGF-alpha or prokineticin 2 have recently been suggested to play a role in the control of the circadian rhythm of locomotor activity

(Kramer *et al.*, 2001; Cheng *et al.*, 2002). However, the above-mentioned transplantation studies also indicated that this simple way of distribution could not be exclusive. In contrast with behavioral functions, the rhythmicity of endocrine functions, such as gonadal function or daily cortisol secretion, was not restored in SCN-grafted animals (Lehman *et al.*, 1987; Meyer-Bernstein *et al.*, 1999). Therefore, SCN neuronal connections onto other brain targets seem to be essential to transmit the full circadian information throughout the entire body. Indeed, recently De La Iglesia *et al.* (2003) very elegantly demonstrated the necessity for a neural rather than a humoral SCN output for the daily control of neuroendocrine rhythms. Nevertheless, these first observations supported the idea that the SCN may guard time-homeostasis within the body, using different tools of distribution. As opposed to behavioural rhythms, endocrine daily rhythms require direct and precise regulation, which could be provided by neuronal projections to diverse target areas. SCN projections were extensively studied in the past by tracing and electrophysiological studies (Watts & Swanson, 1987; Watts *et al.*, 1987). Restricted to only a small part of the central nervous system, SCN projections are concentrated within the medial hypothalamus: medial preoptic area (MPOA), paraventricular nucleus (PVN), subparaventricular nucleus (sPVN), and dorsomedial nucleus of the hypothalamus (DMH). In addition, outside the hypothalamus, the SCN only projects onto the paraventricular nucleus of the thalamus and the lateral geniculate nucleus.

In the following paragraphs I will summarise physiological and anatomical evidence previously obtained by our group and others, showing that the SCN uses variable means of signal distribution for each specific endocrine function. In the distribution of its temporal message to the endocrine system the SCN employs a highly specialized set of projections to 3 different types of neurons within the medial hypothalamus. SCN fibers directly contact both endocrine and autonomic neurons. In addition, the SCN can influence endocrine rhythms via its connection to intermediate neurons projecting to endocrine neurons. Moreover, the diversity of neurotransmitters present in the projecting SCN fibers indicates the possibility of a further differential regulation.

SCN output to endocrine neurons

One of the means the SCN uses to control endocrine rhythms involves direct and indirect control of endocrine neurons. The SCN was shown to be able to affect, directly or indirectly, the activity of both magnocellular neurons, liberating VP or oxytocin directly within the blood stream, and of parvocellular hypophysiotropic neurons, containing releasing hormones such as corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) or gonadotropin-releasing hormone (GnRH).

Direct functional connections between SCN and magnocellular neurons have been demonstrated in the supraoptic nucleus of the hypothalamus (SON) (Cui *et al.*, 1997), as well as in the PVN (Hermes *et al.*, 1996). In addition, also the daily rhythm of plasma vasopressin (VP), as measured with a concentration peak during midday, suggests that magnocellular neurons have a diurnal activity (Terwel *et al.*, 1992; Windle *et al.*, 1992). Evidence for direct, but limited, connections between SCN and hypophysiotropic neurons containing releasing hormones has also been presented (Buijs *et al.*, 1993b; Vrang *et al.*, 1995; Van der Beek *et al.*, 1997; Kalsbeek *et al.*, 2000b). Although both magnocellular and hypophysiotropic neurons are connected to the clock, the influence (direct or indirect) of the SCN differs depending on the endocrine function.

In an initial study on clock control of the hypothalamo-pituitary-adrenal (HPA) axis, we showed a strong inhibitory effect of SCN-derived VP on corticosterone release in the PVN/DMH area (Kalsbeek & Buijs, 1992). Both the sPVN and DMH contain γ-amino-butyric acid (GABA) neurons projecting to the medial parvocellular part of the PVN (Roland & Sawchenko, 1993), and are prominent target areas of SCN projections. Since VP is, as reported so far, a stimulatory neurotransmitter, and since direct contacts between SCN and hypophysiotropic neurons in the PVN are limited, we proposed that the SCN inhibits the HPA axis mainly by using indirect VPergic inputs on inhibitory intermediate neurons in the sPVN and DMH. This idea was confirmed later on by two successive studies. In the first of these two studies, we showed that local infusions of VP within the DMH immediately decreased plasma corticosterone, as well as the adrenocorticotropin hormone (ACTH) levels, in both SCN-intact and SCN-lesioned animals (Kalsbeek *et al.*, 1992). In addition, application of a VP-antagonist in the same structure caused a strong increase of plasma corticosterone levels, but only in intact animals, confirming that the VP signalling involved in the inhibition of the HPA axis derives directly from the SCN. In the second study (Kalsbeek *et al.*, 1996c), VP-antagonist application at different times of the light/dark cycle revealed the existence of an additional stimulatory input from the SCN to the HPA axis. Moreover, using electrophysiological techniques we were able to show, *in vitro*, that VP increases the GABA-ergic inhibition of PVN neurons (Hermes *et al.*, 2000). These results led to a hypothesis, where the corticosterone circadian rhythm results from combined inhibitory (via VPergic release onto GABA-ergic interneurons) and stimulatory SCN outputs of unknown nature (Kalsbeek & Buijs, 1996). In contrast to the indirect connections to the hypophysiotropic neurons, the SCN seems to have a direct influence on magnocellular neurons of the PVN. Indeed, specific VP depletion in the SCN affects the VP content of magnocellular *versus* parvocellular (i.e. hypophysiotropic neurons) PVN neurons in opposite directions (Gomez *et al.*, 1997). VP content increases in magnocellular PVN neurons, whereas it decreases in parvo-

cellular neurons, suggesting decreased release of VP in the general circulation, and an increased release of VP (and CRH) into the portal circulation. Regarding the fact that elevated plasma ACTH levels were measured after such SCN-VP depletion, these results suggest a major inhibitory action of the SCN on the HPA axis. Plasma VP (and oxytocin) levels, however, were not measured, and a physiological confirmation of the stimulatory effect of SCN-VP on the magnocellular system is thus lacking. In the meantime the inhibitory effect of VP on corticosterone release as just described has been reported by a number of other laboratories as well (Wotjak *et al.*, 1996; Gomez *et al.*, 1997), but still the effect is very surprising to many (endocrinologists), since the inhibitory action of VP is completely opposite to the previously described and better known stimulatory role of VP in the HPA stress response. The stimulatory action of VP on ACTH and corticosterone release was definitely established in the early eighties (Gillies *et al.*, 1982; Rivier & Vale, 1983; Rivier *et al.*, 1984). It turned out that VP strongly potentiated the stimulatory action of the then recently discovered true corticotrophin releasing factor, i.e. CRH (Vale *et al.*, 1981). Indeed, shortly afterwards it was shown that CRH and VP are co-localized in the parvocellular neurons of the PVN (Tramu *et al.*, 1983; Kiss *et al.*, 1984; Sawchenko *et al.*, 1984) and that many stimuli that cause an activation of the HPA-axis, such as immobilization, novelty, and hypoglycemia, go along with a depletion of the CRH as well as the VP content in the external zone of the median eminence (Berkenbosch *et al.*, 1989; De Goeij *et al.*, 1992; Bartanusz *et al.*, 1993; Romero *et al.*, 1993). The emerging view is that VP is the principal, regulated, variable that puts a situation-specific drive on the HPA-axis, whereas CRH serves mainly to impose a stimulatory tone (Kovacs *et al.*, 2000); i.e. under resting conditions the parvocellular VP neurons of the PVN do not contain detectable amounts of VP immunoreactivity or mRNA. The logical explanation for the apparently opposite actions of VP, of course, is that the different effects are caused by two completely different and separated VP systems. The inhibitory effect concerns VP-containing neurons located in the SCN projecting to the PVN and DMH, whereas the stimulatory effect of VP is derived from the parvocellular VP neurons located in the PVN that project to the median eminence. In addition, comparing the responses of ACTH and corticosterone in more detail also reveals that the two systems employ different mechanisms to affect the HPA-axis. The circadian variation in HPA-axis activity is mainly controlled by changing the sensitivity of the adrenal cortex to ACTH, whereas the stress response of the HPA-axis relies heavily on the release of ACTH. A further discussion of the different VP systems and the close relation between VPergic systems, glucocorticoids and stress can be found in Kalsbeek *et al.* (2002, 2003).

The VP-mediated SCN control of the HPA axis is not exclusive; in addition, the SCN is thought to use its main peptidergic output, i.e. VP, to control the hypothalamo-pituitary-gonadal (HPG) axis as well, in a direct and indirect manner. The two essential

brain areas for maintaining the LH surge are the MPOA and the SCN (Gray *et al.*, 1978). The MPOA contains a dense concentration of estrogen receptors and GnRH neurons, as well as a dense VP innervation originating from the SCN (Hoorneman & Buijs, 1982; de Vries *et al.*, 1984). Once every 4-5 days, an interaction between the peripheral endocrine estrogen signal and the circadian output leads to a strong GnRH release in the portal circulation, and consequently to a pro-oestrus surge of LH. Direct connections between the SCN and neurons in the MPOA have been shown (de la Iglesia *et al.*, 1995; Watson *et al.*, 1995; Van der Beek *et al.*, 1997); 11-13% of GnRH neurons and up to 30% of the neurons containing estrogen-receptors receive direct efferent projections from the SCN. Since VP infusions within the MPOA are able to restore the LH surge in SCN-lesioned animals, irrespective of the time-of-day (Palm *et al.*, 1999; Palm *et al.*, 2001b), and since, *in vitro*, GnRH release exhibits a significant circadian rhythm in phase with the circadian release of VP from the SCN (Funabashi *et al.*, 2000), we concluded that the SCN uses its VP-ergic output as a stimulatory signal for the control of the HPG axis. We proposed that the SCN would stimulate the release of GnRH indirectly via oestrogen-receptor containing neurons of the MPOA. Interestingly, VP administration within the MPOA could stimulate the LH surge in intact animals only during a restricted time window, whereas in SCN-lesioned rats the LH surge could be induced at any time. These results also suggest that apart from the main stimulatory input, the SCN also sends an inhibitory input of unknown nature during the first part of the day to restrain the GnRH system. Since neurons in the MPOA are also contacted by the SCN in an indirect way via the subparaventricular zone of the PVN or the retrochiasmatic area, and since direct projections from SCN neurons containing vasoactive intestinal polypeptide (VIP) onto the endocrine GnRH-containing neurons have also been demonstrated (van der Beek *et al.*, 1993; Van der Beek *et al.*, 1997), further investigations will be necessary to understand fully the SCN control of the GnRH system. Also for a further discussion of the multiple interactions of central VP systems and reproductive hormones see Kalsbeek *et al.* (2002).

Other examples of the differential SCN regulation of endocrine neurons can be found in the clock control of prolactin release and the hypothalamo-pituitary-thyroid (HPT) axis. In order to control the pro-oestrus daily surge of prolactin (PRL) the SCN also uses multiple outputs. Like the LH surge, the PRL surge is induced by a concomitant positive estrogen feedback and SCN signalling. We propose that the SCN uses several ways to impose its main inhibitory input. During the first part of the light period, PRL release is mainly inhibited by VP release from SCN terminals in the MPOA (Palm *et al.*, 2001a), probably by stimulating MPOA projections to dopaminergic neurons in the tuberoinfundibular area (Mai *et al.*, 1994). Moreover, two successive studies have revealed direct SCN connections, especially VIP-containing fibers,

onto hypothalamic dopaminergic neuroendocrine neurons that may also have an important role in the inhibitory control of the PRL surge (Horvath, 1997; Gerhold *et al.*, 2001). A number of hypothalamic transmitters have been proposed to function as prolactin-releasing factors, such as VIP, serotonin or oxytocin (Johnston & Negro-Vilar, 1988; Arey & Freeman, 1989; Murai *et al.*, 1989; Mai & Pan, 1990). However, it is not yet known which one is "used" by the SCN to stimulate the pro-oestrous PRL surge.

In addition, hypothalamic TRH neurons also show a daily rhythmicity in TRH mRNA (Covarrubias *et al.*, 1988) and in peptide content (Collu *et al.*, 1977; Martino *et al.*, 1985). Moreover, *in vitro* TRH release has also shown diurnal variations (Covarrubias *et al.*, 1994). Recently, we showed the existence of direct contacts between SCN fibers and TRH neurons in the parvocellular part of the PVN as well as their functionality in the control of thyroid hormone rhythmicity (Kalsbeek *et al.*, 2000b). The results of this study indicate that the SCN has a stimulatory action on thyroid-stimulating hormone (TSH) and the secretion of thyroid hormones during the daytime. The daytime increase of plasma TSH levels corresponds with the time period of maximum VP release from the SCN, but so far we have been unable to find effects of VP administration or withdrawal on plasma TSH concentrations (Kalsbeek et al., unpublished observations). Next to the direct and indirect connections to endocrine neurons, the SCN also seems to use another signalling pathway to guard the temporal homeostasis of endocrine functions. Connections between the SCN and pre-autonomic neurons in the PVN that are involved in the autonomic innervation of the thyroid gland and ovary indicate that the SCN might also use the autonomic nervous system to control the circadian activity of the HPT (Kalsbeek *et al.*, 2000b) and HPG axis (Gerendai *et al.*, 1998; 2000), respectively.

SCN output to pre-autonomic neurons

Next to its output towards endocrine neurons, the SCN utilizes a second pathway to control the endocrine temporal homeostasis throughout the body, the autonomic nervous system. Recent evidence shows the importance of the autonomic nervous system in mediating the SCN control of endocrine rhythms. For example, in the HPA axis, the initial indications for a circadian neuronal control of ACTH sensitivity of the adrenal gland through the autonomic nervous system (Kaneko *et al.*, 1980; Kalsbeek *et al.*, 1996c; Buijs *et al.*, 1997) have been confirmed more recently by further physiological and transneuronal tracing studies. Specific lesions of adrenal gland sympathetic innervation eliminate the diurnal corticosterone rhythm and suggest that the diurnal rhythm in corticosterone secretion results in part from neuronal inhibitory control (Jasper & Engeland, 1994). Later on, thanks to transneuronal tracing, we established

the neuronal pathway connecting the SCN and the adrenal cortex, which successively relays to the PVN and the spinal cord (Buijs *et al.*, 1999). Moreover, we showed that activation of the SCN by light immediately inhibits corticosterone, but not ACTH, secretion. Thus, in addition to the endocrine control of the circadian rhythm of corticosterone via an inhibitory action onto endocrine neurons, the SCN also controls the daily corticosterone rhythm by setting adrenal gland ACTH sensitivity via its autonomic innervation. Besides the combined endocrine and neuronal regulation of the HPA axis, also other endocrine systems, such as the HPT- and HPG-axis, may be controlled by the SCN in such a "dual" fashion. However, in these systems a clear example of the "sensitivity-setting" influence of the SCN is still lacking.

As illustrasted in Figure 3, next to the combined control by endocrine and neuronal inputs, there are also examples of endocrine systems in which SCN control seems to occur mainly via its autonomic connections. The circadian control of metabolic hormones such as insulin, glucagon and leptin provide a good example of the control of endocrine rhythms via the autonomic nervous system. In the past 5 years we have provided a large amount of evidence for such an SCN control. Using the regular

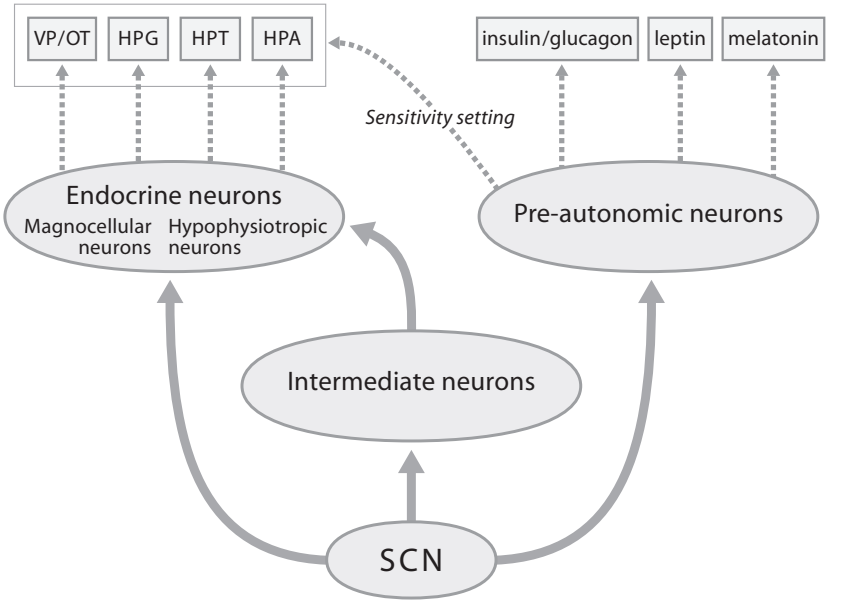

Figure 3 Review scheme of the various SCN projections controlling the rhythmicity of endocrine functions. Three main types of SCN connections can be discerned: connections with endocrine, pre-autonomic and intermediate neurons. In addition to this diversity of connections the SCN uses different neurotransmitters in order to fine-tune its control of each specific endocrine function.

schedule feeding method we were able to prove that insulin responses, plasma glucose, plasma glucagon, and plasma leptin levels follow daily rhythms, independent of acute effects of feeding activity (Kalsbeek & Strubbe, 1998; La Fleur *et al.*, 1999; Kalsbeek *et al.*, 2001, 2003; Ruiter *et al.*, 2003). Using the retrograde tracing technique we were able to demonstrate neuronal connections relaying information from the SCN to the peripheral organs secreting these hormones, i.e. the pancreas, the liver and the adipose tissue (Kalsbeek *et al.*, 2000b; la Fleur *et al.*, 2000). Moreover, additional tracing studies realized after trans-section of either the sympathetic or parasympathetic autonomic innervation of these different organs revealed that both divisions of the autonomic nervous system could be involved in the circadian control of metabolic hormones (Buijs *et al.*, 2001; Kreier *et al.*, 2002). Very recently we were able to demonstrate that, even within the SCN, different neurons are dedicated to the control of the sympathetic and parasympathetic branches of the autonomic nervous system (Buijs *et al.*, 2003a).

But the best-known example for a total control of the rhythmicity solely via autonomic connections can be found in the daily release of melatonin by the pineal gland. Many studies revealed the close relation between this endocrine function and the biological clock. As early as the beginning of the nineteenforties, Bargman (1943) suggested that the endocrine function of the pineal gland was regulated by light, via the central nervous system. In 1960, Kappers revealed a clear sympathetic innervation of the pineal gland in the rat. Soon the endocrine function of this gland was linked to the external light/dark cycle and its influence on reproductive functions (Kappers, 1971). In the late 1950s, the hormone synthesized and released by the pineal was identified as N-acetyl-5-metoxytryptamine, and named melatonin by Lerner *et al.* (1958). Guided by the pathways of light into the brain 15 years later, the endogenous biological clock was located in the SCN (Hendrickson *et al.*, 1972; Moore & Eichler, 1972; Moore & Lenn, 1972; Stephan & Zucker, 1972). Meanwhile, pineal melatonin content, with low levels during the day and high levels during the night, was among the first biological rhythms described and characterized as a true circadian rhythm (Lynch, 1971; Ralph *et al.*, 1971). In the meantime, the functional importance of the sympathetic innervation of the pineal gland in melatonin synthesis had been demonstrated in rat (Wurtman *et al.*, 1967; Klein *et al.*, 1971). Later on, the crucial role of the sympathetic innervation on melatonin synthesis has been further confirmed in many species (Wurtman *et al.*, 1967; Axelrod, 1974; Kneisley *et al.*, 1978; Moore, 1978; Reiter *et al.*, 1982; Bowers *et al.*, 1984; Li *et al.*, 1989; Drijfhout *et al.*, 1996b). Besides the discovery of the sympathetic control of melatonin synthesis, numerous findings helped us to understand the noradrenalin (NA)-induced neurochemical synthesis pathway of melatonin itself (see for review, Simonneaux & Ribelayga, 2003). Really early, Klein & Weller (1970) have revealed that one of the enzymes responsible for melatonin syn-

thesis, the arylalkylamine N-acetyltransferase (AA-NAT), also showed a clear day-night rhythm. Later on, further studies have confirmed that the activity of AA-NAT was also dependent on noradrenergic stimulation, mainly through β_1-adrenergic receptors associated with a potentiative action of α_1-adrenergic receptors (Klein *et al.*, 1971; Yuwiler *et al.*, 1977; Klein & Moore, 1979; Klein *et al.*, 1983b). Further studies have shown additionally that once the NA-induced melatonin synthesis pathway was activated, it caused a 150-fold increase of the expression of the *Aa-nat* gene as well as a 50 to 70-fold increase of AA-NAT enzyme activity, inducing an immediate 10-fold rise in the synthesis and secretion of melatonin (Reiter, 1991b; Borjigin *et al.*, 1995; Roseboom *et al.*, 1996). Melatonin is then immediately released into the extracellular fluidand diffuses into the circulation (Fig. 4). Although the role of the sympathetic pineal innervation in the synthesis of melatonin was clearly demonstrated, it was only recently that the total pathway could be mapped, using the retrograde trans-synaptic virus *in vivo* tracing technique (Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999). The SCN is connected to the pineal gland via neurons of the PVN projecting to preganglionic neurons in the intermediolateral nucleus of the thoracic spinal cord, which subsequently project to the noradrenergic neurons of the superior cervical ganglion (SCG). Although the total neuronal pathway was clearly defined by virtue of the viral tracing studies, the respective roles of each of the relay stations in the control of the melatonin synthesis rhythm still remain to be determined. Besides, the different neurotransmitters used in each step of the pathway, as well as their specific daily pattern of release, remain to be brought to light.

The necessity of the SCN for the daily rhythmicity of melatonin synthesis was rapidly shown in the first SCN lesions studies (Moore & Klein, 1974; Bittman *et al.*, 1989; Tessonneaud *et al.*, 1995). VP being released by the SCN with a phase which is the reverse of that of melatonin release from the pineal gland, i.e. with a peak release during the light period, became the first neurotransmitter proposed to have an inhibitory role in the control of melatonin rhythm. Nevertheless, the first studies using VP-deficient Brattleboro rats did not reveal any difference in the pineal melatonin synthesis except for the phase of the rhythm (Schroder *et al.*, 1988; Reuss *et al.*, 1990). In addition, local VP application within the PVN did not decrease nocturnal melatonin release (Kalsbeek *et al.*, 1993), suggesting the involvement of another neurotransmitter. Subsequently, due to the light sensitivity of the VIP neurons in the SCN as described in the foregoing, and the well-known inhibitory effects of light on melatonin release, the possible involvement of the VIP neurons was investigated. But again no clear functional role for this peptide could be found (Schroder *et al.*, 1989; Simonneaux *et al.*, 1990; Kalsbeek *et al.*, 1993). In the meantime, more and more evidence pointed at the potential importance of GABA in SCN functioning. GABA was shown to be abundantly present in the SCN, even in projecting cells (van den Pol & Gorcs, 1986;

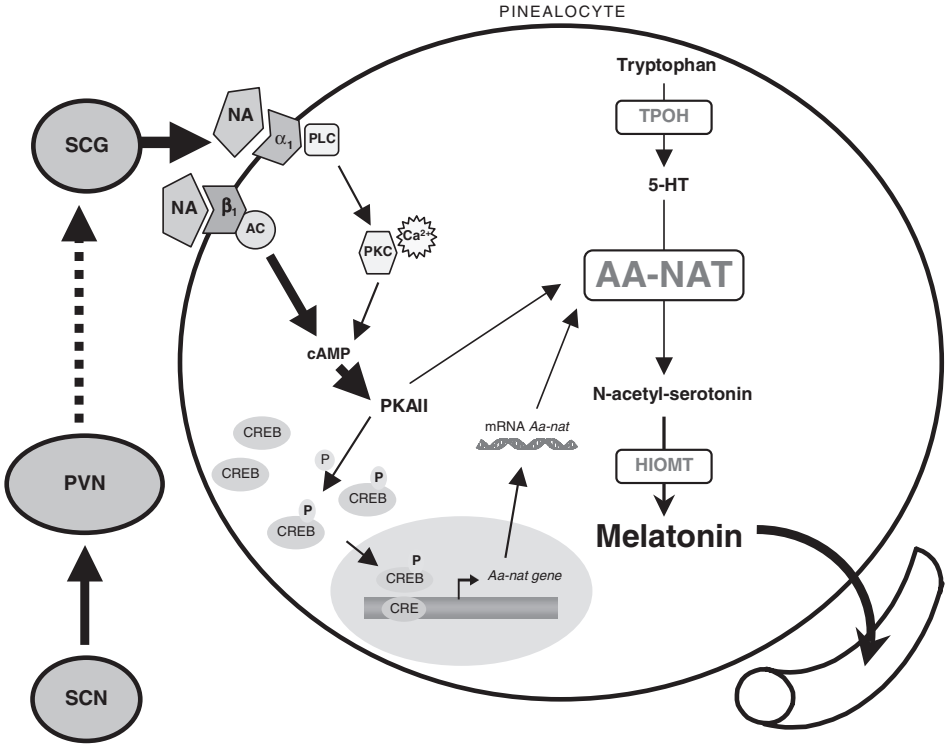

Figure 4 Neurochemical pathway of melatonin synthesis. The enzyme tryptophan hydroxylase (TPOH) transforms tryptophan in serotonin (5-HT), which in turn is transformed by the AA-NAT in N-acetyl-serotonin. In a last step, the enzyme hydroxyindole-O-methyltransferase (HIOMT) O-methylates the N-acetyl-serotonin to form the N-acetyl-5-methoxytryptamine or melatonin, immediately secreted. The activation of the enzyme AA-NAT is the rate-limiting factor of this neurochemical pathway and depends on noradrenergic stimulation. The β1-adrenergic receptor stimulation by NA increases cAMP accumulation via adenylate cyclase (AC) activation. Increased cAMP levels activate protein kinases (i.e. PKAII) that, in one hand, induce the phosphorylation of CREB that can interact with the CRE domain of the *Aa-nat* gene and thereby induce transcription. On the other hand, PKAII also stimulates AA-NAT enzymatic activity. In addition, α1-adrenergic receptors potentiate the synthesis of melatonin by increasing cAMP levels via activation of phospholipase C, protein kinase C and Ca2+ mobilisation.

Okamura *et al.*, 1989; Moore & Speh, 1993; Buijs *et al.*, 1994; Hermes *et al.*, 1996), and we decided to test the functionality of this GABA-ergic output. As a first step we were able to mimic the inhibitory effect of nocturnal light exposure by administration of the GABA-agonist Muscimol to the PVN (Kalsbeek *et al.*, 1996a). In a follow-up study, we were able to prevent the inhibitory effect of light on nocturnal melatonin release by infusing the GABA antagonist Bicuculline within the PVN, which demon-

strated that GABA release in the PVN is necessary for the light-induced inhibition of melatonin synthesis in rat (Kalsbeek *et al.*, 1999). Finally, we were able to show that GABA was also involved in the circadian inhibition of melatonin synthesis, i.e. independent of its role in the direct inhibitory effect of light. Indeed, blocking GABA-ergic transmission in the PVN during (subjective) daytime increases melatonin synthesis (Kalsbeek *et al.*, 2000c). These results strongly suggest that the SCN controls the rhythm of melatonin synthesis by imposing an inhibitory GABA-ergic signal onto the PVN→pineal pathway during (subjective) daytime, assuming a constant stimulatory role for the PVN.

SCN output and peripheral oscillators

The identification of clock genes in numerous tissues other than the SCN, including neuroendocrine cells (Kriegsfeld *et al.*, 2003) and peripheral endocrine tissues (Fukuhara *et al.*, 2000; Bittman *et al.*, 2003), has forced us (i.e. chronobiologists) to consider a completely new view on the role of the SCN in the control of peripheral rhythms. Instead of viewing the periphery as a passive tissue awaiting the signals from the SCN in order to anticipate changes in the outside environment, it now seems possible that peripheral tissues can oscillate independently from the SCN and, in fact, anticipate the output from the SCN. In the foregoing we proposed that the SCN controls peripheral rhythms via a dual mechanism by using its connections to both the endocrine and pre-autonomic neurons in the hypothalamus. For instance, the daily corticosterone peak is controlled not only by inducing the release of CRH from the endocrine neurons in the hypothalamus, and the subsequent release of ACTH from the pituitary, but also by setting, at the same time, the sensitivity of the adrenal cortex to ACTH via the autonomic pathway (Jasper & Engeland, 1994; Buijs *et al.*, 1999). In a similar way, just before the onset of a period of increased activity during the waking hours of the L/D-cycle, the SCN not only increases insulin sensitivity in order to enable the necessary increased glucose uptake by skeletal and heart muscles, but also stimulates hepatic glucose production in order to prevent hypoglycemia due to the increased glucose uptake (la Fleur *et al.*, 2001). The existence of peripheral oscillators suggests that these changes in sensitivity (i.e. for ACTH or insulin) could also be due to cell-autonomous oscillations of peripheral tissues (Young & Kay, 2001; Kita *et al.*, 2002; Young, 2003), instead of SCN control. However, the rhythmic capacity of peripheral tissues disappears in SCN-lesioned animals (Akhtar *et al.*, 2002; Terazono *et al.*, 2003) and, *in vitro* the peripheral oscillator rhythms dampen after 2-7 days (Yamazaki *et al.*, 2000). In addition, grafts of SCN tissue are sufficient to restore rhythms even in genetically arrhythmic mice, i.e. lacking central as well as peripheral oscillators (Sujino *et al.*, 2003). Therefore, peripheral oscillators are clearly dependent

on SCN inputs to maintain their rhythmicity. At present we see two important functions for the peripheral oscillators: 1) local clocks will help to enhance the SCN signal and enable peripheral tissue to maintain rhythmicity during a short-term absence of SCN input (Young & Kay, 2001), and 2) local clocks help to synchronize the activity of an organ and as a consequence they also help to enhance the feedback signal to the hypothalamus (Buijs & Kalsbeek, 2001). Taking into consideration the multiplicity of SCN outputs, as well as the multiplicity of their targets, opens the door to a better understanding of the way the SCN is able to distribute its time-of-day information throughout the body, and "body guard" homeostasis.

C. SCOPE OF THE THESIS

The daily rhythm of melatonin release as mentioned above was discovered long ago, as well as the necessity of SCN functioning to maintain this rhythm. However, the exact way in which the SCN controls the melatonin synthesis rhythm (i.e. the exact effect of the SCN on melatonin synthesis as well as the nature of the transmitters involved in this control) remained completely unknown until a few years ago. The experiments described in this thesis were initiated to elucidate further the circadian control of the daily melatonin rhythm and to test the above mentioned "inhibition" hypothesis launched in 2000 and illustrated in Fig.5, stating that: "The SCN controls the daily rhythm of melatonin synthesis by imposing an inhibitory signal onto the PVN→pineal pathway during (subjective) daytime".

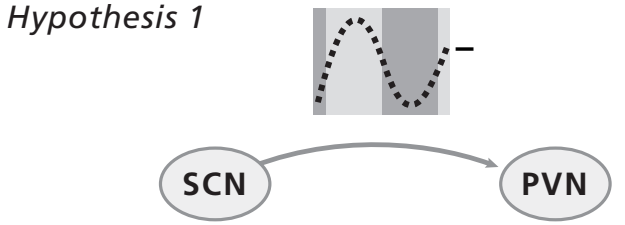

Figure 5 Schematic illustration of the "inhibition" hypothesis.

One major assumption for this hypothesis to work is that the PVN provides a constant stimulatory input to the sympathetic system that innervates the pineal gland. A major prediction that can be deduced from this hypothesis states that SCN lesions will cause a continuous release of melatonin at a level equal to the nighttime levels of SCN-intact animals. However, previous studies measuring either plasma melatonin concentration, enzymatic activity or gene expression of the main enzyme involved in the melatonin synthesis pathway, i.e. arylalkylamine N-acetyltransferase (AA-NAT), in SCN-lesioned animals, have, so far, only reported low to intermediate levels of pineal activity, and therefore seem to contradict this prediction (Raisman & Brown-Grant, 1977; Reppert *et al.*, 1981; Bittman *et al.*, 1989; Tessonneaud *et al.*, 1995; Kalsbeek *et al.*, 1996a). Nevertheless, so far no study has reported a comparison of the respective implications of the 3 main structures involved in the SCN-pineal pathway, i.e. SCN, PVN, and SCG, within one study and using the same method. Therefore, in the first study, presented in Chapter 2, we realised this comparison by lesioning or removing specifically either the SCN, the PVN or the SCG, and compared for the first time, thanks to the *in situ* hybridisation technique, their respective effects on *Aa-nat*

gene expression, which reflects melatonin synthesis the best. Interestingly, in contrast with our "inhibition" hypothesis, the results obtained revealed that both acrophase and nadir of the melatonin rhythm are driven by the SCN, and that PVN and SCG play a simple role of relay-station to the pineal gland. Consequently, we had to renew our concept and proposed that in order to control the daily rhythm of melatonin synthesis the SCN uses a combination of both inhibitory (during daytime) and stimulatory signals towards the PVN→pineal pathway.

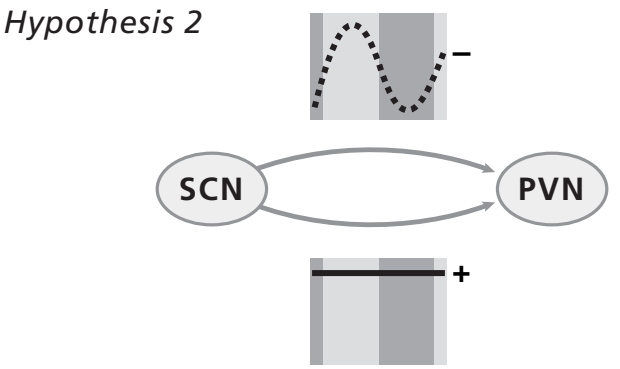

Figure 6 Schematic illustration of the new "combination" hypothesis.

The results of this first study indicated, also for the first time, a functional role for the nocturnal activity of the SCN. However, the SCN had until then been considered a daytime functioning structure, due to the fact that its main activity period (metabolic and electric) was situated in the (subjective) light period. Hence, it appeared necessary to check the concept of a nocturnal stimulatory SCN output in an acute and *in vivo* situation. In addition, it was of great interest to identify the (chemical) nature of this stimulatory signal coming from the SCN. These two points were successively addressed in the second study, presented in Chapter 3, using the multiple microdialysis technique. At first we tested the effect of an acute and temporary shutdown of either the PVN or the SCN during the dark period, on melatonin release. In addition, we examined the role of glutamatergic signalling from the SCN to the PVN-pineal pathway on the melatonin rhythm generating system.

Another assumption of the original "inhibition" hypothesis is that the early morning decline of melatonin release is due to the increased release of GABA onto pre-autonomic PVN neurons. According to the new "combination" hypothesis, the morning decline of melatonin would be due to a combination of increasing GABA release and a withdrawal of glutamate release onto the pre-autonomic PVN neurons. In order

to test further the importance of the rhythmic GABA release in both hypotheses we infused the GABA-antagonist Bicuculline in the PVN during the dark/light transition (i.e. ZT21-ZT4). According to the original "inhibition" hypothesis should administration of the GABA-antagonist be sufficient to delay the early morning decrease of melatonin until at least ZT4. However, yet another possibility for explaining the early morning decrease of melatonin is to assume that, next to GABA and glutamate, another signal might be involved in the control of the daily melatonin rhythm. For instance, previously it has been suggested that the intrapineal accumulation of ICER could be responsible for the arrest of melatonin synthesis at the end of the dark period. Therefore, it was crucial to first check *in vivo* that the decline of melatonin at the end of the night was actually induced by a clock arrest of the sympathetic release of NA. Therefore, we artificially prolonged the noradrenergic stimulation of the pineal gland, and measured its ability to produce melatonin at the transition from dark to light. These experiments are described in Chapter 4.

During the course of the above experiments a slow increase of evidence was notable in the literature for multiple clock outputs as well as for a clear compartmentalisation of the SCN. Moreover, with specific subdivisions of the SCN showing *Per* genes expression along the light/dark cycle (Yan & Okamura, 2002), it became of great interest to couple the activation of specific subsets of SCN cells with the onset/offset of melatonin synthesis. This aim was pursued in Chapter 5. Since it takes at least 5 days for the nocturnal peaks of AA-NAT activity and melatonin secretion to reappear after an 8-h phase advance of the L/D cycle (Illnerova, 1989; Kennaway, 1994; Drijfhout *et al.*, 1997; Kalsbeek *et al.*, 2000a), and since such an abrupt shift also affects the spatial and temporal distribution of *Per* gene expression in the SCN (Nagano *et al.*, 2003), we decided to use this experimental paradigm to investigate whether we could correlate *Per* gene expression in a subpopulation of SCN neurons with the reappearance of either the onset or offset of the melatonin peak.

Finally, Chapter 6 summarises the results and conclusions, and discusses them in the framework of the most recent (i.e. December 2003) existing literature. Chapter 6 sums up what the present experiments have added to our knowledge on the output mechanisms of the SCN, and what new questions have evolved and remain to be resolved for a more detailed understanding of the SCN output mechanisms. Altogether, the studies in this thesis present a new concept of the control of the daily melatonin rhythm by the biological clock. In fact, this concept may also facilitate the understanding of other physiological and hormonal rhythms controlled by the biological clock.

New concept

Multiple clock outputs control the daily rhythm of melatonin

CHAPTER 2

Suprachiasmatic control of melatonin synthesis in rat: inhibitory and stimulatory mechanisms

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Abstract

The suprachiasmatic nucleus (SCN) controls the circadian rhythm of melatonin synthesis in the mammalian pineal gland by a multisynaptic pathway including, successively, preautonomic neurons of the paraventricular nucleus (PVN), sympathetic preganglionic neurons in the spinal cord and noradrenergic neurons of the superior cervical ganglion (SCG). In order to clarify the role of each of these structures in the generation of the melatonin synthesis rhythm, we first investigated the day- and night-time capacity of the rat pineal gland to produce melatonin after bilateral SCN lesions, PVN lesions or SCG removal, by measurements of arylalkylamine N-acetyltransferase (AA-NAT) gene expression and pineal melatonin content. In addition, we followed the endogenous 48-h-pattern of melatonin secretion in SCN-lesioned vs. intact rats, by microdialysis in the pineal gland. Corticosterone content was measured in the same dialysates to assess the SCN lesions effectiveness. All treatments completely eliminated the day/night difference in melatonin synthesis. In PVN-lesioned and ganglionectomised rats, AA-NAT levels and pineal melatonin content were low (i.e. 12% of night-time control levels) for both day- and night-time periods. In SCN-lesioned rats, AA-NAT levels were intermediate (i.e. 30% of night-time control levels) and the 48-h secretion of melatonin presented constant levels not exceeding 20% of night-time control levels. The present results show that ablation of the SCN not only removes an inhibitory input but also a stimulatory input to the melatonin rhythm generating system. Combination of inhibitory and stimulatory SCN outputs could be of a great interest for the mechanism of adaptation to day-length (i.e. adaptation to seasons).

Introduction

In mammals, most physiological functions (such as hormone release, metabolism, body temperature) show endogenous circadian rhythms that are controlled by the

biological clock located in the suprachiasmatic nucleus (SCN) (Buijs & Kalsbeek, 2001) and synchronised to the light/dark environment via the retino-hypothalamic tract (Klein & Moore, 1979). One of these functions is the nocturnal synthesis of melatonin in the pineal gland. The SCN is connected to the pineal gland by a multi-synaptic pathway which successively includes neurons of the paraventricular nucleus of the hypothalamus (PVN), sympathetic preganglionic neurons of the intermedio-lateral cell column of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion (SCG) (Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999). In rodents, the release of noradrenaline into the pineal gland during the night (Drijfhout *et al.*, 1996c) stimulates the melatonin synthesis pathway. The activation of β_1-adrenergic receptors (Reiter, 1991a) causes a 150-fold increase of the gene expression of the arylalkylamine N-acetyltransferase (AA-NAT) and a 50-70-fold increase of its enzymatic activity. This process immediately induces a 10-fold rise in the synthesis and secretion of melatonin (Roseboom *et al.*, 1996).

The brain regions involved in the control of melatonin synthesis were identified by previous lesion (Moore & Klein, 1974; Klein *et al.*, 1983a; Lehman *et al.*, 1984; Bittman *et al.*, 1989; Tessonneaud *et al.*, 1995; Garidou *et al.*, 2001) and tracing studies (Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999). However, the respective roles of these structures in the control of melatonin synthesis generating rhythm have not been elucidated yet. Besides, the different neurotransmitters used in each step of the pathway, as well as their specific daily pattern of release, remain to be brought to light. Recently, we demonstrated that, in rats, γ-aminobutyric acid (GABA) release in the PVN is involved in the light-induced inhibition of melatonin synthesis (Kalsbeek *et al.*, 1999) and that blocking GABA-ergic transmission from the SCN to the PVN during (subjective) daytime increases melatonin synthesis (Kalsbeek *et al.*, 2000c). These results suggest that the SCN controls the rhythm of melatonin synthesis by imposing an inhibitory GABA-ergic signal onto the PVN→pineal pathway during (subjective) daytime. We consequently hypothesised that the PVN would sustain a constant stimulatory drive to the pineal gland, using the SCG as a simple relay.

This study aimed to clarify the respective role of the structures controlling the melatonin generating rhythm system. For the first time within the same experiment, we compared the effect of SCN lesions, PVN lesions and SCG removal on day- and night-time pineal AA-NAT gene expression in rats. In addition, in order to understand further the daily pattern of SCN outputs, we determined and compared the effect of SCN lesions on long-term (i.e. 48-h) melatonin and corticosterone secretion profile. Following our hypothesis, the removal of the influence of the SCN (inhibitory output) would result in a constant stimulation of the pineal activity and, consequently, in constantly elevated levels of melatonin release. Preliminary data have been presented in abstract form (Perreau, 2001).

Materials and methods

Animals

In all experiments we used male Wistar rats (Harlan, Zeist, the Netherlands) kept in a temperature-controlled environment under 12 h light/12 h dark conditions and fed *ab libitum*. Surgery was performed under anaesthesia (see below) 1 week after the arrival of the rats. During this week they could acclimatize to the new laboratory environment, housed at five animals per cage. After surgery, they were housed individually. After each surgical procedure and after the postanaesthesia arousal of the rats, we subcutaneously injected them with a depressant analgesic opiate (buprenorfine hydrochloride, 0.324 mg in 50 mg dextrose; Temgesic®, Reckitt & Colman Products Ltd, Kingston-Upon-Hull, UK; 0.03 mL/100 g) in order to reduce postoperative pain. All our experiments were conducted under the approval of the Animal Care Committee of the Royal Netherlands Academy of Sciences.

Experimental set-up

EXPERIMENT 1 (EXP1)

This experiment aimed to study the respective role of each of the following brain areas in the control of melatonin synthesis: SCN, PVN and SCG. We therefore studied the effect of bilateral thermic lesions of either the SCN or the PVN, and the effect of ganglionectomy on the day and night AA-NAT mRNA and pineal melatonin levels. We divided the animals in five groups: a group of SCN-lesioned rats, a group of PVN-lesioned rats, a group of ganglionectomised rats, a group of Sham-operated rats (electrodes lowered at the SCN coordinates but not heated), and a group of intact (nonoperated) rats. Half of the animals in each group were killed (anaesthetised with CO_2/O_2 and decapitated) in the middle of the light period (from Zeitgeber time, ZT5 to ZT7) and the other half in the middle of the dark period (from ZT17 to ZT20). We collected the brains taking care that each pineal gland stayed in place. The rostral part of the brain, containing the hypothalamic area, was put in fixative (paraformaldehyde 4%), while the caudal part, with the pineal gland, was frozen on liquid nitrogen steam.

EXPERIMENT 2 (EXP2)

In the second experiment, we followed the daily pattern of melatonin release by microdialysis in the pineal gland (Drijfhout *et al.*, 1993) of SCN-lesioned vs. intact rats during 48-h, in order to investigate the SCN influence on long-term melatonin release. The experiment consisted of two consecutive parts. First, dialysate samples were collected every hour for 30 h, from ZT6 to ZT36. Then, in order to control the effective placement of the pineal probe, we prolonged the sampling throughout the second night while we artificially stimulated the pineal gland with the β-adrenergic agonist isoproterenol (ISO, 10^{-6} M), from ZT36 to ZT51, by reverse dialysis. To assess

the effectiveness of the SCN lesions, we also measured the corticosterone content of the dialysate samples.

Lesions and ganglionectomy
SCN (EXP1, n= 39; EXP2, n= 40) and PVN bilateral thermic lesions (EXP1, n= 20) were performed in rats of 180-200 g. The animals were anaesthetised with a combination of a neuroleptanalegesic mix (fentanyl citrate, 0.315 mg/mL, and fluanisone, 10 mg/mL; Hypnorm®, Jansen Pharmaceutical Ltd, Oxford, England; 0.06 mL/100 g i.m.) and a benzodiazepine (midazolam hydrochloride, 5 mg/mL; Dormicum®, Roche Nederland B.V., Mijdrecht, the Netherlands; 0.04 mL/100 g s.c.) and placed in a David Kopf stereotact (David Kopf Instruments, Tujunga, CA, USA). SCN lesions were performed as previously described (Kalsbeek *et al.*, 2000c) while the different parameters of the PVN lesion protocol were determined in a pilot study. Thermic stainless electrodes were lowered bilaterally to the stereotaxic coordinates of either the SCN (tooth bar set at + 5.0 mm; angle of <6°; coordinates: 1.4 mm rostral to bregma; 1.1 mm to midline; 8.3 mm below the brain surface) or the PVN (tooth bar set at - 3.3 mm; angle of 10°; coordinates: - 2 mm rostral to bregma; + 2 mm to midline; 7.4 mm below the brain surface). The tips of the electrodes were heated for 60 s, at a temperature of 80 °C for SCN lesions and 60 °C for PVN lesions. In order to be able to make a preselection of the effective SCN-lesions we measured food and water-intake after a recovery period of two weeks. The rats that drank ≥ 33% of their 24-h water-intake during 8-h in the middle of the light period (from ZT2 to ZT10) were considered arrhythmic. Until the final histological analysis we assumed that these rats had a correct lesion of the SCN and we consequently selected them to be part of the SCN-lesioned group.
Bilateral ganglionectomies were performed (n = 12) as described previously by Garidou *et al.* (2001) in rats of 200-250 g, 10 days before they were killed. The success of the surgery was checked by observing the ptosis of both eyelids.

Hybridisation
The dissected and frozen caudal parts of the brain, containing the pineal gland in place, were stored at –80 °C until sectioning. Two series of eight coronal sections of 20 µm of the pineal were realised at the cryostat to use for the *in situ* hybridisation and the remainder of the pineal, which approximately corresponds to half of the pineal gland, was collected out of the brain and then sonicated in 500 µl sodium phosphate buffer to measure the melatonin levels by radio immunoassay. *In situ* hybridisation was performed on 20-µm-thick coronal brain sections mounted onto gelatine-coated slides as previously described (Garidou *et al.*, 2001). Briefly, the riboprobes were synthesised from the linearised pBluescript-cytomegalovirus phagemid containing

the cDNA encoding AA-NAT (1311 bp) (Roseboom *et al.*, 1996) using either T7 (antisense) or T3 (sense) RNA-polymerase (MAXIscript transcription kit, Ambion Clinisciences, Montrouge, France; α[^{35}S]-UTP, 1250 Ci/mmol, NEN-Dupont, Zaventem, Belgium). Both probes were hydrolysed by alkaline treatment to generate 200-bp-long fragments. The prehybridisation steps included fixation, acetylation and dehydration, at room temperature. Hybridisation was performed overnight at 54 °C with 120 µl/slide in a hybridisation medium containing 80 amole/µl of antisense or sense probe. Non-specific signal was removed following a wash in 0.02 Kunitz unit/mL type X-A ribonuclease (Sigma) for 30 min at 37 °C. Finally, brain sections were washed, dehydrated and air-dried before 48-h of exposure to an autoradiographic film (Hyperflim MP, Amersham, Orsay, France). Quantitative analysis of the autoradiograms was performed using the computerised analysis system Biocom-program RAG 200. Specific hybridisation was determined as the difference between total (antisense) and nonspecific (sense) hybridisation.

Microdialysis

In the second experiment we measured pineal melatonin release in preselected SCN-lesioned (n = 10) and SCN-intact rats (n = 10). The microdialysis perfusion started after a recovery period of 6 days. Probes were perfused with Ringer, or ISO (10^{-6} M) at a flow rate of 3 µl/min. Dialysate samples were collected every hour and stored at –20 °C until melatonin and corticosterone assay.

Radioimmunoassays

EXPERIMENT 1

As explained above, after sectioning half of the pineal gland, we took the other half and sonicated it in 500 µl sodium phosphate buffer. Melatonin content was measured in 100 µl of pineal homogenate by radioimmunoassay as previously described (Simonneaux *et al.*, 1993). Protein content was measured in 100 µl, following the protocol of Lowry *et al.* (1951), using a bovine serum albumin as standard.

EXPERIMENT 2

Melatonin concentration of dialysates was measured in duplicate by radioimmunoassay using [^{125}I] melatonin (Amersham, Bucks, UK; specific activity 2000 Ci/mmol) and rabbit antiserum (AB/R/03, Stockgrand Ltd, Guildford, UK) with a final dilution of 1 : 200 000. Stock melatonin (Sigma Chemicals) was stored at a concentration of 1 mg/mL. The standard range of dilutions extended from 3.75 pg/mL to 4 ng/mL, and the minimum detection limit level was 4 pg/mL. The intra-assay coefficients of variation were 19, 13 and 13% for standards containing 60, 300 and 1500 pg/mL, respectively, and the interassay coefficient of variation between seven assays was 22, 13 and 3%, for the 3 concentrations mentioned before (Barassin *et al.*,

1999). Corticosterone concentrations in the dialysates were measured in duplicates by radioimmunoassay using commercially available ^{125}I-labelled corticosterone radioimmunoassay kit (ICN Biomedical Division, Carson, CA, USA). The intra-assay and interassay (at 50% binding of the standard curve) coefficients of variation were 7.4% and 6.7%, respectively. The lower limit of sensitivity using this method is 5 ng/mL.

Immunocytochemistry

After fixation, the hypothalamus was sectioned on a vibratome. Hypothalamic sections were stained for vasopressin (VP), vasoactive intestinal peptide (VIP) or oxytocin (OT) in order to analyse the extent of the lesions. If VP- or VIP-containing neurons were detected in the SCN area, the animals were removed from the group of SCN-lesioned rats. PVN-lesioned rats that still presented some VP- or OT-containing neurons in the PVN area were considered as partially lesioned.

Data analysis

Data from both experiments are expressed as mean ± SEM. For all analysis realised in this study $P < 0.05$ was considered significant.

EXPERIMENT 1

Since the distribution of the data was strongly different from a normal distribution (Shapiro-Wilk normality test, $W= 0.63$, $P < 0.0001$), we chose to analyse the data of this experiment with the nonparametric Kruskal-Wallis test followed by multiple comparisons (Conover, 1980).

EXPERIMENT 2

In order to avoid the factor of microdialysis probe placement, we only analysed the data of animals that had a mean ≥200 pg/mL/h of melatonin release during the dark period (for SCN-intact rats) or during the ISO perfusion (for SCN-lesioned rats). The data were analysed using a repeated-measures analysis of variance (ANOVA) with the factors 'lesion' and 'time' followed by a Student-Newman-Keuls posthoc test if necessary.

In order to measure the individual circadian rhythmicity of either melatonin or corticosterone the data points of individual animals were subjected to a statistical analysis by the cosinor method (Table Curve™, Jandel Scientific, CA, USA) using only a 24-h period as the fundamental rhythm.

Results

Respectively 11 and 10 SCN-lesioned rats were preselected for the two experiments on the basis of their drinking behaviour. Their respective mean percentages of water intake during 8-h of the light period were 34.1 ± 0.6% and 34.0 ± 1.0%. In PVN-le-

sioned rats and in Sham-lesioned rats this percentage was, respectively, 13.9 ± 1.4%, 11.8 ± 0.7%.

In the first experiment, we also measured the food intake of the animals. The total 24-h food intake did show a significant difference between groups ($F_{2,127} = 7.67$, $P < 0.001$). No significant difference (posthoc tests; $P = 0.155$) was present between SCN-lesioned rats (20.19 ± 0.37 g) and intact rats (22.17 ± 0.52 g), but food intake significantly increased (posthoc tests; $P < 0.05$) in PVN-lesioned rats (24.53 ± 0.74 g). In addition, the pattern of feeding of the animals significantly changed after treatment ($F_{5,68} = 27.60$, $P < 0.001$). Indeed, daytime food consumption as observed in intact rats (0.15 ± 0.04 g/h) significantly increased in both SCN-lesioned rats (1.03 ± 0.04 g/h) and PVN-lesioned rats (0.67 ± 0.08 g/h).

Confirmation of the correct size and site of the lesion was obtained at the end of the experiment after histological examination using VP and VIP staining for the SCN lesions and VP and OT for the PVN lesions (Fig.1). The analysis of the sections enabled us to select the rats which presented a complete bilateral lesion of the SCN (EXP1,

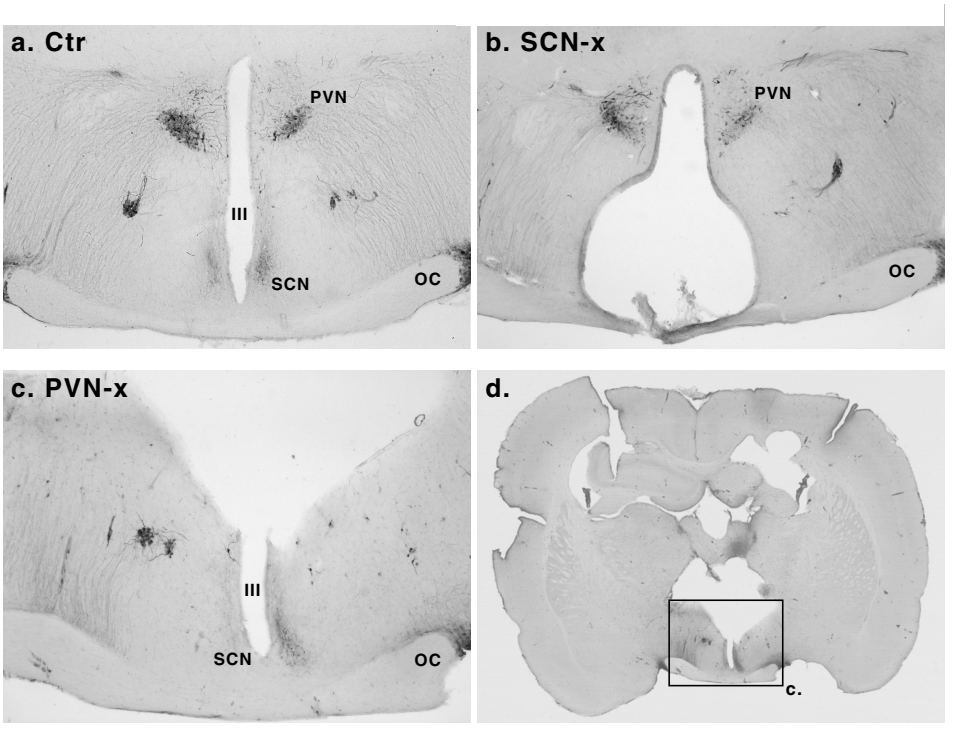

Figure 1 Coronal brain sections of hypothalamus immuno-stained for VP in control rats (a), SCN-lesioned rats (b) and PVN-lesioned rats (c, d). Note that the SCN-lesioned rats present a complete SCN lesion and intact PVN, and that PVN-lesioned rats present a complete PVN lesion without any damage on the SCN. OC, Optic chiasma; III, third ventricule.

9 out of 11 rats; EXP2, 8 out of 10 rats). Even though none of the PVN-lesioned rats (n = 20) showed a complete lesion, in all animals dorsal and ventral parts of the PVN (which contain the preautonomic neurons) were lesioned.

Experiment 1: day/night AA-NAT gene expression

Since no significant difference of either daytime ($U = 2$, $P = 0.06$) or night-time ($U = 8$, $P = 0.39$) AA-NAT gene expression levels between Sham-lesioned rats and intact (non-operated) rats were found following Mann-Whitney tests, these two groups were combined as one control group (n = 20).

The nonparametric analysis on AA-NAT levels of expression revealed highly significant differences between the 8 different groups (Kruskal-Wallis Test; $P < 0.001$) we tested together. In control rats, AA-NAT mRNA levels presented a clear day/night rhythm with a typical rise of about 100-fold at night-time ($P < 0.001$). This day/night rhythm completely disappeared in all other groups, i.e. SCN-lesioned rats ($P = 0.35$), PVN-lesioned rats ($P = 0.10$) and ganglionectomised rats ($P = 0.79$) (Fig. 2A). In SCN-lesioned rats, both day- and night-time AA-NAT gene expression showed a more than 10-fold increase as compared to the daytime control group ($P < 0.001$). In addition, the multiple comparison tests, following the nonparametric analysis of our data, revealed that the night-time AA-NAT mRNA levels of SCN-lesioned animals were significantly 3-times higher than the levels measured in PVN-lesioned and ganglionectomised rats ($P < 0.001$). Still, the increased values of the SCN-lesioned animals only amounted to 30% of night-time control levels. No significant difference of AA-NAT gene expression could be observed between the different groups of PVN-lesioned rats and ganglionectomised rats (multiple comparison tests; $0.18 < P < 1$). These levels of AA-NAT gene expression were significantly higher than daytime controls, although the absolute levels were relatively low, i.e. 12% of night-time controls. Melatonin levels measured in the pineal glands of these animals showed the same fluctuations than the AA-NAT mRNA levels (Fig. 2B).

Experiment 2: pattern of melatonin secretion

One out of the eight SCN-lesioned rats and 2 out of the 10 intact rats were removed from the final analysis because the levels of melatonin they presented were < 200 pg/mL/h, probably due to a microdialysis probe misplacement.

Over the 48-h of sampling, intact rats showed two peaks of melatonin and of corticosterone secretion (Fig. 3A, B). The peak of melatonin secretion presented a 16-fold rise in the middle of the night while the corticosterone peaks presented a 24-fold rise at the day/night transition. Due to the stimulation of the pineal gland with ISO perfusion, the area under the curve of the two melatonin peaks was significantly different ($P = 0.003$). In contrast, the second peak of corticosterone was not affected by the ISO

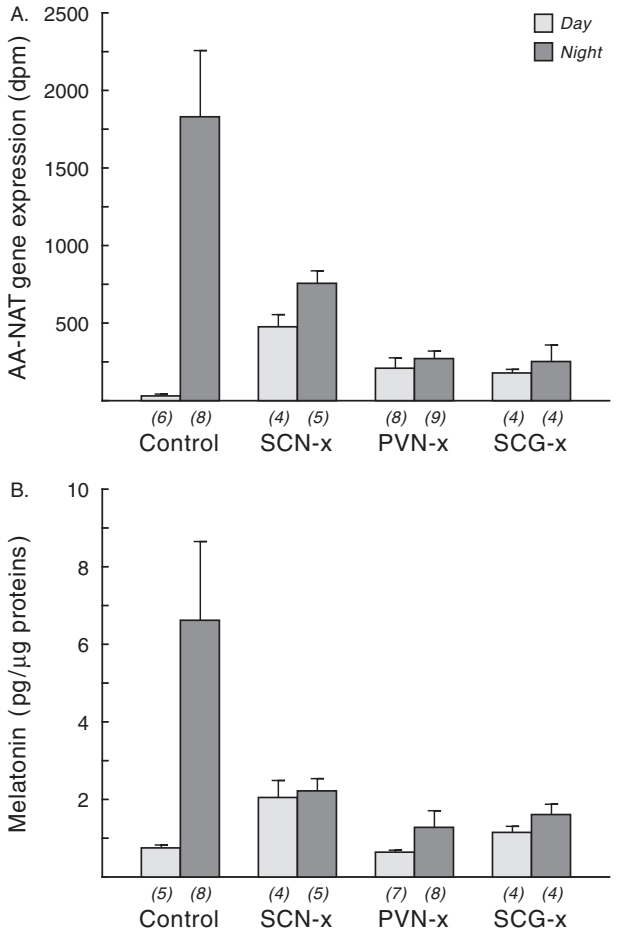

Figure 2 Day/night AA-NAT gene expression (A) and pineal melatonin content (B) in the pineal gland of Control rats vs. SCN-lesioned (SCN-x), PVN-lesioned (PVN-x) and ganglionectomised (SCG-x) rats. The numbers in brackets correspond to the number of animals of each group.

perfusion and was not significantly different from the first peak (P = 0.61).

In contrast to the intact rats, the 24 h rhythm of melatonin and corticosterone release was completely abolished in SCN-lesioned rats. The typical nocturnal peak of melatonin release was deficient during the first dark period and the levels of melatonin measured throughout this period were not significantly different from the levels measured throughout the 12 daytime hours that followed ($F_{1,141} = 0.613$, P = 0.435). Although a small increase is apparent in the mean levels of the SCN-lesioned animals, no significant difference was found between ZT5-ZT12 and ZT28-ZT36 ($F_{1,128} = 2.51$, P = 0.12). Albeit relatively low, i.e. 20% of nocturnal levels in control animals, these

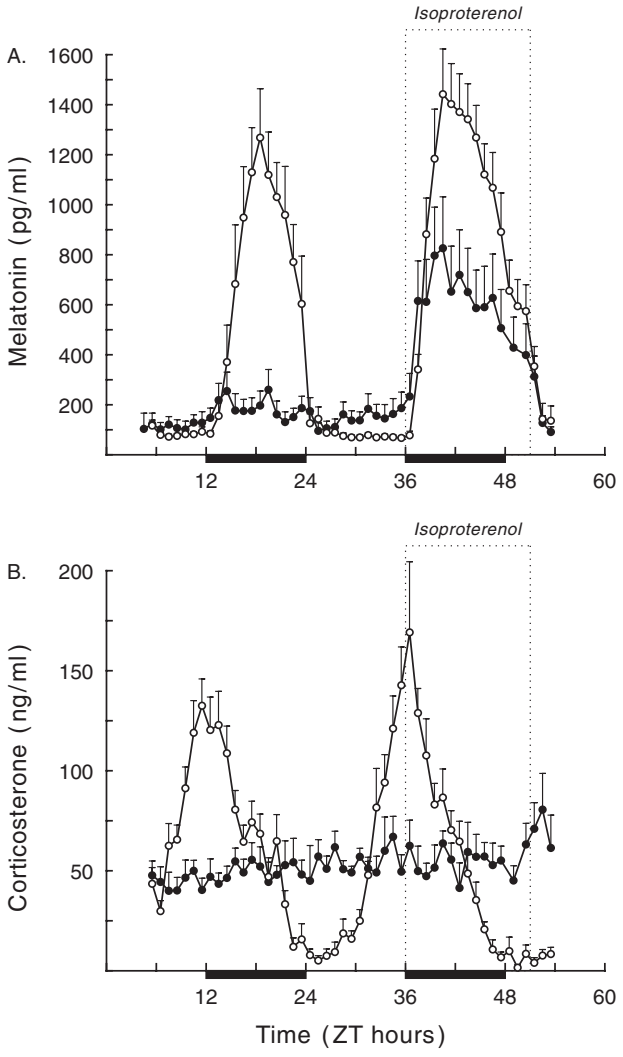

Figure 3 Long-term secretion pattern of melatonin (A) and corticosterone (B) in intact (open circles) vs. SCN-lesioned (closed circles) rats measured by microdialysis in the pineal gland. A solution of ISO was applied through the microdialysis probe in order to artificially stimulate the pineal gland and by this way check the capacity to measure the release of melatonin. These graphs represent mean data (± SEM) of 8 intact rats and SCN-lesioned rats. (A) Note that the relatively low melatonin levels measured in SCN-lesioned increase after ISO perfusion, showing that (1) the pineal is able to synthesize melatonin and (2) that our probes were correctly implanted in the gland.

levels were significantly higher than the basal daytime values measured in intact rats ($F_{1,15} = 66.57$, $P < 0.001$). During the second part of the experiment, stimulation with ISO perfusion induced a ≈5-fold increase of melatonin secretion. In both SCN-lesioned and control rats, the dose of ISO we applied (10^{-6}M) was not sufficient to sustain constant high levels of melatonin synthesis at the end of the dark period. This decrease of potency is probably due to a desensitisation of the β-adrenergic receptors of the pineal gland. Corticosterone secretion in SCN-lesioned rat was constant with intermediate levels (33% of peak levels in intact rats). This secretion in SCN-lesioned rats was also not affected by the ISO perfusion within the pineal gland.

The cosinor analysis was realized on the 48-h of sampling for corticosterone and only on the first 30-h of sampling for melatonin. The results are summarised in Table 1. All but one (r46) of the control animals showed very significant fits of both their melatonin and corticosterone rhythm, i.e. assuming a 24-h cosine rhythm explained > 60% of the variation observed. The acrophases of melatonin and corticosterone rhythms were located at ZT19.50 and ZT13, respectively. Unlike control rats, in the SCN-lesioned group only 4 and 1 animals showed a significant fit for, respectively, their melatonin and corticosterone rhythms. However, no animal showed a significant fit of both rhythms. In addition, in the lesioned animals showing significant fits, the amplitude of the rhythm was strongly decreased and the acrophase was shifted.

Discussion

The first experiment of the present study allowed us to establish, for the first time, a ratio in the control of melatonin synthesis between the different structures involved in the SCN→pineal pathway. The constant intermediate levels of AA-NAT gene expression and melatonin release observed in SCN-lesioned rats reveal three essential points about the control of the melatonin rhythm. First, without any SCN input a basic stimulatory effect on pineal gland activity (30% of night-time AA-NAT mRNA levels) is maintained by the PVN. Second, the intrinsic activity of the sympathetic PVN neurons is silenced by an inhibitory SCN input during the light period, and third, the SCN is also responsible for the stimulation of melatonin synthesis during the night. This last point was confirmed clearly by the second experiment showing that in the absence of the SCN, the noradrenergic fibres originating from the SCG are not stimulated during the dark phase.

The significantly higher levels of AA-NAT gene expression and melatonin content measured during daytime in SCN-lesioned rats (compared to PVN-lesioned and ganglionectomised animals), are in line with our previous results (Kalsbeek *et al.*, 2000c), and confirm that the SCN sustains an inhibitory output on the activity of the PVN→pineal gland pathway. It is likely that this is a direct effect of the SCN on autonomic

Table 1 Cosinor analysis of individual melatonin and corticosterone secretion patterns of SCN-lesioned and intact rats.

Rat number	r^2	F-value	Daily mean (pg/ml/h)	Amplitude (%)	Acrophase (ZT)
Melatonin secretion					
Intact rats					
r41	0.67	22.29	391.57	137.78	19.4
r42	0.78	39.54	659.69	118.76	18.7
r43	0.55	12.4	418.1	137.56	19.5
r45	0.77	36.94	453.91	113.3	19.2
r46	0.13	1.62	140.35	-	-
r47	0.64	19.79	361.01	137.61	20
r48	0.72	27.97	390.34	101.9	18.7
r49	0.78	39.37	724.65	128.81	19.5
Mean	**0.63**	**24.99**	**442.45**	**125.1**	**19.29**
SEM	**0.08**	**4.84**	**64.19**	**5.35**	**0.18**
SCN-lesioned rats					
r6	0.32	5.22	280.08	29.73	16.5
r11	0.53	12.25	46.64	39.92	15.1
r24	0.06	0.71	52.54	-	-
r25	0.01	0.2	171.41	-	-
r28	0.27	4.12	218.4	44.8	18
r29	0	-	252.13	-	-
r38	0.57	14.49	57.76	22.78	21.6
Mean	**0.25**	**6.17**	**137.8**	**34.31**	**17.8**
SEM	**0.09**	**2.43**	**40.77**	**4.96**	**1.4**
Corticosterone secretion					
Intact rats					
r41	0.75	64.53	35.27	110.09	13.1
r42	0.75	67.51	70	126.63	13
r43	0.71	53.25	52.99	92.7	13.7
r45	0.8	93.26	54.13	112.93	12.8
r46	0.72	52.72	76.64	98.94	12.5
r47	0.83	115.33	79.22	98.49	13.7
r48	0.75	69.04	43.01	111.49	13.3
r49	0.79	90.01	48.19	89.65	11.6
Mean	**0.76**	**75.71**	**57.43**	**105.11**	**12.96**
SEM	**0.01**	**7.74**	**5.7**	**4.36**	**0.24**
SCN-lesioned rats					
r6	0.06	1.42	47.25	-	-
r11	0	-	46.95	-	-
r24	0.01	0.25	67.65	-	-
r25	0.03	0.67	44.77	-	-
r28	0.02	0.6	43.53	-	-
r29	0.3	8.77	69.95	23.75	20.5
r38	0.03	0.61	49.83	-	-
Mean	**0.06**	**2.05**	**53.83**	-	-
SEM	**0.04**	**1.35**	**4.82**	-	-

Amplitude (equal to one-half the total extent of the sinusoid and expressed as a percentage relative to the daily mean) and acrophase (peak time of the fitted cosine) are only shown for animals showing a significant fit.

PVN neurons, because viral tracing studies from the pineal only show second order labelling in the hypothalamus in these autonomic PVN neurons before the SCN neurons are infected (Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999). As till now no spontaneous activity in PVN neurons projecting to the spinal cord has been detected, although PVN neurons projecting to the brainstem did show such a spontaneous activity (Stern, 2001). The lack of spontaneous activity in 'sympathetic' PVN neurons might be due to the presence of an extrinsic tonic inhibitory input to those neurons (Stern, 2001). The present results indicate that the SCN could be one of these extrinsic sources.

On the other hand, in the absence of the SCN, the AA-NAT and melatonin levels did not reach the maximum levels as observed in control rats at night. The histological controls as well as the corticosterone data of the SCN-lesioned animals support that the lesions were restricted to the SCN area. Thus, these intermediate levels suggest that the PVN, on its own, is by no means able to maximally stimulate pineal gland activity. Therefore, the present results clearly reveal that the SCN, in addition to its inhibitory role, also has a crucial role in stimulating melatonin synthesis.

The relatively low levels of AA-NAT gene expression (i.e. 12% of control night-time AA-NAT mRNA levels) observed during day- and night-time after PVN lesion and SCG ablation confirm that both structures are essential for the nocturnal stimulation of pineal gland activity. The basal rate of pineal activity maintained after removal of either the PVN or the SCG corresponds to the results observed previously by Garidou *et al.* (2001) after SCG ablation. On the other hand, the fact that these levels are significantly higher than control daytime levels implies the necessity of an inhibitory signal via the PVN and the SCG to lower melatonin and AA-NAT expression to day-time levels. These results thus confirm that the PVN and the SCG act as a relay of inhibitory and stimulatory signals.

At first glance, it seems attractive to assume daytime inhibition and night-time stimulation of the autonomic PVN neurons by SCN efferents. However, after application of a GABA-antagonist in the PVN during daytime (Kalsbeek *et al.*, 2000c), melatonin release increased almost to night-time levels. The present study shows that complete ablation of the SCN results in relatively low melatonin synthesis and release compared to intact night-time levels. Although the two studies were not conducted within the same experiment it is clear that removing the entire SCN is different from simply blocking its inhibitory output towards the PVN→pineal pathway. Consequently, we conclude that the SCN provides a stimulatory output not only at night but also during the daytime.

Therefore, we propose that the control of the melatonin rhythm is composed of a combination of a constant stimulatory and a rhythmic inhibitory SCN output (Fig.4).

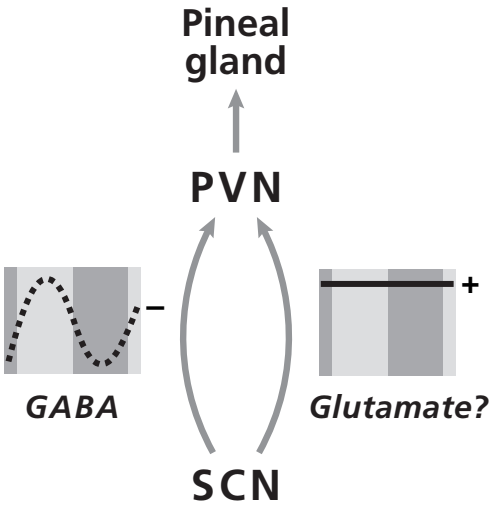

Figure 4 SCN control of pineal gland activity through the preautonomic PVN neurons. We propose that the pineal gland activity rhythm is controlled by combined rhythmic inhibitory (sustained by a GABA-ergic output) and a constant stimulatory SCN output (witch could be sustained by a glutamatergic output).

It may seem surprising to propose a stimulatory output of the SCN during the dark period since, as reported so far, SCN activity seems to be mainly restricted to (subjective) daytime (Schwartz & Gainer, 1977; Inouye & Kawamura, 1979; Shibata *et al.*, 1982; Bos & Mirmiran, 1990). However, *in vitro* studies measuring the activity of individual SCN neurons have also shown evidence for SCN neurons with an opposite-phase of activity (Herzog *et al.*, 1997; Nakamura *et al.*, 2001). It is likely that due to the small number of these night-time active SCN cells, multi-unit activity recording prevents detection of their activity. Furthermore, Nakamura *et al.* (2001) recently reported that 16% of the SCN neurons recorded with a multielectrode plate were not rhythmic. Such SCN neurons, showing activity throughout 24 h, could support a constant stimulatory input. In addition, the lower firing rate during the dark period could still be sufficient to release small neurotransmitters such as GABA and glutamate (Hermes *et al.*, 2000).

The concept of simultaneous inhibitory and stimulatory SCN outputs during daytime is interesting. Although the stimulatory SCN output during (subjective) daytime is not apparent in control conditions, the constant presence of a stimulatory input to the melatonin rhythm generating system might reveal its utility in case of a phase-advance of the dark period. Thanks to the presence of a constant stimulatory input, removal of the inhibitory signal will result in the immediate start of melatonin syn-

thesis, as clearly illustrated by our previous study (Kalsbeek *et al.*, 2000c). Without the presence of a constant stimulatory input an earlier onset of melatonin synthesis can only occur after the stimulatory input has shifted to the night-time. A quicker adaptation to a modification of the light/dark environment is, of course, of great interest for the mechanism of adaptation to day-length (i.e. seasons).

The present study provides a new insight for a nocturnal role of the SCN. This study clearly proves that the rhythm of melatonin synthesis is not formed by a single circadian (daytime) inhibitory signal derived to the PVN from the SCN, but by a combination of this inhibitory signal with a stimulatory input to the PVN, also derived from the SCN. As it was proposed previously for the control of corticosterone by the SCN (Kalsbeek & Buijs, 1996), the SCN also seems to use multiple outputs for the control of melatonin synthesis. However, looking at the mean levels of corticosterone and melatonin in SCN-lesioned animals in comparison with the peak levels in intact animals, the stimulatory SCN output seems more important in the generation of melatonin rhythm than in the generation of corticosterone rhythm. On the basis of the recent electrophysiological evidence (Hermes *et al.*, 1996; Csaki *et al.*, 2000; Cui *et al.*, 2001), we propose SCN-derived glutamate release within the PVN as the main candidate for stimulation of melatonin synthesis. Further experiments, using, for instance, NMDA-antagonists, need to be done to confirm this idea.

Acknowledgements

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CHAPTER 3

Glutamatergic clock output stimulates melatonin synthesis at night

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Abstract

The rhythm of melatonin synthesis in the rat pineal gland is under the control of the biological clock, which is located in the suprachiasmatic nucleus of the hypothalamus (SCN). Previous studies demonstrated a daytime inhibitory influence of the SCN on melatonin synthesis, by using γ-aminobutyric acid (GABA) input to the paraventricular nucleus of the hypothalamus (PVN). Nevertheless, a recent lesion study suggested the presence of a stimulatory clock output in the control of the melatonin rhythm as well. In order to investigate further this output in acute *in vivo* conditions, we first measured the release of melatonin in the pineal gland before, during and after a temporary shutdown of either SCN or PVN neuronal activity, using multiple microdialysis. For both targets, SCN and PVN, the application of Tetrodotoxin (TTX) by reverse dialysis in the middle of the night decreased melatonin levels. Because of recent evidence of the existence of glutamatergic clock output, we then studied the effect on melatonin release of glutamate antagonist application within the PVN in the middle of the night. Blockade of the glutamatergic input to the PVN significantly decreased melatonin release. These results demonstrate that (1) neuronal activity of both PVN and SCN is necessary to stimulate melatonin synthesis during the dark period, and that (2) glutamatergic signalling within the PVN plays an important role in melatonin synthesis.

Introduction

In mammals, the endogenous circadian rhythms of physiological functions (such as hormone release, metabolism, and body temperature) are controlled by a central biological clock, located in the suprachiasmatic nucleus of the hypothalamus (SCN) (Buijs & Kalsbeek, 2001). The daily synthesis of melatonin in the pineal gland, which exclusively occurs during the dark phase of the light-dark cycle, represents a stable and reliable output of the biological clock. The SCN is connected to the pineal gland

by a multisynaptic pathway, including, successively, neurons of the paraventricular nucleus of the hypothalamus (PVN), sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion (SCG) (Moore & Klein, 1974; Klein *et al.*, 1983a; Lehman *et al.*, 1984; Bittman *et al.*, 1989; Tessonneaud *et al.*, 1995; Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999; Garidou *et al.*, 2001). Recently, we suggested that, in rat, the SCN uses a combination of inhibitory and stimulatory signals towards the PVN for that purpose (Perreau-Lenz *et al.*, 2003). In the present study, we tested this concept for the first time in acute *in vivo* conditions. In view of the fact that a stimulatory role of the SCN at night was suggested, and that the main neuronal activity of the SCN is reported during daytime (Schwartz & Gainer, 1977; Inouye & Kawamura, 1979; Shibata *et al.*, 1982; Bos & Mirmiran, 1990), we measured the effect of a temporary shutdown of the neuronal activity of either the SCN or the PVN, using a local tetrodotoxin (TTX) application by reverse dialysis, on melatonin release. Both interventions resulted in a diminished melatonin secretion, as measured by microdialysis in the pineal gland. We previously showed that blocking GABA-ergic transmission to the PVN lifts the inhibition on melatonin synthesis in the pineal gland (Kalsbeek *et al.*, 1999; Kalsbeek *et al.*, 2000c), which suggested that GABA represents the inhibitory signal of the SCN to the PVN during daytime. On the other hand, the melatonin decrease observed after the shutdown of the SCN neuronal activity demonstrates the presence of a stimulatory signal during nighttime. Interestingly, recent studies indicate that both GABA and glutamate may be used, respectively, as inhibitory and stimulatory SCN inputs to regions of the preoptic area involved in the control of sleep/wake rhythm (Sun *et al.*, 2000; Sun *et al.*, 2001). In addition, evidence of glutamate immunoreactivity within presynaptic boutons in the PVN (van den Pol, 1991), as well as evidence of a specific glutamate release from the SCN onto (preautonomic) PVN neurons (Hermes *et al.*, 1996; Csaki *et al.*, 2000; Cui *et al.*, 2001) shore up the idea of a glutamatergic SCN input to the PVN as well. On the basis of this evidence, we proposed that SCN-derived glutamate release within the PVN is responsible for stimulation of melatonin synthesis. Hence, we investigated the effect on melatonin release of nightly glutamate signalling blockage within the PVN.

Material and methods

Animals

Male Wistar rats (Harlan, Zeist, The Netherlands) were kept in a temperature-controlled environment under 12-h light/12-h dark conditions, and fed *ad libitum*. Time is expressed in Zeitgeber Time (ZT) with ZT12 corresponding to the beginning of the dark period. For the experiments requiring sampling during the dark phase of

the animals, the rats were kept in reverse light/dark conditions during 4 weeks before surgery, in order to assure a total adaptation to the new light/dark regime. Rats weighed 125-150 g and were housed at five animals per cage upon their arrival. For the experiments realized during the light period, the animals (250-275 g) arrived one week before their operation. After surgery, the rats were housed individually. All experiments were conducted under the approval of the Animal Care Committee of the Royal Netherlands Academy of Sciences.

Experimental set-up

IN VIVO ELECTRICAL ACTIVITY SHUTDOWN IN THE SCN OR THE PVN

The first part of the study aimed at measuring the effect on melatonin release of a temporary shutdown of the nighttime electrical activity of either the SCN or the PVN. We used a multiple microdialysis technique in order to follow the secretion of melatonin, by transpineal microdialysis, as described previously by Drijfhout et al. (1993), before, during, and after a 2 hours bilateral application, by reverse microdialysis, of the Na^{2+} channel blocker Tetrodotoxin (TTX) within either the PVN (experiment A) or the SCN (experiment B) in the middle of the dark-period. For both experiments, a Ringer solution (NPBI BV, Emmer-Compascum, The Netherlands) was perfused during 3 consecutive days (Ctr1, ExpTTX, Ctr2) from the beginning to the end of the dark period (from ZT12 to ZT24) through the microdialysis probe implanted in the pineal gland and the ones implanted in either the PVN or the SCN. Besides, during the second dark period (ExpTTX), TTX was added to the Ringer solution (concentration of 10^{-6} M) and perfused through the hypothalamic probes from ZT17 to ZT19. In addition, in order to measure the effect of the hypothalamic Ringer perfusion itself on melatonin release, Ringer was perfused only through the pineal probes on an extra-control-day (CTR).

In addition, we measured the effect of a temporary shutdown of SCN neuronal activity during the light period (experiment C). For this purpose we followed the secretion of melatonin during two daytime periods. Therefore, Ringer solution was perfused within the pineal gland and the SCN from ZT1 to ZT18 for the first day (Ctr), and from ZT1 to ZT11 for the second light period (ExpTTX). We followed the melatonin release during one dark period in order to confirm the correct placement of the microdialysis probe. In addition, during the second light period (ExpTTX), TTX was added to the Ringer solution (concentration of 10^{-6} M) and perfused from ZT4-ZT8 within the SCN through the hypothalamic probes.

BLOCKAGE OF GLUTAMATE SIGNALLING WITHIN THE PVN AND MELATONIN RELEASE

The second part of this study aimed at investigating the role of the glutamatergic input to the PVN with regard to the rhythm of melatonin synthesis. Therefore, we also used

the multiple microdialysis technique to measure the secretion of melatonin before, during and after the application of an N-methyl-D-aspartate (NMDA) receptor-specific glutamate antagonist in the middle of the night. Ringer was perfused through the pineal gland and the bilateral PVN for three consecutive days, from the beginning to the end of the dark period. During the second dark period (ExpMK801), the NMDA antagonist (+/-) MK-801 was added to the Ringer (concentration of 10^{-4} M) and applied by reverse microdialysis from ZT17 to ZT19 within the PVN.

Microdialysis Probes and Chemicals

Two kinds of microdialysis probes were used in this study. The microdialysis probes implanted in the pineal gland, or transversal probes, were made as previously described (Drijfhout et al., 1993), using the microdialysis membrane Hospal (AN69; cut-off: 35-40 kDa). The hypothalamic probes, or U-shaped probes, used for reverse microdialysis in the hypothalamus, were made as previously described (Engelmann *et al.*, 1992; Barassin *et al.*, 2002) using the microdialysis membrane C-DAK™ Artificial Kidney cut-off: 6 kDa (135 SCE catalogue nr. 201-8000, CD Medical Inc., Miami Lakes, Florida, USA).

Different chemicals were applied by reverse dialysis in the SCN or PVN. TTX (Tocris Cookson Ltd, Avonmouth, Bristol, UK) was used at a concentration of 10^{-6} M, and (+/-) MK-801 (Research Biomedical International, Natrick, MA, USA) was used at a concentration of 10^{-4} M.

Surgeries

Surgeries were performed in rats of 300-350 g. The animals were anaesthetized with a neuroleptanalegesic mix (fentanyl citrate, 0.315 mg/ml, and fluanisone, 10 mg/ml; Hypnorm®, Jansen Pharmaceutical Ltd., Oxford, England; 0.06 ml/100 g i.m.) and a benzodiazepine (midazolam hydrochloride, 5mg/ml; Dormicum®, Roche Nederland B.V., Mijdrecht, The Netherlands; 0.04ml/100 g s.c.) and placed in a David Kopf stereotaxic apparatus. The transpineal microdialysis probes were implanted transversally in the pineal gland as described previously by Drijfhout et al. (1993). In addition, U-shaped hypothalamic microdialysis probes were implanted bilaterally within either the SCN (tooth bar set at +5.0 mm; angle of -6°; coordinates: 1.4 mm rostral to bregma; 1.3 mm to midline; 8.5 mm below the brain surface) or the PVN (tooth bar set at –3.3 mm; angle of -10°; coordinates: 2 mm caudal to bregma; 2 mm to midline; 7.8 mm below the brain surface). In order to reduce post-operative pain, all rats were subcutaneously injected with depressant analgesic opiate (buprenorfine hydrochloride, 0.324 mg in 50 mg dextrose; Temgesic®, Reckitt & Colman Products Ltd., Kingston-Upon-Hull, UK; 0.03 ml/100 g) after post-anaesthesia arousal.

Microdialysis
After a recovery period of 6-8 days, the microdialysis probes were connected to the system of microinjection pumps as described previously (Bothorel *et al.*, 2002). Ringer was perfused through the dialysis probes at a flow rate of 3 µl/min during experimental periods and 1 µl/min during resting periods. Dialysate samples were collected every hour or every half-hour (during the application of TTX and glutamate antagonist) from the pineal probe, and stored at - 20° C until melatonin and corticosterone assay. Chemical solutions (such as Ringer, TTX or glutamate antagonist) were perfused through the hypothalamic probes at a flow rate of 3 µl/min.

Radio Immunoassays
Melatonin concentration of dialysates was measured in duplicate by radioimmunoassay using [^{125}I] melatonin (Amersham, Bucks, UK; specific activity 2000 Ci/mmol) and rabbit antiserum (AB/R/03, Stockgrand Ltd, Guildford, UK) with a final dilution of 1:200,000. Stock melatonin (Sigma Chemicals) was stored at a concentration of 1 mg/ml. The standard range of dilutions extended from 3.75 pg/ml to 4 ng/ml, and the minimum detection limit level was 4 pg/ml. The intra-assay coefficients of variation were 19, 13 and 13 % for standards containing 60, 300 and 1,500 pg/ml, respectively, and the inter-assay coefficient of variation between seven assays was 22, 13 and 3%, for the 3 concentrations mentioned before (Barassin *et al.*, 1999). Corticosterone concentrations in the dialysates were measured in duplicates by radioimmunoassay using a commercially available [^{125}I] corticosterone radioimmunoassay kit (ICN Biomedical Division, Carson, CA, USA). The intraassay and interassay (at 50% binding of the standard curve) coefficients of variation were 7.4% and 6.7%, respectively. The lower limit of sensitivity using this method is 12.5 pg/ml.

Immunocytochemistry
In order to check the placement of the probes, the animals were perfused with fixative (paraformaldehyde 4%) at the end of each experiment, and immunocytochemical staining was performed on the brain sections obtained with a vibratome apparatus. Alternating hypothalamic sections were processed for a Nissl counterstaining or to detect the presence of vasopressin or vasoactive intestinal peptide containing neurons and fibers.

Data analysis
Data from all experiments are expressed as mean ± SEM. In order to avoid the factor of microdialysis probe placement, we only analysed the data of animals that had a mean ≥ 200 pg/ml/hr of melatonin release during the control dark periods. The data were analysed using two-way MANOVA with both treatment (i.e. 3 levels, Ctr1,

ExpTTX or MK-801, and Ctr2) and time of day (depending on the experiment 10 or 14 levels) as repeated measures within-subject factors. MANOVA tests were followed, if F-values were appropriate, by a Student-Newman-Keuls post-hoc test. For all analyses realized in this study $P < 0.05$ was considered significant.

Results

The MANOVA analysis applied for the comparison of the different control days (CTR, Ctr1 and Ctr2) measured in 5 animals did not reveal any significant effect of the Ringer perfusion within the hypothalamic probes on melatonin release (Treatment, $F(2,6)=2.65$, $P = 0.15$; Time, $F(13, 39)=3.56$, $P < 0.001$; Interaction, $F(26,78)=0.89$, $P = 0.62$).

In vivo shutdown of electrical activity of PVN/SCN and melatonin release

In the first place, we tested *in vivo* the effect of the shutdown of neuronal activity of the PVN in the middle of the night on pineal melatonin release (experiment A). For this experiment 18 rats were operated. Due to a too strong decrease of their body weight (more than 10%) one week after the operation, or to an obstruction of the pineal probe, 5 animals were not connected to the perfusion system of dialysis. Of the 13 rats perfused only 11 were perfused for the entire experiment. Of these 11 rats, 3 more animals had to be excluded due to the low level of melatonin measured, suggesting misplacement or damage of the pineal microdialysis probe. All hypothalamic probes of the 8 remaining animals were located in or directly adjacent to the PVN as observed after histological analysis of the brain sections (for an example see Fig.1).

As shown in Figure 2A, melatonin release quickly, strongly and significantly (Table 1A) decreased following the application of TTX within the PVN (from ZT17 to ZT19). Levels started to decrease 30 minutes after the start of the TTX perfusion and reached daytime basal levels already after 1h. This effect was reversible, and melatonin levels rose to control levels 2 hours after the end of the TTX perfusion.

Next, we tested the *in vivo* effect of shutting-down neuronal activity within the SCN in the middle of the night, on melatonin release in the pineal gland (experiment B). For this experiment 20 rats were operated and only 13 animals were connected to the perfusion system of dialysis for the same reasons explained above. Of the 13 rats perfused, 11 were perfused for the entire experiment. Due to too low levels of melatonin concentration, 2 additional animals were excluded. Of the 9 remaining animals, 6 showed a significant drop of melatonin after TTX application (>80%) and 3 did not. Post-hoc analysis of the brain sections revealed a placement clearly anterior of the SCN for the probes of these 3 animals. As shown in Figure 2B for the 6 remaining animals, TTX application in the middle of the night within the SCN also strongly,

quickly and significantly (Table 1A) decreased melatonin levels, as observed after TTX application at the level of the PVN. Levels started to decrease 30 minutes after the start of the TTX perfusion. Like in the PVN, the effect of TTX application in the SCN was quickly reversible after the end of the perfusion. Indeed, melatonin levels reached control levels 2 hours after the end of the TTX perfusion.

In order to test the *in vivo* effect of shutting-down the neuronal activity of the SCN on melatonin release in the pineal gland during the light period, we performed a third experiment (experiment C). For this experiment 14 animals were operated. Of these 14 rats 3 were not connected to the perfusion system of dialysis for the same reasons explained above, and 8 of the 11 rats remaining were perfused for the entire experiment. Of these 8 rats, 3 were excluded, due to either the misplacement of the hypothalamic microdialysis probe (n=1) or due to too low levels of melatonin release

Table 1 Statistical effects of neuronal activity shutdown of either the PVN (experiment A) or the SCN (experiment B and experiment C) on melatonin release in the pineal gland (A) or on corticosterone release (B). Data result from the MANOVA analyses (See Material and Methods). "F" indicates the degree of freedom and "p" the probability for each factor (Time, Treatment, and the interaction between Time and Treatment). The significance threshold is $p<0.05$.

A. Melatonin	**Factors**	**F**	**p**
Experiment A	Time	$F(13,91) = 4.45$	$p<0.001$
	Treatment	$F(2,14) = 5.86$	$p<0.05$
	Interaction	$F(28,182) = 3.75$	$p<0.001$
Experiment B	Time	$F(13,52) = 10.23$	$p<0.001$
	Treatment	$F(2,8) = 0.93$	$p=0.434$
	Interaction	$F(26,104) = 2.24$	$p<0.005$
Experiment C	Time	$F(9,36) = 2.08$	$p=0.058$
	Treatment	$F(1,4) = 0.04$	$p=0.851$
	Interaction	$F(9,36) = 1.66$	$p=0.136$
B. Corticosterone			
Experiment A	Time	$F(13,78) = 3.08$	$p<0.001$
	Treatment	$F(1,6) = 0.99$	$p=0.358$
	Interaction	$F(13,78) = 0.74$	$p=0.72$
Experiment B	Time	$F(13,65) = 2.39$	$p<0.05$
	Treatment	$F(1,5) = 27.88$	$p<0.005$
	Interaction	$F(13,65) = 2.80$	$p<0.005$
Experiment C	Time	$F(9,9) = 1.47$	$p=0.286$
	Treatment	$F(1,1) = 90.48$	$p=0.07$
	Interaction	$F(9,9) = 2.43$	$p=0.101$

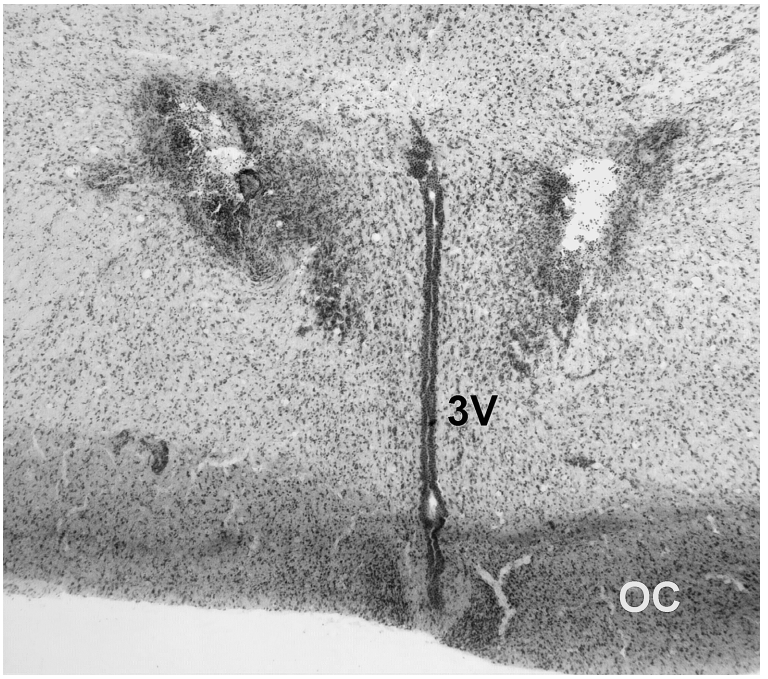

Figure 1 A representative example of the microdialysis probe placement in the PVN. All probe placements were verified by examining transversal vibratome sections treated with an immunocytochemical or Nissl counterstaining. In this example the tips of the microdialysis probes are shown reaching the dorsal part of the PVN in a Nissl stained section. OC: optic chiasma, 3V: third ventricle.

(n=2). The melatonin data of the remaining 5 rats are shown in Figure 2C. Interestingly, daytime TTX application within the SCN had no effect on melatonin release. Indeed, melatonin levels were not significantly different (Table 1A) from the daytime basal levels measured during the control day.

In order to assess the local specificity and efficiency of the TTX administration, we also measured the corticosterone levels in the same dialysate samples collected for these 3 experiments. TTX application in either the PVN or the SCN caused very different effects regarding corticosterone release (Fig.3).

TTX in the PVN did not cause a change in corticosterone release as compared to Ctr1, but TTX in the SCN caused a clear and significant increase of corticosterone release during the dark period (Table 1B). During the light period, the TTX application increased the levels of corticosterone, but due to the small number of samples (the samples of 3 animals were lost) the statistical analysis did not reach significance (Table 1B).

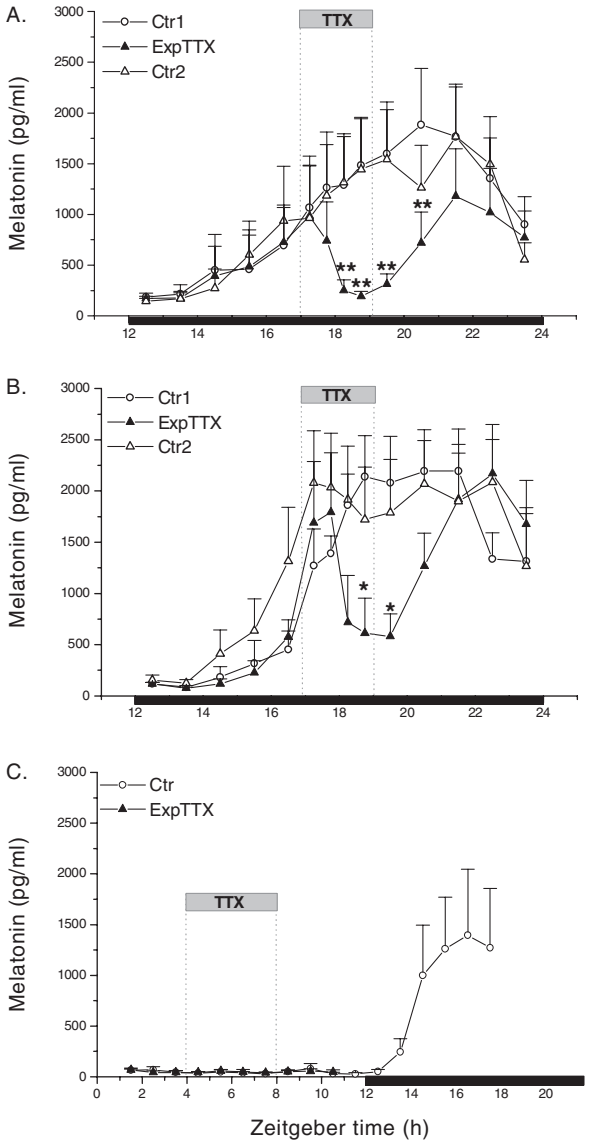

Figure 2 Effect of a temporary shutdown of the neuronal electrical activity of the PVN or the SCN on melatonin release in the pineal gland. A local 2h perfusion of TTX in the middle of the dark period within either the PVN (A) or in the SCN (B) decreased the release of melatonin when applied between ZT17-ZT19. But, a 4h perfusion of TTX within the SCN during the middle of the light period, from ZT4 to ZT8, has no effect on melatonin synthesis (C). Data are expressed in mean levels of melatonin concentration for each time point of 5 to 8 rats, error bars represent SEM. Student-Newman-Keuls post-hoc tests realized after a MANOVA indicate the time-points that for the day of TTX perfusion were significantly different compared to the first control day. Significant differences $P < 0.05$ and $P < 0.001$ are respectively indicated by * and **.

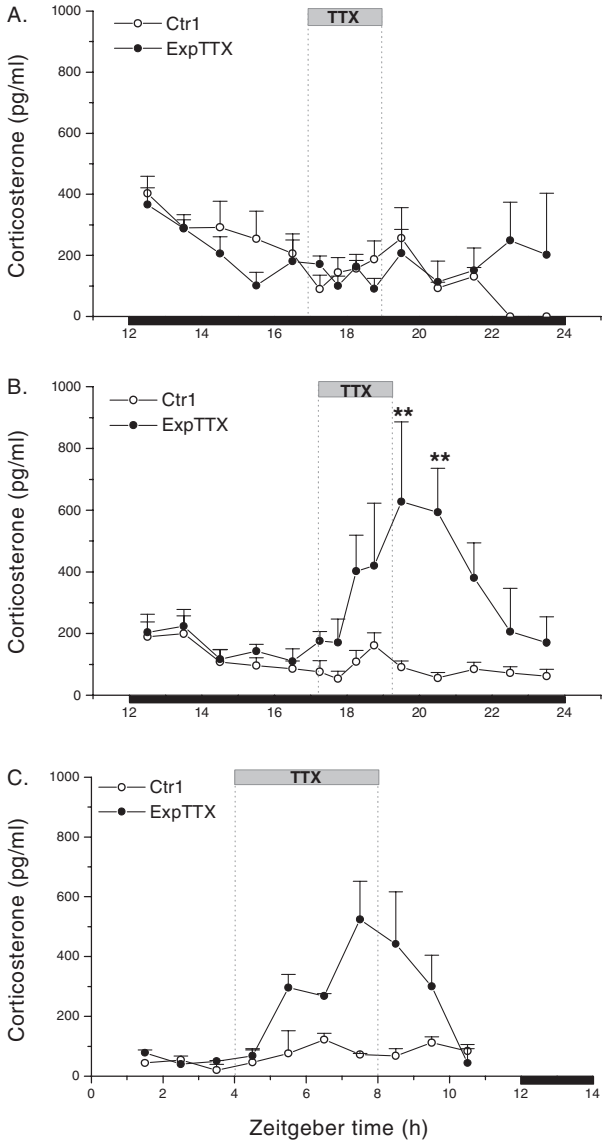

Figure 3 Effect of a temporary shutdown of the neuronal electrical activity of the PVN or the SCN on Corticosterone release. A local 2h perfusion of TTX during the middle of the dark period, from ZT17 to ZT19, did not affect corticosterone release when applied within the PVN (A) but did strongly increase it when applied within the SCN (B). A 4h perfusion of TTX within the SCN during the middle of the light period, from ZT4 to ZT8, also strongly increased the release of corticosterone (C). Data are expressed in mean levels of corticosterone concentration for each time point of 2 to 8 rats, error bars represent SEM. Student-Newman-Keuls post-hoc tests realized after a MANOVA indicate the time-points that for the day of TTX perfusion were significantly different compared to the first control day. Significant differences $P<0.001$ are indicated by **.

Blockage of glutamate signalling within the PVN and melatonin release

In a second series of experiments, we tested the role of endogenous glutamate release within the PVN on melatonin release. For this purpose, we measured the effect of a glutamate transmission blockage (application of a glutamatergic antagonist) in the PVN on the nocturnal release of melatonin in pineal gland. For this experiment 12 rats were implanted with two bilateral hypothalamic and one transpineal microdialysis probe. Due to a too strong decrease of their body weight (more than 10%) one

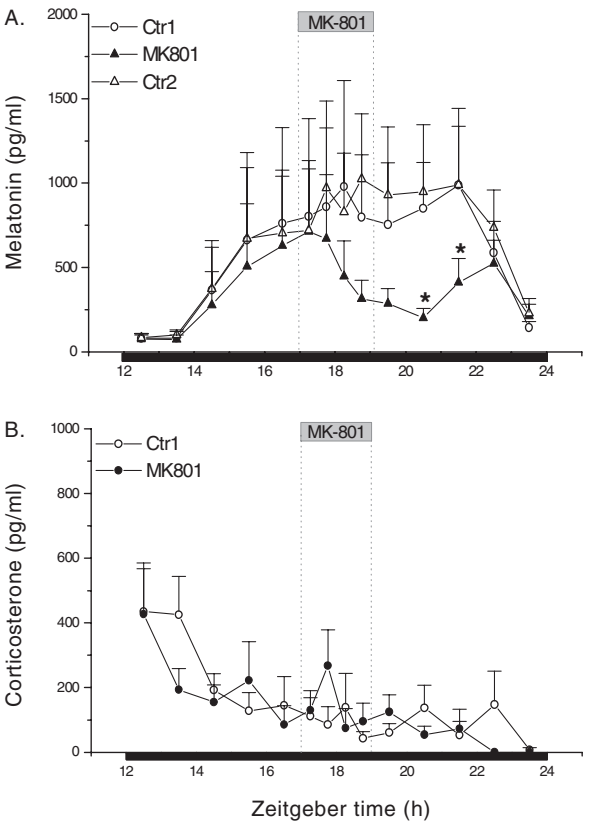

Figure 4 Effect of a temporary blockade of the glutamatergic signalling within the PVN on the nocturnal melatonin release in the pineal gland (A) and on corticosterone release (B). A local 2h perfusion of glutamate antagonist, +/-MK-801, within the PVN during the middle of the dark period (ZT17-ZT19) decreased the release of melatonin in the pineal gland (A), but did not affect the corticosterone secretion (B). For each time point, data of melatonin and corticosterone concentration are expressed in mean levels of 6 rats, error bars representing the SEM. Student-Newman-Keuls post-hoc tests realized after a MANOVA indicate the time-points that were significantly different for the day of TTX perfusion as compared to the first control day. Significant differences $P < 0.05$ are indicated by *.

Table 2 Statistical effects of glutamatergic signalling blockade on nocturnal melatonin release in the pineal gland (A) and on corticosterone release (B). Data result from the MANOVA analyses (See Material and Methods). "F" indicates the degree of freedom and "p" the probability for each factor (Time, Treatment, and the interaction between Time and Treatment). The significance threshold is $p<0.05$.

A. Melatonin	**Factors**	**F**	**p**
	Time	$F(13,65) = 1.97$	$p<0.05$
	Treatment	$F(2,10) = 3.27$	$p=0.081$
	Interaction	$F(26,130)= 2.46$	$p<0.001$
B. Corticosterone			
	Time	$F(13,65) = 4.44$	$p<0.001$
	Treatment	$F(1,5) = 0.15$	$p=0.718$
	Interaction	$F(13,65) = 1.08$	$p=0.389$

week after the operation, or to an obstruction of the pineal probe, 2 animals were not connected to the dialysis system of perfusion. Of the 10 rats perfused, 4 animals were excluded due to either an instability between the different control days, suggesting damage of the pineal microdialysis probe (n=2), or due to an incomplete sampling pattern (n=2). The melatonin release patterns of the 6 remaining animals are shown in Figure 4A.

The glutamate antagonist infusion within the PVN significantly decreased melatonin release (Table 2A). The effect of MK-801 on melatonin is not as strong and as rapid as the effect observed after TTX application. But just as with TTX, this effect is also reversible, and the levels of melatonin increased again and reached the level of the control days 3 hours after the end of the application. As shown in Figure 4B and Table 2B, the perfusion of the glutamate antagonist did not have any significant effect on the release of corticosterone.

Discussion

The present *in vivo* experiments (1) demonstrate for the first time that nocturnal neuronal activity of both the SCN and the PVN is crucial to stimulate melatonin synthesis in the rat pineal gland, and (2) clearly reveal that glutamate is an essential neurotransmitter used by the SCN to stimulate the PVN→pineal pathway and consequently the melatonin synthesis.

Acute *in vivo* shutdown of the neuronal activity of both SCN and PVN during the dark period caused, indeed, an immediate diminution of melatonin release in the pineal gland. The effect of silencing nocturnal neuronal activity in the PVN could be

expected from previous lesions studies (Klein *et al.*, 1983a; Lehman *et al.*, 1984; Hastings & Herbert, 1986; Bittman *et al.*, 1989; Larsen *et al.*, 1998). However, the present results for the first time establish the importance of PVN output for a proficient melatonin production in an *in vivo* experimental setting and after an acute and reversible shutdown of its neuronal activity. Nocturnal melatonin release was also decreased in animals with permanent thermal lesions of the SCN. But, despite its strong decrease nocturnal levels of mRNA expression of the limiting enzyme for melatonin synthesis, arylalkylamine N-acetyltransferase, and melatonin release in SCN-lesioned animals remained significantly elevated as compared to basal daytime levels in control animals (Bittman *et al.*, 1989; Tessonneaud *et al.*, 1995; Kalsbeek *et al.*, 2000c; Perreau-Lenz *et al.*, 2003). The intermediate levels of melatonin release in SCN-lesioned animals and the complete arrest of melatonin release after a temporal shutdown of nocturnal SCN neuronal activity, confirm the dual role of the SCN (i.e. inhibitory and stimulatory) in the control of melatonin synthesis.

It could be argued that due to the very similar effects of TTX administration in the PVN and the SCN on melatonin release, TTX diffusion from one target affects the other target. However, the analysis of corticosterone release, in the same samples, showed a clear differentiation of PVN and SCN infusions. Indeed, silencing PVN neuronal activity in the middle of the night did not affect the corticosterone levels, as expected, while silencing SCN neuronal activity in the middle of the night, clearly increased the corticosterone levels. Apparently, SCN neuronal activity is already necessary in the middle of the night to inhibit corticosterone suggesting that the decrease of corticosterone secretion at this time is not a simple passive mechanism, as previously suggested (Kalsbeek *et al.*, 1996c). Consequently, a shutdown of the SCN removed its inhibitory influence on the corticotropin-releasing-hormone neurons of the PVN and then on corticosterone secretion. On the other hand, in control conditions corticotropin-releasing-hormone neurons in the PVN apparently show little activity during the dark period, and an additional shutdown of its activity by TTX administration in the PVN does not affect corticosterone levels. Thus, the diminution of melatonin release observed after TTX application within the SCN is site specific and cannot be explained by a possible diffusion of the TTX to the PVN. The present *in vivo* results, therefore, show a clear functional evidence of nocturnal neuronal activity of the SCN, which is not only crucial for melatonin synthesis in the pineal gland, but also for the inhibition of corticosterone secretion.

It may seem surprising to propose a significant output of the SCN during the dark period since, as reported so far, the SCN is mainly active during (subjective) daytime (Schwartz & Gainer, 1977; Inouye & Kawamura, 1979; Shibata *et al.*, 1982; Bos & Mirmiran, 1990). However, more recent *in vitro* studies measuring the activity of individual SCN neurons have shown evidence for SCN neurons with an opposite-

phase of activity (Herzog *et al.*, 1997; Nakamura *et al.*, 2001). In addition, Saeb-Parsy & Dyball (2003a) have shown recently that defined cell groups in the rat SCN have different day/night rhythms of single-unit activity *in vivo*, suggesting that for different subpopulations of SCN cells the peak time of their activity differs. Therefore, it is likely that, although the general neuronal activity recorded within the SCN at night is lower than during the day, the activity of selected populations of SCN neurons will be responsible for the stimulation of melatonin secretion in the pineal gland and for the inhibition of corticosterone secretion.

In addition, the present study, further confirms our previous data (Perreau-Lenz *et al.*, 2003) showing that, during the light period, the SCN sustains both an inhibitory and a stimulatory input to the PVN at the same time. We have previously shown that blocking GABA input to the PVN during 4 hours in the (subjective) day allows the stimulation of melatonin synthesis and release in the pineal gland (Kalsbeek *et al.*, 2000c). On the other hand, the present results revealed that silencing all SCN neuronal activity during daytime has no such effect on melatonin release, although the TTX solution was applied during a period long enough (i.e. 4 hours) for a neo-synthesis of melatonin. Moreover, although no effect could be observed on melatonin release, the strong increase of corticosterone following TTX application in the SCN attests for an effective infusion. Thus, we can conclude that during daytime the effect of a total SCN neuronal activity shutdown is different from the effect of only a blockade of its inhibitory (GABA-ergic) output to the PVN. These results show that the stimulatory SCN input to the PVN→pineal pathway is also present and functional during the light period, and is silenced in case of a TTX application in the SCN but not by a blockade of GABA signalling in the PVN. In addition, as already shown by previous studies (Buijs *et al.*, 1993a; Kalsbeek & Buijs, 1996; Kalsbeek *et al.*, 1996c), the present results confirm that the SCN control of corticosterone release at this time of the day (middle of light-period) is mainly inhibitory.

The present evidence of glutamate as a SCN neurotransmitter responsible for the stimulation of melatonin secretion fits very well with previous studies showing glutamate as a dominant central excitatory neurotransmitter with an important role within the hypothalamus (van den Pol *et al.*, 1990; van den Pol, 1991). Moreover, Sun et al. (2000; 2001) recently proposed that SCN projections to the ventromedial and ventrolateral regions of the preoptic area use both GABA and glutamate as inhibitory and stimulatory inputs, respectively, for the control of the sleep/wake rhythm. In addition, several recent studies revealed specific glutamate release from the SCN onto (preautonomic) PVN neurons (Hermes *et al.*, 1996; Csaki *et al.*, 2000; Cui *et al.*, 2001). In the present study, we clearly demonstrated that glutamatergic signalling in the PVN is involved in the stimulation of melatonin synthesis in the pineal gland. Indeed, the blockade of glutamatergic transmission (at least via NMDA receptors)

within the PVN selectively inhibits melatonin synthesis. At present we cannot exclude the involvement of either other glutamatergic receptors or other stimulating neurotransmitters or neuropeptides in the nocturnal stimulation of melatonin release. Accordingly, further studies will be necessary to complete our knowledge on the stimulating SCN input towards the PVN. However, we can already clearly conclude from the present study that release of glutamate within the PVN at night is necessary to stimulate melatonin synthesis. The present results, obtained with a non-competitive NMDA receptor antagonist, MK-801, infused specifically in the PVN, confirm and can explain previous results obtained in hamsters after an intra-peritoneal injection of MK-801, at ZT19, showing that MK-801 induces a dose-dependent inhibition of melatonin production (Vuillez *et al.*, 1998). It is likely that in this experiment the glutamate antagonist reached the PVN.

In conclusion, the present study demonstrates that even if the general neuronal activity of the SCN is rather weak at night, it is sufficient, and, even more, necessary, to stimulate melatonin synthesis and to inhibit corticosterone at the same time. Furthermore, the present study clearly demonstrates that glutamate release within the PVN is a major component for the stimulation of melatonin synthesis at night, suggesting a clear functionality for direct glutamatergic SCN inputs to the preautonomic neurons of the PVN. These results consequently bring new insight on the way the biological clock can control diverse physiological functions. In future experiments it will be interesting to investigate further the pattern of release of this SCN stimulatory signal and to identify the subpopulation of SCN neurons that sustains a stimulatory output to the pineal gland.

Acknowledgements

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CHAPTER 4

In vivo evidence for controlled offset of melatonin synthesis by the suprachiasmatic nucleus in the rat

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Submitted

Abstract

The daily rhythm of melatonin synthesis in the rat pineal gland is controlled by the central biological clock, located in the suprachiasmatic nucleus (SCN), via a multisynaptic pathway involving, successively, neurons of the paraventricular nucleus of the hypothalamus (PVN), sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion (SCG). Recently, we showed that, in the rat, the SCN uses a combination of daytime inhibitory and nighttime stimulatory signals towards the PVN-pineal pathway in order to control the daily rhythm of melatonin synthesis, γ-aminobutyric acid (GABA) being responsible for the daytime inhibitory message and glutamate for the night time stimulation. In the present study we tested the involvement of these inhibitory and stimulatory SCN outputs in the early morning circadian decline of melatonin release. We hypothesised that, at dawn, the increased release of GABA onto pre-autonomic PVN neurons results in a diminished noradrenergic stimulation of the pineal, and ultimately an arrest of melatonin release. First, we established that prolonged noradrenergic stimulation of the pineal gland was indeed sufficient to prevent the early morning decline of melatonin release. Next, we measured the effect of the application of a GABA-antagonist within the PVN on melatonin release. Since blockade of GABA-ergic signalling could not entirely prevent the early morning decline of melatonin, we suggest that, concomitant with an increased release of GABA, an arrest of the SCN-derived glutamate release is a prerequisite for the circadian decline of melatonin release.

Introduction

Melatonin, also called the night hormone, is exclusively synthesised in and released from the pineal gland in large amounts during the night, in a circadian manner, and in proportion to the night-length. Thereby, melatonin is a perfect output of the central

clock (Reiter, 1993). Besides its help in enforcing the circadian message distributed by the central biological clock, the main function of melatonin is that of a photoperiodic-message distributor, supporting the synchronisation of numerous physiological functions after environmental changes (Pévet, 1987). In mammals the central biological clock, located in the suprachiasmatic nucleus (SCN), controls the daily rhythm of many physiological functions via endocrine and/or neuronal pathways, (see for review Perreau-Lenz *et al.*, in press). The daily rhythm of melatonin synthesis is controlled by the SCN through a multi-synaptic pathway involving, successively, neurons of the paraventricular nucleus of the hypothalamus (PVN), sympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord, and noradrenergic sympathetic neurons of the superior cervical ganglion (SCG) (Moore & Klein, 1974; Klein *et al.*, 1983a; Lehman *et al.*, 1984; Bittman *et al.*, 1989; Tessonneaud *et al.*, 1995; Larsen *et al.*, 1998; Teclemariam-Mesbah *et al.*, 1999; Garidou *et al.*, 2002a). In rats, the release of noradrenalin (NA) into the pineal gland by the sympathetic fibres stimulates melatonin synthesis. The activation of adrenergic receptors (Reiter, 1991b) causes a 150-fold increase of the gene expression of the arylalkylamine N-acetyltransferase (*Aa-nat*) and a 50 to 70-fold increase of its enzymatic activity, inducing an immediate 10-fold rise in the synthesis and secretion of melatonin (Borjigin *et al.*, 1995; Roseboom *et al.*, 1996). *In vivo* evidence showed a clear coupling between noradrenalin release and melatonin secretion in rats (Drijfhout *et al.*, 1996b; Drijfhout *et al.*, 1996c), suggesting that the daily rhythm of melatonin synthesis totally depends on the activation or inactivation of sympathetic fibres by the SCN. However, previous studies in rats (Sassone-Corsi, 1998) and hamsters (Garidou *et al.*, 2003) suggested that the early morning decline of melatonin synthesis was not the direct consequence of a withdrawal of noradrenalin release at the end of the night, but would rather be induced by the accumulation of an intra-pineal factor.

Recently, we showed that, in the rat, the SCN uses a combination of daytime inhibitory and nighttime stimulatory signals towards the PVN-pineal pathway, in order to control the daily rhythm of melatonin synthesis (Kalsbeek *et al.*, 2000c; Perreau-Lenz *et al.*, 2003; Perreau-Lenz *et al.*, 2004), GABA being responsible for the daytime inhibitory message and glutamate for the night time stimulation. Furthermore, we revealed that a stimulatory input to the PVN-pineal pathway is also present during the light period, but under normal conditions its stimulatory effect is overwhelmed by the GABA-ergic output of the SCN (Kalsbeek *et al.*, 2000c; Perreau-Lenz *et al.*, 2004). At present it is not clear whether the stimulatory SCN output to the PVN is derived from one continuously firing population of SCN neurons or whether it is derived from two separate populations of SCN neurons (i.e. one which is active during the light period and one during the dark period). Previously, we hypothesised that the early morning decline of melatonin release would be caused by an increased release of GABA onto

the pre-autonomic PVN neurons, resulting in an arrest of the stimulatory sympathetic input to the pineal gland. The discovery of a stimulatory as well as an inhibitory SCN output, however, presented another possible mechanism for the early morning decline of melatonin release, i.e. a withdrawal of the glutamatergic stimulation of the pre-autonomic PVN neurons. In addition, of course, also a combination of these two mechanisms is possible. In order to test these concepts, we first had to prove that the early morning melatonin decline is really due to the termination of the nocturnal pineal sympathetic input, and not the result of an intra-pineal factor. Therefore, we measured, *in vivo*, the effect of an artificial prolongation of the noradrenergic stimulation on melatonin release, by administering β-adrenergic and α-adrenergic receptor agonists into the pineal gland through reverse microdialysis. In a second experiment, we infused the GABA-antagonist bicuculline (BIC) in the PVN during the dark/light transition in order to test the result of the absence of an increased GABA-ergic input to the PVN-pineal pathway on the early morning decline of melatonin release. According to our initial hypothesis, administration of the GABA-antagonist should prevent the early morning decrease of melatonin. On the other hand, if the early morning decline of melatonin is also dependent on the withdrawal of glutamate release, blockade of GABA signalling would not be sufficient to prevent the melatonin decline.

Material and Methods

Animals

Male Wistar rats (Harlan, Zeist, The Netherlands) were kept in a temperature-controlled environment under 12-h light/12-h dark conditions, fed *ad libitum*, and housed at five animals per cage. For practical reasons, just after their arrival the rats were subjected to a new light-dark regime with the transition from dark to light, or Zeitgeber Time 0 (ZT0), scheduled at 1300 h (i.e. lights on from 1300 h to 0100 h). Surgeries were done four weeks after acclimatisation of the animals to the new laboratory environment. After surgery, all rats were housed individually. All our experiments were conducted under the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

Experimental set-up

EXPERIMENT 1

The first experiment was realised in order to test, *in vivo*, the capacity of the pineal to respond to a prolonged noradrenergic stimulus and produce melatonin at the dark to light transition. Therefore, we investigated whether an artificial continuation of the noradrenergic stimulation at the end of the night is able to prevent the offset of melatonin synthesis. In a first group of rats (group A, n=12), we measured *in vivo*

intra-pineal melatonin release by microdialysis during three subsequent days. On the first day (Ctr1), dialysate samples were collected from ZT14 to ZT26. On the second day (ISO), dialysate samples were collected from ZT14 to ZT29. A β-adrenergic receptor agonist, isoproterenol, was added to the pineal perfusate from ZT21 to ZT26. Finally, on the third day (Ctr2), serving as a post-treatment control day, dialysate samples were collected again from ZT14 to ZT26. In a second group of animals (group B, n=12), we tested the effect of a combined infusion of a β1-adrenergic and a α1-adrenergic receptor agonist, isoproterenol and phenylephrine, respectively, on the offset of melatonin release. In this group, dialysate samples were collected during two consecutive days from ZT20 to ZT31. On the first day (Ctr), only Ringer was perfused through the pineal. On the second day (ISO+Phe), Ringer was also infused from ZT20 to ZT31 but now isoproterenol and phenylephrine were added to the perfusate from ZT21 to ZT26.

EXPERIMENT 2

In the second experiment, we aimed to test whether the early morning arrest of melatonin release was due to an increased GABA-ergic signalling from the SCN to the PVN. Therefore, we measured the effect of a blockade of GABA-ergic signalling in the PVN at dawn on melatonin release in the pineal gland. The release of melatonin was followed during two consecutive days in 16 rats previously implanted with bilateral PVN microdialysis probes and a transversal pineal probe. For both experimental days, pineal gland dialysate samples were collected from ZT20 to ZT30. In addition, Ringer was also perfused in the PVN through the hypothalamic microdialysis probes, from ZT20 to ZT30. On the second experimental day, BIC was added to the PVN perfusate from ZT21 to ZT28.

Surgeries

Surgeries were performed in rats of 300-350 g. The animals were anaesthetized with a neuroleptanalgesic mix (fentanyl citrate, 0.315 mg/ml, and fluanisone, 10 mg/ml; Hypnorm®, Jansen Pharmaceutical Ltd., Oxford, England; 0.06 ml/100 g i.m.) and a benzodiazepine (midazolam hydrochloride, 5mg/ml; Dormicum®, Roche Nederland B.V., Mijdrecht, The Netherlands; 0.04ml/100 g s.c.) and placed in a David Kopf stereotaxic apparatus. The transpineal microdialysis probes were implanted transversally in the pineal gland as described previously (Drijfhout *et al.*, 1993). In the second experiment, the animals were also implanted with U-shaped microdialysis probes aimed at the PVN (tooth bar set at –3.3 mm; angle of -10°; coordinates: 2 mm caudal to bregma; 2 mm to midline; 7.8 mm below the brain surface), as described previously (Perreau-Lenz *et al.*, 2004). In order to reduce post-operative pain, all rats were subcutaneously injected with depressant analgesic opiate (buprenorfine hydro-

chloride, 0.324 mg in 50 mg dextrose; Temgesic®, Reckitt & Colman Products Ltd., Kingston-Upon-Hull, UK; 0.03 ml/100 g) after post-anesthesia arousal. The transversal microdialysis probes implanted in the pineal gland, were made as previously described (Drijfhout *et al.*, 1993), using the microdialysis membrane Hospal (AN69; cut-off: 35-40 kDa). Also U-shaped microdialysis probes implanted in the PVN were made as previously described (Engelmann *et al.*, 1992; Barassin *et al.*, 2002) using the microdialysis membrane C-DAK™ Artificial Kidney cut-off: 6 kDa (135 SCE catalogue nr. 201-8000, CD Medical Inc., Miami Lakes, Florida, USA).

Microdialysis and chemicals

After a recovery period of 6-8 days, the microdialysis probes were connected to the system of microinjection pumps as described previously (Bothorel *et al.*, 2002). Ringer was perfused through the dialysis probes at a flow rate of 3 µl/min during experimental periods and 1 µl/min during resting periods. Dialysate samples from the pineal probe were collected every hour, and stored at - 20° C until melatonin assay. The chemicals isoproterenol (10^{-6} M), L-phenylephrine (10^{-3} M) and BIC (10^{-4} M) were applied to the pineal gland and the hypothalamus, respectively, by reverse dialysis. Chemical solutions were perfused through the hypothalamic probes at a flow rate of 3 µl/min.

Radio Immunoassays

Melatonin concentration of dialysates was measured in duplicate by radioimmunoassay using [^{125}I] melatonin (Amersham, Bucks, UK; specific activity 2000 Ci/mmol) and rabbit antiserum (AB/R/03, Stockgrand Ltd, Guildford, UK), with a final dilution of 1:200,000. Stock melatonin (Sigma Chemicals) was stored at a concentration of 1 mg/ml. Standard dilutions ranged from 3.75 pg/ml to 4 ng/ml, and the minimum detection limit level was 4 pg/ml. The intra-assay coefficients of variation were 19%, 13% and 13% for standards containing 60, 300 and 1,500 pg/ml, respectively, and the inter-assay coefficient of variation between seven assays was 22, 13 and 3%, for the 3 concentrations mentioned before (Barassin *et al.*, 1999).

Histology

In order to check the placement of the hypothalamic probes, the animals were perfused with fixative (paraformaldehyde 4%) at the end of each experiment and Nissl counterstaining was performed on the hypothalamic sections obtained with a vibratome apparatus.

Data analysis

Data from both experiments are expressed as mean ± SEM. For every analysis realized in this study $P < 0.05$ was considered significant. In order to avoid the factor of microdialysis probe placement, we only analysed the data of animals that had a mean ≥ 200 pg/ml/hr of melatonin release during the control session dark period. Data were analysed using a two-way repeated-measures analysis of variance (ANOVA) with the factors "treatment" and "time", followed by a Student-Newman-Keuls post-hoc test if appropriate.

Results

Experiment 1

At the time of the experiments, one animal of group A and 3 animals of group B were disconnected and excluded from the experiment due to their weak health status. One more animal of group B was disconnected due to a block of its microdialysis probe after a few hours of sampling. After the melatonin assay, 4 more animals (3 rats of group A and 1 rat of group B) were removed from the final analysis due to their low levels of melatonin, suggesting a misplacement of the transpineal microdialysis probe.

In the remaining 8 rats of group A, a clear effect of isoproterenol administration at the end of the dark period on melatonin release could be measured (Fig. 1A). ANOVA also revealed a highly significant effects of time ($F_{12, 84}=25.02$; $P < 0.0001$), treatment ($F_{2, 14}=8.66$; $P < 0.01$) and interaction ($F_{24, 168}=2.81$; $P < 0.0001$). The infusion of isoproterenol indeed postponed the complete arrest of melatonin synthesis as shown by the significantly higher levels of melatonin collected between ZT23 and ZT25. In addition, these results show that even in the presence of light, isoproterenol is able to stimulate melatonin synthesis. Indeed, at the beginning of the light period (from ZT24 to ZT26) melatonin levels of ISO animals are also significantly higher than the ones measured at Ctr1. However, when comparing the day-time melatonin levels during the perfusion of isoproterenol (ZT1 and ZT2 time-points) with the peak-time nocturnal levels of melatonin (ZT21 and ZT22 time-points) during control days, post-hoc tests revealed significant differences as well ($P < 0.001$ in all cases).

The data analysis on the remaining 7 rats of group B also revealed a strong effect of the mixed solution of isoproterenol and phenylephrine on the early morning release of melatonin, as shown by the highly significant effects of time, treatment and interaction revealed by ANOVA ($F_{10, 60}=19.65$, $P < 0.0001$; $F(1, 6)=30.04$, $P < 0.01$; $F(10, 60)=10.21$, $P < 0.0001$, respectively). As shown in Fig. 1B, the simultaneous administration of a α- and a β-adrenergic agonist in the pineal gland completely prevented the offset of melatonin release. Melatonin levels measured in the last hour of the night period were significantly higher during ISO+Phe than during the control day. Also dur-

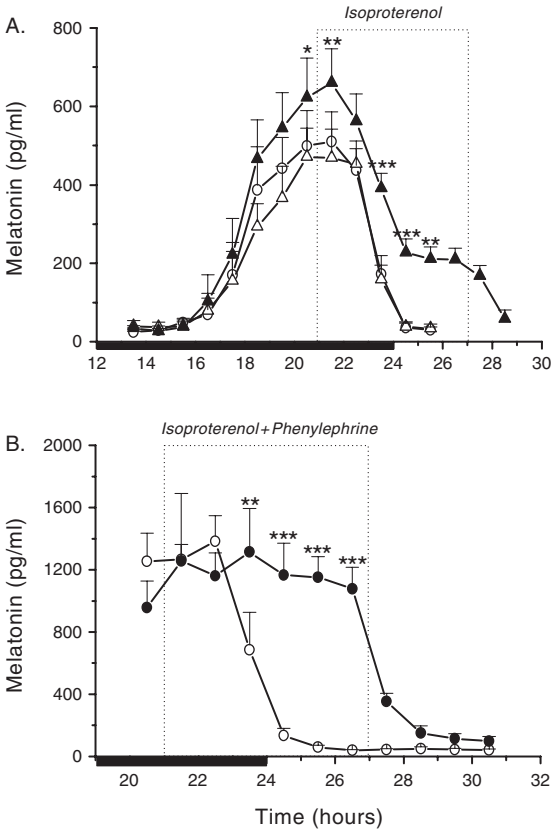

Figure 1 Melatonin profiles at the transition from dark to light measured before, during and after the stimulation of the pineal gland with a β1-adrenergic agonist (A) or with a combination of a β1-adrenergic and a α1-adrenergic agonist (B). In both groups A and B, on the control days Ctr1 (open circles), Ctr2 (open triangles) and Ctr (open circles), melatonin declines before the onset of the light period, whereas on the experimental days (closed symbols) the morning decline is partially (A) or totally (B) prevented. Error bars represent S.E.M. and the *, **, *** indicate significant differences between time-points of experimental days (ISO and ISO+Phe) *versus* the first control day (Ctr1 or Ctr) for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, according to post-hoc analysis.

ing the first three time-points in the light period melatonin levels were significantly higher during ISO+Phe than during Ctr. Moreover, during ISO+Phe, the melatonin levels measured during the first 3 hours of the light period were not significantly different from the peak release measured at ZT23 on the same day ($P = 0.97$, $P = 0.96$, $P = 0.87$ for ZT25, ZT26 and ZT27, respectively) or on the control day ($P = 0.77$, $P = 0.85$, $P = 0.63$ for ZT25, ZT26 and ZT27, respectively).

Experiment 2

From the 16 rats operated upon for the second experiment one died during the operation and 4 animals had to be excluded from the experiment due to their weak health (n=3) or the blockade of a microdialysis probe (n=1). In addition, 3 more rats were excluded from the analysis due to too low melatonin levels.

The data analysis on the remaining 8 animals revealed a significant effect of BIC infusion in the PVN on pineal melatonin release, as shown by the highly significant effects of time, treatment and interaction revealed by the ANOVA ($F_{9, 63}$=8.31, P <0.0001; F(1, 7)=13.65, P <0.01; F(9, 63)=8.12; P < 0.0001, respectively). As shown in Fig. 2, the BIC infusion in the PVN increased melatonin levels during the night period. Indeed, melatonin levels measured in the samples collected between ZT22 and ZT24 were significantly higher during BIC administration in the PVN than during the control day or before the start of the BIC infusion (post-hoc tests: P < 0.001 for both time-points). In addition, melatonin levels measured in the last hour of the dark period during BIC administration were significantly higher than the one measured in control conditions. However, melatonin levels measured during BIC infusion during this final hour of the dark period are still significantly lower than the one measured during the two preceding hours (post-hoc tests: P < 0.001 for both time-points), or than the one measured before the start of the BIC infusion (post-hoc tests: P < 0.05). One hour after lights on melatonin levels measured during BIC did no longer differ significantly from those measured during the control day (post-hoc tests: P = 0.098), despite the still somewhat higher mean level.

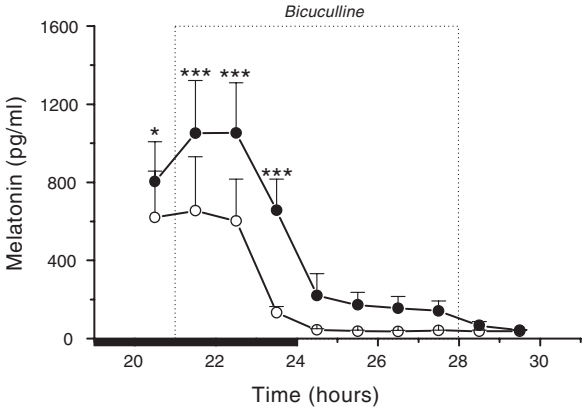

Figure 2 Melatonin profiles at the transition from dark to light measured before, during and after BIC infusion in the PVN. On the control day (open circles) as well as on the experimental day (closed circles) melatonin declines before the onset of light, despite the higher mean levels of melatonin on the experimental day. Error bars represent S.E.M. and the *, *** indicate significant differences between time-points of the experimental day *versus* the control day for P < 0.05 and P < 0.001, respectively.

Discussion

The results of the present study provide clear *in vivo* evidence that in rats the early morning decline of the melatonin rhythm is not due to the accumulation of an intra-pineal inhibitory factor, but is solely due to the arrest of the sympathetic input to the pineal. Therefore, also the circadian decline of the nocturnal melatonin release depends on a direct control by the central pacemaker in the SCN. It is concluded that the early morning withdrawal of the sympathetic input to the pineal is due to a concomitant, SCN-mediated, increased GABA-ergic inhibition and decreased glutamatergic stimulation of the pre-autonomic neurons in the PVN.

Melatonin offset is induced by noradrenergic pineal-activation termination

In order to test the ability of the pineal gland to synthesise melatonin at the end of the night we prolonged the noradrenergic stimulation of the β-adrenergic, or both the β-adrenergic and α-adrenergic receptors of the pineal gland, during the final 3-h of the night and during the first 3-h of the light period. These experiments showed that an artificial extension of the sympathetic stimulation at the end of the dark period was able to rescue the high nocturnal levels of melatonin release, despite the onset of the light period. Interestingly, in the present experimental conditions, the early morning decline was prevented completely when both β- and α-adrenergic pineal receptors were stimulated. Previously it has been shown that the sympathetic stimulation of the pineal gland results not only in the activation of the *Aa-nat* gene, but also in the accumulation of an inhibitory factor such as ICER (Takahashi, 1994; Stehle *et al.*, 1995). It was hypothesised that once this inhibitory factor would have reached a certain threshold, i.e. after several hours of sympathetic stimulation, it would inhibit the activation of the *Aa-nat* gene and thereby the synthesis of melatonin. Moreover, it has recently been shown that, in Syrian hamsters, the daily rhythm of melatonin synthesis is framed by inhibitory and stimulatory intra-pineal transcription factors (Garidou *et al.*, 2003). It was proposed that the necessary synthesis of a stimulatory protein could explain the long delay (7-8 h) between the onset of the dark period and a significant effect on melatonin synthesis in this species. In addition, the inability to stimulate melatonin synthesis during daytime in that species was explained by proposing the synthesis of an inhibitory protein (probably following noradrenalin stimulation), starting at the end of the night.

The present results, however, contradict, at least in rats, an exclusive role for ICER or other intra-pineal inhibitory factors as determining agents for the early morning decline of melatonin. The prolonged stimulation of β-adrenergic receptors alone was sufficient to prevent a total decline of melatonin synthesis, even in the presence of light. In addition, when stimulating both β-adrenergic and α-adrenergic receptors, we were able to prevent totally, for the time of the infusion, the decline of melatonin

synthesis. In both cases, melatonin levels decreased within one hour and returned to basal daytime levels within 2 hours after the arrest of the infusion. These results agree with the findings in the CREM knock-out mice (Foulkes *et al.*, 1996). On the one hand this study confirmed the inhibitory role of ICER on *Aa-nat* expression, with null mutant animals showing a significant increase of nocturnal AA-NAT transcripts, with an earlier rise, higher peak levels and a longer persistence than in wild type animals. However, on the other hand, CREM knock-out-animals still displayed a clear circadian rhythm of the AA-NAT message, clearly indicating that ICER was not involved in the early morning decline of melatonin.

The co-administration of an α-adrenergic receptor agonist clearly potentiated the action of the β-adrenergic agonist, as also reported previously for enzyme activity of AA-NAT and melatonin synthesis (Klein *et al.*, 1983b; Drijfhout *et al.*, 1993). However, α-adrenergic antagonists do not decrease *Aa-nat* gene transcription (Roseboom *et al.*, 1996; Garidou *et al.*, 2002b). Consequently, it is likely that the high levels of melatonin measured during the infusion of adrenergic agonists are mainly due to the stimulation of AA-NAT activity. Indeed, the prolongation of noradrenergic activation of the pineal certainly prevents the decrease of cAMP levels and consequently the rapid degradation of AA-NAT proteins induced by proteasomal proteolysis and dephosphorylisation by protein phosphatase, as shown after arrest of noradrenergic stimulation after light exposure (Klein & Weller, 1972; Klein *et al.*, 1978; Illnerova *et al.*, 1979; Gastel *et al.*, 1998). As soon as the infusion of the adrenergic agonists is terminated, this degradation process is unleashed and low daytime melatonin levels are reached within 2 hours. Our results further confirm the functionality of the coupling between noradrenaline release and melatonin release in the pineal gland as previously shown by Drijfhout *et al.* (1996b).

The clock signals the end of the night to the pineal gland

In the second experiment, we showed that, also at the end of the dark period, local administration of the GABA-ergic antagonist BIC in the PVN has a stimulatory effect on melatonin release, as previously reported for the light period (Kalsbeek *et al.*, 2000c). However, clearly, the administration of BIC was not able to prevent the circadian decrease of melatonin release, as previously BIC was unable to restore nighttime melatonin levels completely during light period infusions. On the other hand, during the subjective light period, BIC infusions in the PVN are able to completely restore melatonin release, i.e. reaching nocturnal release levels (Kalsbeek *et al.*, 2000c). The inability of BIC to restore melatonin release completely during the normal light period is probably due to an incomplete blockade of all GABA-ergic receptors. During a normal light period GABA is released from SCN terminals not only through a circadian mechanism, but also by a direct light-mediated stimulation of the SCN neurons.

However, the inability of BIC in the present study to prevent the circadian decrease of melatonin release cannot be attributed to a direct inhibitory effect of light since even before lights on, i.e. ZT23-24, we noticed a significant decrease of melatonin release. Apparently, the circadian decline of melatonin is not due solely to an increased inhibition of the pre-autonomic PVN neurons by GABA released from SCN terminals. At present we see two possibilities. First, next to GABA, an additional inhibitory SCN transmitter is operative, or, secondly, concomitant with the increased GABA release in the PVN, the stimulatory input of the SCN to the pre-autonomic neurons is withdrawn. The first possibility would imply that the additional inhibitory SCN transmitter is only released for a few hours during the dark/light transition, because for all other time-points tested so far, BIC alone is sufficient to elicit the release of melatonin (Kalsbeek *et al.*, 2000c). The withdrawal of the stimulatory input could be accomplished in two different ways. First, it is possible that a continuously active subpopulation of glutamatergic SCN neurons is transiently inhibited during the dark/light transition. This possibility, too, would imply that, next to GABA, another inhibitory SCN transmitter is involved. Moreover, this hypothetical inhibitory SCN transmitter could even inhibit the pre-autonomic PVN neurons and the glutamatergic SCN neurons at the same time. Previously we have shown that vasopressin administration during the final 4 hours of the dark period was not able to induce a premature decline of melatonin release, therefore this third SCN transmitter implicated in the control of the circadian melatonin rhythm is clearly not vasopressin (Kalsbeek *et al.*, 2000c). On the other hand, the withdrawal of the stimulatory input could also be accomplished by an incomplete overlap of two populations of SCN neurons, active either during the light or the dark period, and each responsible for the stimulation of the pre-autonomic PVN neurons.

Recent results obtained in rats showed the existence of a factor, dependent on an intact sympathetic input to the pineal gland, that inhibits *Aa-nat* gene expression during the daytime (Garidou *et al.*, 2001; Perreau-Lenz *et al.*, 2003). During the light period, pineal *Aa-nat* mRNA levels were higher in rats that had their superior cervical ganglia (SCG) removed than in sham-operated animals. These increased levels were not induced by a direct effect of circulating catecholamines on the pineal receptors (Garidou *et al.*, 2001). Because neuropeptide-Y is co-localised with noradrenalin in the sympathetic fibres (Schon *et al.*, 1985; Reuss & Moore, 1989) and it has been reported to decrease β-adrenergic-induced cAMP accumulation (Olcese, 1991; Simonneaux *et al.*, 1994) and pineal AA-NAT activity (Reuss & Schroder, 1987), it may function as an inhibitory sympathetic factor. At present, we cannot exclude the possibility that this inhibitory factor derived from the SCG plays a significant role during the dark/light transition. Measurement of pineal NPY release by microdialysis will be necessary to determine the precise role of this peptide in the melatonin generating rhythm system.

In conclusion, the present results clearly show that the early morning decline of melatonin release cannot be attributed solely to the accumulation of an inhibitory pineal factor or the increased release of GABA from SCN terminals onto the pre-autonomic PVN neurons. Maybe even a third SCN transmitter, next to GABA and glutamate, is involved in the control of daily melatonin rhythm. However, a definitive elucidation of the mechanism(s) responsible for the circadian decrease of early morning melatonin release can be obtained only by the measurement of the exact daily patterns of GABA and glutamate release within the PVN or the identification of a third SCN transmitter that is released specifically during the dark/light transition.

Acknowledgements

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CHAPTER 5

Feeding conditions affect vasopressin and Per2, but not Per1, gene expression in the rat suprachiasmatic nucleus

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Submitted

Abstract

Like many other biological functions, the rhythmic nocturnal synthesis of melatonin in the pineal gland is controlled by the circadian pacemaker, located in the suprachiasmatic nucleus of the hypothalamus (SCN). Recently we demonstrated that in order to control the daily rhythm of melatonin synthesis, the SCN sends a combination of inhibitory and stimulatory signals to the paraventricular nucleus of the hypothalamus (PVN). The present study was initiated in order to identify the sub-population(s) of SCN cells responsible for these stimulatory and inhibitory signals. Therefore, we analysed and compared the spatial and temporal distribution of *Per1* and *Per2* gene expression within the SCN, using two experimental paradigms, i.e. an 8-h advance of the light/dark-cycle, and a time-restricted-feeding regime. Firstly, these specific conditions revealed the necessity for a periodic absence of *Per* gene expression within the dorsal SCN in order to enable the expression of the melatonin rhythm. Secondly, the present study clearly reveals that feeding conditions feed back to the molecular clock by changing the normal expression of the *Per2* gene. Finally, we revealed a close and specific correlation between *Per2* and VP gene expression in the SCN.

Introduction

The biological clock, located in the suprachiasmatic nucleus of the hypothalamus (SCN), controls the circadian rhythm of many biological functions and sustains cyclic expression of clock genes (i.e. *Per1* and *Per2* genes) interacting with each other in transcriptional-translational positive and negative feedback loops (see for review Reppert & Weaver, 2001; Albrecht & Eichele, 2003). Until recently, the SCN was seen as a rather homogeneous structure, mostly active during (subjective) daytime (Green & Gillette, 1982; Schwartz *et al.*, 1983; Shibata & Moore, 1988), and distributing one global timing signal. However, neuroanatomical and physiological studies on clock-controlled endocrine rhythms (e.g. melatonin and corticosterone release)

have revealed that both inhibitory and stimulatory signals emanate from the SCN at different moments of the day (Kalsbeek *et al.*, 1996a; Kalsbeek *et al.*, 1996b; Kalsbeek *et al.*, 2000b; Perreau-Lenz *et al.*, 2003; Perreau-Lenz *et al.*, 2004). Moreover, recent electrophysiological studies strongly suggest the existence of different subpopulations of SCN cells, active at different times of the L/D-cycle (Quintero *et al.*, 2003; Saeb-Parsy & Dyball, 2003b). In addition, a differentiated temporal and spatial distribution has been shown for *Per1* and *Per2* gene expression within the SCN (Hamada *et al.*, 2001; Dardente *et al.*, 2002; Yan & Okamura, 2002). Recently, an elegant study demonstrated that, after an abrupt shift in the day/night cycle, a total synchrony between the dorsomedial and ventrolateral subdivisions of the SCN is necessary for a complete resynchronisation of the locomotor activity rhythm (Nagano *et al.*, 2003).

Although vasopressin (VP) neurons (involved in the inhibitory part of the clock-controlled circadian corticosterone rhythm) are restricted to a clearly defined dorsal subdivision of the SCN, the majority of SCN neurons seem to be able to synthesise γ-aminobutyric acid (GABA) and/or glutamate, respectively responsible for the inhibitory and stimulatory clock outputs involved in the melatonin-rhythm generating system (Kalsbeek *et al.*, 2000b; Perreau-Lenz *et al.*, 2004). This study was therefore initiated to identify specific subsets of SCN neurons responsible for the onset or offset of melatonin synthesis. Since, it takes at least 5 days for the nocturnal peaks of arylalkylamine-*N*-acetyltransferase activity and melatonin secretion to reappear after an 8-h phase advance of the L/D cycle (Illnerova, 1989; Kennaway, 1994; Drijfhout *et al.*, 1997; Kalsbeek *et al.*, 2000a), and since such an abrupt shift also affects the spatial and temporal distribution of *Per* genes expression in the SCN (Nagano *et al.*, 2003), we investigated whether the activation of a particular subpopulation of SCN neurons could be correlated with the reappearance of either the onset or offset of the melatonin peak. In addition, since a restricted-feeding protocol delays the reappearance of the melatonin rhythm after such a phase-advance even further (Kalsbeek *et al.*, 2000a), we also included a restricted-feeding group, to differentiate further *Per* gene expression changes in the SCN. Radioactive and non-radioactive *in situ* hybridisation techniques were used to evaluate spatial and temporal expression of *Per1* and *Per2* genes in the SCN. In addition, in the same animals we also measured the SCN VP mRNA content and the plasma melatonin concentration.

Material and methods

Animals

Male Wistar rats (Harlan, Zeist, The Netherlands) of 275-300 g, housed at five animals per cage, were kept in a temperature-controlled environment under 12-h light/12-h dark conditions and fed *ad libitum* during one week after their arrival in order to

acclimatize to the new laboratory environment. Subsequently animals were housed singly and maintained either in *ad libitum* or in time-restricted feeding conditions for at least 3 weeks. In the time-restricted feeding conditions, rats had a daily access to food for 2 hours (starting 4 hours after the onset of the light period (Zeitgeber Time 4, ZT4; ZT0 being the beginning of the light period). Experiments were conducted under the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts ands Sciences.

Experimental set-up

Animals were divided in 2 groups maintained in *ad libitum* (n=36) or time-restricted feeding conditions (n=27). The *ad libitum* group was subdivided in two subgroups. One subgroup, designated as the control group (AL; n=18), remained in the same L/D-conditions throughout the experiment and did not experience any phase-shift of the L/D cycle. The second subgroup (ALs; n=18) was subjected to a phase-advance of 8-h. The group of animals maintained in time-restricted feeding conditions was also subdivided in two subgroups. One subgroup (RFs; n=18) was also subjected to an 8-h advance of the L/D cycle, while for the second subgroup, considered as the control group (RF; n=9), no shift was applied and the restricted-feeding regime continued. The 8-h advance of the L/D cycle was realised by shutting down the light at ZT4, 4 weeks after the start of the restricted-feeding regime. From this first day of shift (D0), the RFs animals were provided with food *ad libitum*. Starting at ZT10 on day 5 after the shift (D5) and ZT2 for the non-shifted groups, the animals of the 4 groups were maintained in constant darkness under red-dim-light until the end of the experiment. The animals of the AL, ALs, and RFs groups were perfused every 4-hours at six different time points starting at CTs14 on D5 (CTs14 indicating CT14 after the shift and corresponding to ZT6 in non-shifted time). Animals of the RF control group were perfused at three time points, 2-h before (CT2), at the end (CT6) and 4-h after (CT10) the 2-h feeding period. For each time-point animals were perfused with fixative (4% paraformaldehyde, 75 mM lysine, 10mM sodium periodate in 100 mM phosphate buffer, pH 7.4). After anaesthesia and before perfusions, an intra-cardiac blood puncture was made. Brains were removed and post-fixed during 2h at 4°C, rinsed with 50% ethanol and embedded in polyethylene glycol after dehydratation as described previously (Klosen *et al.*, 1993). Six series of 10 µm coronal sections were mounted on slides (6 sections per slide covering the rostro-caudal area of the SCN; Superfrost™ Plus, Menzel-Gläser, Germany).

Hybridisation

As previously described elsewhere (Dardente *et al.*, 2002), non-radioactive and radioactive *in situ* hybridisation was realised using rat *Per1* and *Per2* clones, kindly donated

by Prof. H. Okamura (Department of Anatomy and Brain Science, Kobe University School of Medicine, Japan).

NON-RADIOACTIVE *IN SITU* HYBRIDISATION

Hybridisations were carried out using hapten-labeled riboprobes (digoxigenin, Roche Biochemicals, Germany). In short, sections were pretreated with a postfixation step (4% phosphate buffered formaldehyde for 10 min), a digestion step (2 µg/ml of proteinase K in PBS for 30 min at 37° C) and an acetylation step (100 mM triethanolamine in PBS, 0.25% acetic anhydride). The sections were then hybridised for 40h at 54° C with 200 ng/ml of labelled sense or antisense probes in the hybridisation buffer (50% formamide, 5×SSC, 5×Denhardt's, 500 µg/ml denaturated salmon sperm DNA and 0.04% diethylpyrocarbonate). After hybridisation, the probe was washed off in 5×SSC at room temperature for 10 min. Stringency rinses were performed at 72° C for 6×10 min in 0.1 SSC. In addition, RNase treatment (20 µg/ml in 10 mM Tris pH 7.5, 400 mM NaCl, 2.5 mM EDTA) was carried out. Sections were then processed for immunocytochemistry using alkaline phosphatase labelled anti-digoxigenin antibodies. Detection of the hybridisation process was achieved by nitrobluetetrazolium (400 µM) in 100 mM Tris pH 9.5, 100mM NaCl, 5 mM $MgCl_2$ and 5% polyvinyl alcohol.

RADIOACTIVE *IN SITU* HYBRIDISATION

Pretreatments were done as described above for the non-radioactive procedure. Antisense and sense probes were transcribed in presence of α[^{35}S]-UTP (1250 Ci/mmol, NEN-Dupond, Zaventem, Belgium) according to the manufacturer's protocol (MAXIscript, Ambion, USA). After double acetylation, sections were rinsed twice in PBS, dehydrated in a graded ethanol series of 1 min (70%, 90%, 95% (with 250 mM ammonium acetate) and 100%) and dried at room temperature. Sections were hybridised with either antisense or sense cDNA riboprobes in a solution containing 50% deionised formamide, 10% dextran sulphate, 50 mM dithiothreitol, 1×Denhardt's solution, 2×SSC, 1 mg/ml salmon sperm DNA, 1 mg/ml yeast RNA, at 54° C for 16h. After incubation, the sections were rinsed twice for 10 min in 2×SSC before being treated with Ribonuclease A (Sigma, 0.02 Kunitz unit/ml) in 10 mM Tris, 500 mM NaCl, 10 mM EDTA buffer (30 min at 37° C). Slides were then rinsed twice and stringency washes were carried out as described for non-radioactive hybridisation. Finally, sections were dehydrated in a graded ethanol series (70%, 90%, 95% and 100%) and air-dried. Slides were exposed with ^{35}S standards to an autoradiographic film (Hyperfilm MP, Amersham, Orsay, France) for 5 to 15 days.

Radio Immunoassays

Melatonin plasmatic concentration was measured in duplicate by radioimmunoassay using [^{125}I] melatonin (Amersham, Bucks, UK; specific activity 2000 Ci/mmol) and rabbit antiserum (AB/R/03, Stockgrand Ltd, Guildford, UK) with a final dilution of 1:200000. Stock melatonin (Sigma Chemicals) was stored at a concentration of 1 mg/ml. The standard range of dilutions extended from 3.75 pg/ml to 4 ng/ml, and the minimum detection limit level was 4 pg/ml. The intra-assay coefficients of variation were 19, 13 and 13 % for standards containing 60, 300 and 1500 pg/ml, respectively and the inter-assay coefficient of variation between seven assays was 22, 13 and 3 %, for the 3 concentrations mentioned before (Barassin *et al.*, 1999).

Data analysis

Quantitative analyses of the autoradiograms for the first experiment were performed using a computerised analysis system (ImageProRunnablePlus). The optical density of each SCN area for the 6 mounted sections (covering the rostro-caudal axis of the SCN) was measured. The mean background of each slide (one slide/animal) was subtracted from this measurement, and the result was multiplied by the area of the SCN, giving the Integrative Density. Statistical comparisons between groups were assessed using a 2-way analysis of variance (ANOVA) with the factors "*group*" and "*time*". In addition, the time-effect was analysed for each group separately using a one-way ANOVA. Possible correlations were analysed using Pearson analysis. Data from both experiments are expressed as mean ± SEM. For every analysis realized in this study $P < 0.05$ was considered significant.

Results

In the present study, the body-weight time-course of the animals from the AL, ALs and RFs groups before the shift was highly different ($F_{6,102}$=111.08; $P < 0.0001$). Indeed, rats submitted to a 2-h restricted-feeding regime from ZT4 to ZT6 (RFs) maintained their body-weight around 300g, while the rats fed *ad libitum* (AL and ALs) showed a typical growth curve, growing approximately 100g in 4 weeks (post-hoc tests: $P < 0.001$ compared to AL and ALs).

Per genes expression distribution within the SCN

The non-radioactive *in situ* hybridisation revealed a different distribution of *Per* gene expression in the SCN following an 8-h advance of the L/D cycle after prior *ad libitum versus* RF conditions. In *ad libitum* conditions, under a typical L/D=12-h/12-h cycle, we found *Per1* and *Per2* genes expressed with the characteristic daily time course in different regions of the SCN (Yan & Okamura, 2002; Nagano *et al.*, 2003). *Per1*

and *Per2* expression was the highest and the most widespread (i.e. dorsomedial and ventrolateral expression) around CT6 and CT10, respectively. As shown in Fig.1 and Fig. 2, the nocturnal expression of *Per1* and *Per2* is restricted to a few cells located in the centre of the SCN. Five days after an 8-h advance of the L/D cycle, the daily

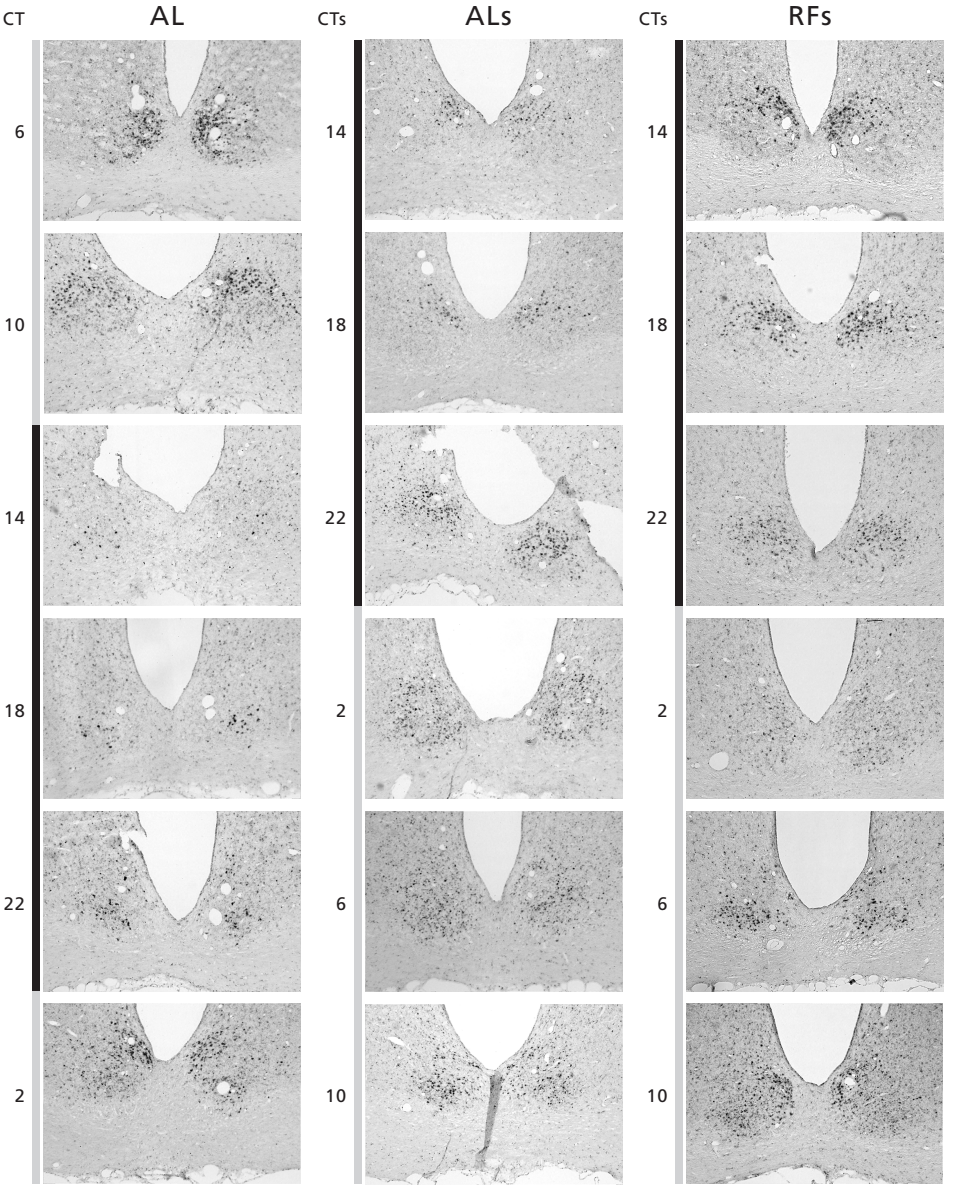

Figure 1 Spatial and temporal distribution of *Per1* gene expression within the SCN, through the L/D cycle, in the AL, ALs and RFs group, as revealed by the non-radioactive immuno-in-situ hybridisation technique. Grey and black bars indicate light and dark portions of the L/D cycle.

rhythm of *Per* gene expression was not yet completely shifted in the ALs group. The highest and most widespread expression of *Per1* and *Per2* genes was found at CTs22 and CTs2-CTs6, respectively, i.e. still 4-8 hours away from the destined acrophase. In animals previously maintained on the restricted feeding regime and subjected to an

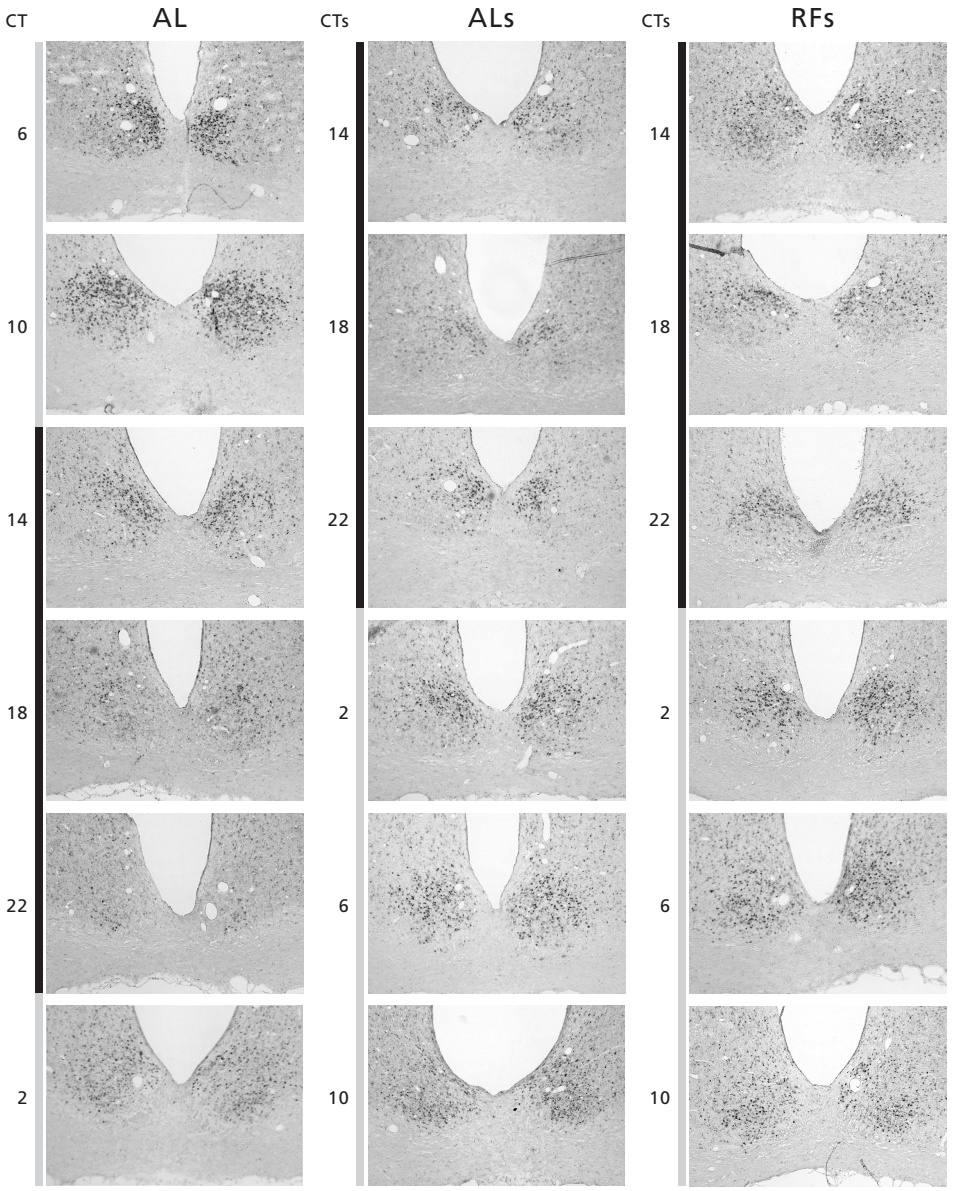

Figure 2 Spatial and temporal distribution of *Per2* gene expression within the SCN, through the L/D cycle, in the AL, ALs and RFs group, as revealed by the non-radioactive immuno-in-situ hybridisation technique. Grey and black bars indicate light and dark portions of the L/D cycle.

8-h advance of the L/D cycle, the expression pattern of the *Per* genes was even more disturbed. Indeed, in these animals, *Per1* expression can be divided in a two periods: one at the beginning of the new night, and characterized by a clear dorsal expression, and one with a predominant ventral expression of *Per1* around CTs6. This second time period corresponds to the timing of the new acrophase once the animals would be synchronized to the new L/D cycle. In these RF shifted animals there is only one time point with an almost complete absence of *Per1* expression, i.e. CTs2. Such a clear subdivision, either in time or in space, is not observed for *Per2* gene expression. Indeed, *Per2* gene expression is distributed almost equally throughout the L/D cycle. Remarkably, none of the 6 time points shows a complete absence of *Per2* expression.

Per genes expression quantification in the SCN

Using the radioactive *in situ* hybridisation technique on adjacent sections, the temporal changes in *Per1* and *Per2* gene expression were also analysed quantitatively (Fig. 3A & B). Concerning *Per1* gene expression, the 2-way ANOVA revealed highly significant effects of *time*, *group* and *interaction* between the 3 groups AL, ALs and RFs (Table 1). Significant differences between values of ALs and RFs *versus* AL groups are illustrated in Fig. 3A. Values of ALs and RFs groups significantly differed only for the time-point CTs10 (post-hoc test: $P < 0.01$). In addition, the one-way ANOVA analysis

Table 1 Statistical analyses of the mRNA quantifications of *Per1*, *Per2*, VP genes and of melatonin concentrations measured respectively in the SCN and in the plasma of AL, ALs and RFs animals.

	Per1	***Per2***	**VP**	**Melatonin**
2-way ANOVA				
time	F(5, 36)=8.81 p<0.0001	F(5, 35)=1.68 p=0.167	F(5, 36)=4.72 p<0.01	F(5, 35)=1.68 p=0.166
group	F(2, 36)=6.62 p<0.01	F(2, 35)=13.81 p<0.0001	F(2, 36)=11.434 p<0.0001	F(2,35)=2.50 p=0.097
time x group	F(10, 36)=8.34 p<0.0001	F(10, 35)=9.74 p<0.0001	F(10, 36)=13.32 p<0.0001	F(10, 35=0.80 p=0.63
1-way ANOVA				
AL	F(5,12)=11.92 p<0.001	F(5,11)=10.22 p<0.001	F(5, 12)=14.80 p<0.001	F(5, 12)=1.41 p=0.29
ALs	F(5, 12)=4.5 p<0.05	F(5,12)=5.27 p<0.01	F(5,12)=6.53 p<0.001	F(5, 11)=0.93 p=0.50
RFs	F(5, 12)=10.94 p<0.001	F(5, 12)=2.84 p=0.07	F(5, 12)=3.50 p<0.05	F(5, 12)=1.05 p=0.44

revealed an effect of *time* of *Per1* gene expression for each group analysed separately (Table 1). In *ad libitum* conditions before the shift (AL) *Per1* expression showed a significant peak at CT6 (post-hoc test: $P < 0.01$ to all other time-points), while after the shift, in the ALs group, the integrative density for *Per1* mRNA was significantly higher at CTs22 compared to CTs18, CTs6 and CTs10 (post-hoc tests: $P < 0.05$ for the three time-points). Besides, in restricted-feeding conditions, higher *Per1* gene expression was also noticed for CTs22, CTs2 and CTs10 time-points *versus* CTs14, CTs18 and CTs6 (post-hoc tests: CTs22: $P < 0.01$ compared to three time-points; CTs2: $P < 0.05$ compared to three time-points; CTs10: $P < 0.01$, $P < 0.05$, and $P < 0.01$ compared to CTs14, CTs18 and CTs6, respectively).

These quantifications also clearly revealed that *Per2* gene expression is differently affected by the restricted-feeding regime. Although the 2-way ANOVA revealed no significant effect of *time* in the expression of *Per2* in the SCN, it showed a highly signifi-

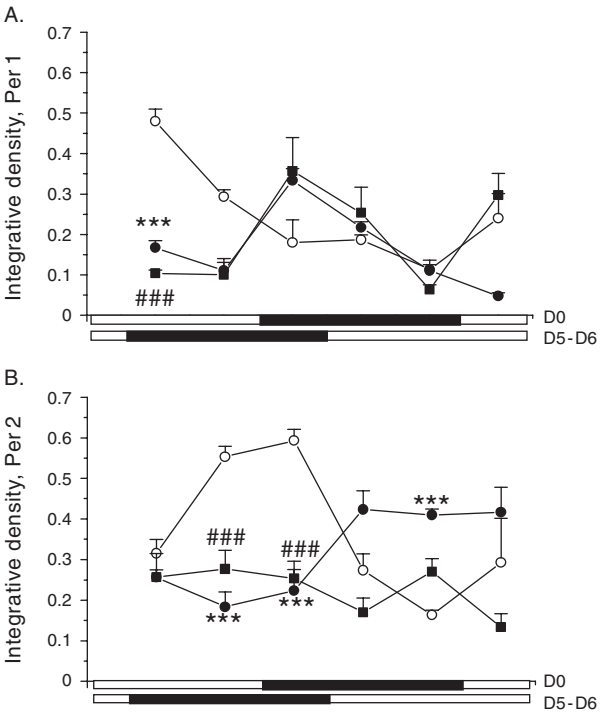

Figure 3 Quantification of *Per1* (A) and *Per2* (B) gene expression in the SCN in AL (open circles) *versus* ALs (closed circles) and RFs (closed squares) groups. Bottom bars indicate the nocturnal periods before and after the 8-advance of the L/D cycle. Relative mRNA amount is expressed as Integrative Density. *** and ### indicate significant differences of density of $P < 0.001$ for ALs *versus* AL groups and RFs *versus* AL groups, respectively.

cant effect of *group* and *interaction* between the 3 groups AL, ALs and RFs (Table 1). In addition, in AL group, *Per2* gene expression showed a significant peak at CT10-CT14 before the shift (post-hoc tests: $P < 0.05$ compared to all other time-points). Five days after an 8-h advance of the L/D cycle, the integrative density for *Per2* mRNA, in animals fed *ad libitum* (ALs), showed significantly higher levels at CTs6 than at CTs14, CTs18 and CTs22 (post-hoc tests: $P < 0.05$ for the three time-points), but no such peak could be measured in the animals with prior RF (Table 1). In addition, *Per2* expression levels were significantly different in ALs animals *versus* AL animals, with lower levels for the time-points CTs18 and CTs22 but higher levels at CTs6. On the other hand, *Per2* gene expression in RFs animals only significantly differed from that in AL animals, showing lower values for two time-points CTs18 and CTs22 (Fig. 3B). These two time points correspond to the acrophase before the shift in AL animals, i.e. the daily peak of *Per2* expression is completely lost in RFs animals.

VP mRNA content in the SCN

In an attempt to further correlate *Per* gene expression and distribution within the SCN with clock outputs, we also measured VP mRNA content in the SCN by *in situ* hybridisation on adjacent sections in the same animals. Vasopressin is one of most extensively studied SCN neurotransmitters and recognized as the one most clearly related to SCN output (Gillette & Reppert, 1987; Cagampang *et al.*, 1994; Kalsbeek *et al.*, 1995). The 2-way ANOVA revealed significant effects of *time*, *group* and *interaction* between the 3 groups AL, ALs and RFs (Table 1). Post-hoc-tests revealed that two time points significantly differed in VP expression between the AL *versus* ALs group (Fig. 4A). The peak of VP mRNA content, measured at CT10 in AL animals, had indeed totally disappeared at CTs18 in ALs animals, whereas VP expression was significantly higher in ALs animals at CTs6 as compared to CT22 in AL animals. In addition, in the AL group we observed a rhythm of VP mRNA content with a peak expression at the end of the day (i.e. CT10). In the ALs group, VP mRNA content also showed a significant daily rhythm. In these animals, VP expression during the middle of the (shifted) light period (CTs6) was significantly higher than at nocturnal time points and than at CTs2 (post-hoc tests: $P < 0.01$ compared to CTs14 and CTs18, $P < 0.05$ for CTs22 and CTs2). These results indicate an almost complete shift of the VP mRNA rhythm five days after the shift of the L/D-cycle. In the RFs group, on the other hand, no such a peak of expression could be observed. Indeed, although ANOVA analysis revealed a small effect of *time* in the RFs group, post-hoc comparison tests only indicated a trough of VP gene expression at CTs6, with only the CTs2 and CTs6 time points differing significantly from each other ($P < 0.05$). In addition, except for the CTs18 time point, VP gene expression in the RFs group did not differ significantly from the VP gene expression measured in AL group.

The similarity of the phase-advance effect on the VP and *Per2* gene expression profiles was confirmed by a correlation analysis for the animals of the AL and ALs groups. No significant correlation could be measured between the expression of *Per1* and VP, either before ($r = 0.43$; $p = 0.09$) or after the 8-h advance ($r = 0.09$; $p = 0.72$). On the contrary, the average integrative density of VP mRNA was positively correlated to the one of the *Per2* gene in both conditions ($r = 0.64$; $P < 0.01$ and $r = 0.65$; $p = 0.01$, respectively).

Melatonin plasma content

By intra-cardiac blood punctures we were able to measure another hand of the clock in the same animals, i.e. plasma melatonin. However, statistical analysis did not reveal any significant differences in plasma melatonin levels (Table 1), although the effect of *group* approached significance. Also no significant effects of *time* were observed for

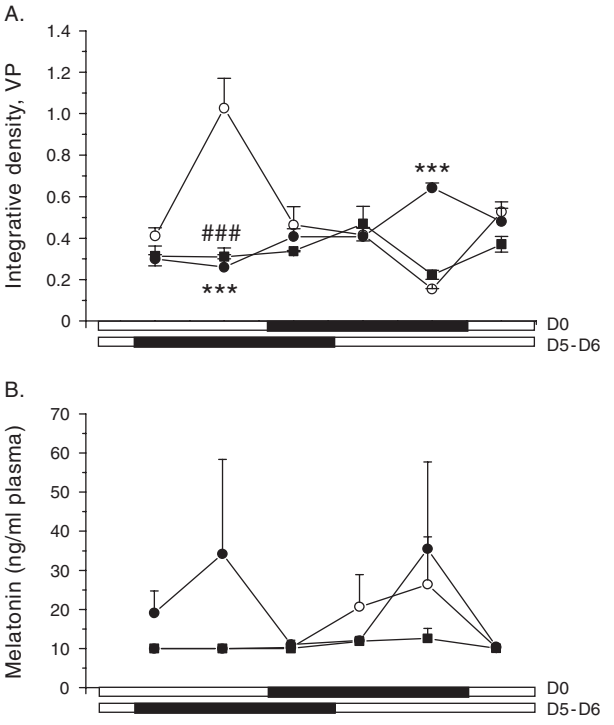

Figure 4 24-h profiles of relative VP mRNA amount in the SCN (A) and plasma melatonin concentration (B) in AL (opened circles) *versus* ALs (closed circles) and RFs (closed squares) groups. Bottom bars indicate the nocturnal periods before and after the 8-advance of the L/D cycle. *** and ### indicate significant differences of density of $P < 0.001$ for ALs *versus* AL groups and RFs *versus* AL groups, respectively.

the data of the individual groups. Most likely this is due to the small numbers of animals and the strong inter-individual differences. However, we did notice some clear differences between the 3 groups in their mean plasma melatonin levels. In the AL group a normal nocturnal increase of plasma melatonin concentrations was observed at CT18-22. On the other hand, after the shift, none of the RFs animals showed increased melatonin levels at any of the 6 time points. In the ALs group some animals showed increased plasma melatonin concentrations during the new dark period, whereas other animals showed increased plasma melatonin levels at a time point corresponding to the previous dark period (i.e. CTs6).

Restricted Feeding

In addition to the differential effects of phase-shifting on *Per* gene expression in the ALs and RFs animals, a clear effect of restricted feeding on *Per1* and *Per2* gene expression could be observed already before animals were exposed to the 8-h phase-advance (Fig.5A & 5B). Indeed, *Per1* integrative density was clearly not affected by the restricted-feeding regime, while the *interaction* effect just escaped significance for the *Per2* gene expression (Table 2), with a tendency for *Per2* to be up-regulated in RF animals just after feeding (CT6) compared to AL animals.

In addition, measurement of the VP mRNA content revealed that the VP expression in the SCN was strongly influenced by the restricted feeding regime (Fig.5C). Indeed, VP gene expression in the RF animals was significantly increased just after

Table 2 Statistical analyses of the mRNA quantifications of *Per1*, *Per2*, *VP*genes in the SCN, in AL and RF animals, at CT2, CT6 and CT10.

	Per1	*Per2*	VP
2-way ANOVA			
time	F(2, 12)=2.76 p=0.10	F(2, 11)=2.79 p=0.10	F(2, 12)=10.85 p<0.01
group	F(1, 12)=2.13 p=0.17	F(1, 11)=0.70 p=0.42	F(1, 12)=0.92 p=0.36
time x group	F(2, 12)=1.01 p=0.39	F(2, 11)=3.71 p=0.06	F(2, 12)=19.84 p<0.001
1-way ANOVA			
AL	F(2, 6)=9.70 p<0.05	F(2, 5)=3.84 p=0.098	F(2, 6)=12.97 p<0.01
RF	F(2, 6)=0.37 p=0.71	F(2, 6)=3.17 p=0.11	F(2, 6)=19.07 p<0.01

feeding-time (CT6) and decreased 4 hours later (CT10) as compared to the *ad libitum* fed animals (Table 2). Moreover, we found that the integrative densities of *Per2* mRNA and VP mRNA were positively correlated in the RF group ($r = 0.82$; $P < 0.01$), whereas no significant correlation could be found between *Per1* and VP mRNA (r=-0.126; P = 0.75).

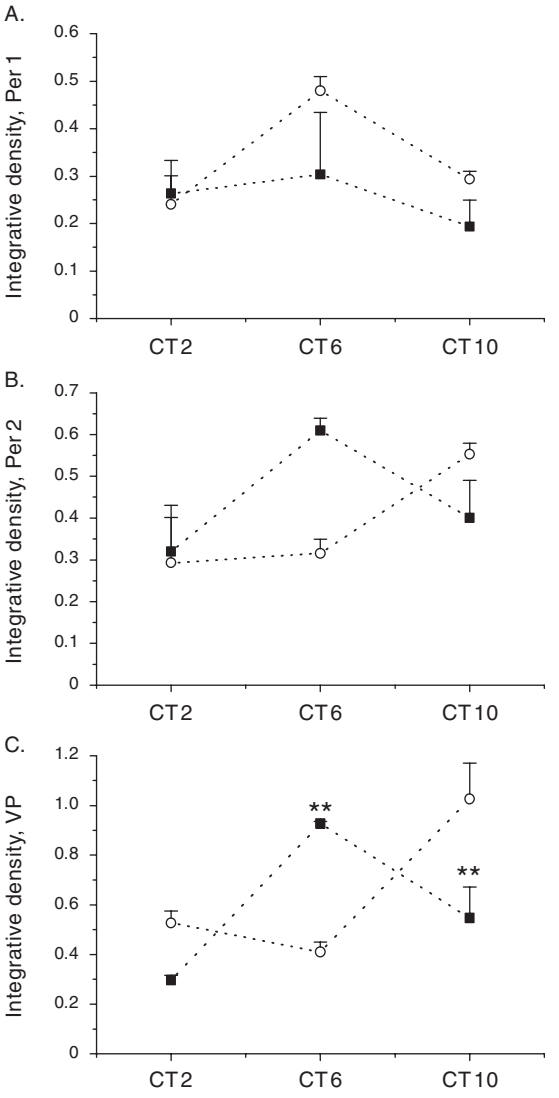

Figure 5: Direct effect of time-restricted feeding for 2 hours (from CT4 to CT6) on *Per1* (A), *Per2* (B) and VP (C) gene expression in the SCN. Significant differences of density from RF (closed squares) *versus* AL (open circles) groups are indicated by ** for P < 0.01.

Discussion

The present results show that 5 days after an abrupt 8-h advance of the L/D cycle, the incomplete recovery of clock outputs, such as the melatonin rhythm, is associated with desynchronised expression of *Per1* and *Per2* genes in the dorsal and ventral SCN subsets. Unfortunately, no specific subpopulation of SCN cells could be attributed to either the onset or offset of the nocturnal melatonin peak, but the complete absence of melatonin release (in the RFs animals) correlated with a continuous expression of the *Per2* gene in the dorsal part of the SCN. In addition, the present results show that *Per2* expression in the SCN is sensitive to feeding conditions and that there is a close relation between the expression of the *Per2* and *VP* gene in the SCN.

Spatial and temporal distribution of Per1 and Per2 gene expression in the SCN

Under entrained conditions the acrophase of *Per1* and *Per2* gene expression occurs during the light period, and the expression of both genes is mainly restricted to the dorsomedial part of the SCN, as described previously also by others (Shigeyoshi *et al.*, 1997; Yan *et al.*, 1999; Reppert & Weaver, 2001; Dardente *et al.*, 2002). Also some minor expression of the *Per* genes was found in the ventrolateral part of the SCN during the first half of the light period, as recently described for the protein expression of *Per2* (Beaule *et al.*, 2003). In *ad libitum* conditions, the 8-h advance of the L/D-cycle affected the expression pattern of both *Per1* and *Per2* genes in the SCN in a similar way. Indeed, the ventrolateral expression of both *Per* genes becomes more apparent and is concentrated in the new lighting period. The ventral expression of *Per1* was already observed at CTs10, while its dorsal expression was mainly restricted to CTs22. Similarly, expression of the *Per2* gene in the ventral SCN peaked at CTs10, while the dorsal expression was strongest at CTs22, CTs2 and CTs6, i.e. the ventrolateral part of the SCN seems to shift faster towards the new time-point of the acrophase than the dorsomedial part of the SCN (Figs.1 and 2). This phenomenon, as also observed by Nagano *et al.* (2003) for *Per1* gene expression, is probably a consequence of light exposure in the preceding days at the time of the original dark period. Since light-induced *Per1* mRNA in the rat SCN is spatially limited to the ventrolateral part of the SCN (Shigeyoshi *et al.*, 1997; Yan *et al.*, 1999; Miyake *et al.*, 2000), and since vasoactive-intestinal-polypeptide and gastrin-releasing peptide, both specifically expressed in the ventrolateral part of the SCN, play a role in mediating photic entrainable signals to the dorsomedial part of the SCN (Watanabe *et al.*, 2000; Aida *et al.*, 2002; Dardente *et al.*, 2002), we can imagine that the light-induced activation of the ventrolateral SCN will cause the dorsolateral SCN to phase-shift. This phenomenon can explain the delay of phasing of the two cell subsets of the SCN after an abrupt phase shift. In agreement with previous studies (Reddy *et al.*, 2002; Nagano *et al.*, 2003), the results of the present study also show that 5 days were not enough for a complete

resynchronisation of the SCN cells to an 8-h phase-advance of the L/D-cycle. However, although the daily rhythm of *Per* gene expression did not completely shift within 5 days, the quantification study did reveal the presence of a new peak of expression for both *Per* genes. Interestingly, due to the strong expression in the ventrolateral SCN, total SCN expression of both *Per* genes did exhibit a significant peak 8-h apart from the pre-shift peak times. Strikingly, the phase-shifting of *Per* genes expression observed in the present study seems to have occurred according to a delay process since it is more similar to the pattern of *Per1* gene expression observed by Nagano *et al.* (2003) after a 10-h phase-delay of the L/D cycle than the one observed after a 6-h phase-advance of the L/D cycle. Illnerova *et al.* (1987) have shown that after an 8-h phase-shift of the L/D cycle, the activity rhythm of the main enzyme responsible for melatonin synthesis, arylalkylamine-*N*-acetyltransferase, adjusts within 5 cycles to the advance shift but within only one cycle to the delay shift. The fact that in the present study no clear rhythm of melatonin can be measured after 5 cycles confirmed therefore that we actually applied a phase advance and not a phase delay. In order to realise the advance of the L/D cycle, Nagano *et al.* (2003) shortened the dark period, whereas in the present study we decided to shorten the light period in order to follow our previous experimental protocol (Kalsbeek *et al.*, 2000a). In addition, in order to prevent any influence of light on *Per* genes expression we additionally put the rats in constant darkness on D5. Taken together these results suggest that although both methods (shortening of the night period or shortening of the day period) lead to an 8-h advance of the L/D-cycle, the subsequent SCN mechanisms involved may differ considerably.

In animals habituated to a time-restricted feeding regime, the 8-h advance induced an even more pronounced disruption of *Per* gene expression. Ventral expression of *Per1* and *Per2* genes could be seen at CTs6 and CTs10 and at CTs2, CTs6 and CTs10, respectively, while for both genes expression in the dorsal SCN was seen throughout the L/D cycle. Besides, the quantification study clearly exposed the differential effect of the RF regimen on *Per1 versus Per2* expression in the SCN. Indeed, except for the CTs10 time point, the effects of prior AL and RF feeding after an 8-h phase-shift were almost identical for the *Per1* gene expression in the SCN, but clearly different for *Per2* gene expression. In AL fed animals *Per2* expression showed a clear daily rhythm, whereas in the RF animals such a daily rhythm was completely absent. In fact, the differential effect of restricted feeding conditions on *Per2* expression already became apparent before the 8-h phase-advance. Animals adapted to a restricted feeding protocol for 3 weeks showed a clear advance, close to significance, of the *Per2* gene expression peak as compared to AL fed animals, whereas no significant differences could be detected for *Per1* gene expression (Fig.5A and 5B). Therefore, habituation to the restricted feeding regime may induce a delay in the resynchronisation of the SCN

to the new L/D cycle by the changes it induces in the rhythm of *Per2* gene expression. Indeed, *Per2* gene expression would first have to recover from the phase-advance induced by the restricted feeding, before being able to adapt to the new L/D cycle. *Per1* gene expression, not affected by the feeding regime, may therefore shift faster.

Clock outputs

The spatial and temporal expression of *Per* genes was clearly affected by the 8-h advance phase-shift in *ad libitum* and in restricted feeding conditions. However, we were unable to correlate the presence of a specific subpopulation of SCN neurons with the onset and/or offset of the melatonin peak after such a shift. Also, within one experimental group, no clear difference could be noticed in the distribution pattern of *Per* genes expression, despite clear differences in their plasma melatonin concentration. Nevertheless, two observations might give more information on the neuroanatomical substrate responsible for the control of the melatonin rhythm. First, during the nocturnal period before the shift, when melatonin synthesis is high, *Per* genes expression could be seen in a few neurons located in the central part of the SCN (Fig.1). While investigating the spatial and temporal regulation of the mitogen-activated protein kinase (MAPK) phosphorylation in the mouse SCN, Nakaya *et al.* (2003) also noticed the presence of a subpopulation of neurons in the central part of the SCN that especially became active during the night. Although the present results do not allow any definitive conclusions yet, we propose that the neurons showing *Per* gene expression and MAPK phosphorylation in the central part of the SCN during the night are responsible for the glutamatergic SCN output to PVN-pineal gland axis, that is necessary to stimulate the nocturnal synthesis and release of melatonin. The second observation concerns the *Per* gene expression in the dorsal SCN, which is high during the light period (when melatonin synthesis is low) and absent during the dark period (when melatonin synthesis is high). After the 8-h phase-advance of the L/D cycle, melatonin is low in all the animals of the RF group. This around-the-clock inhibition of melatonin release coincides with a clear and continuous expression of both *Per* genes in the dorsal SCN. It is tempting to speculate that the absence of "activity" in the dorsal part of the SCN is a prerequisite for the synthesis and release of melatonin. During day-time, both *Per1* and *Per2* genes are mainly expressed in VP-containing cells of the dorsomedial part of the SCN (Hamada *et al.*, 2001; Dardente *et al.*, 2002; Yan & Okamura, 2002). In addition, there is a close match between the daily rhythm of *Per* gene expression and the release of VP from SCN terminals (Kalsbeek *et al.*, 1995). Moreover, part of the VP-containing SCN neurons also produces GABA (Buijs *et al.*, 1995), which is used by the SCN to inhibit daytime melatonin release (Kalsbeek *et al.*, 2000b). Therefore, it is likely that the continuous "activity" of neurons in the dorsal part of the SCN prevents the synthesis and release of melatonin as a

consequence of the continuous inhibition of pre-autonomic PVN neurons by GABA release from the SCN terminals.

Otherwise, the present study revealed a close and specific relation between *Per2* gene expression and VP mRNA content in the SCN. Although also present in *ad libitum* conditions, this correlation became especially apparent during the RF conditions and after the 8-h phase advance. In the pre-shift *ad libitum* conditions, VP gene expression within the SCN showed a clear daily rhythm with a peak of expression during the second part of the daytime period (ZT10) as reported many times before (Burbach *et al.*, 1988; Carter & Murphy, 1992; Cagampang *et al.*, 1994). Five days after the 8-h phase advance, peaks of VP, as well as *Per1* and *Per2* expression were found during the "new" light period in the ALs animals. However, in the RFs animals only *Per1*, but not VP or *Per2*, showed a peak during the "new" light period. Moreover, also in the RF animals before the shift a close correlation was found between the expression of *Per2* and VP. Like *Per2* gene expression, but unlike *Per1*, VP mRNA content in the SCN was strongly increased just after the feeding period (ZT6) and significantly decreased 4-h later, indicating a 4-h phase-advance of the expression of both genes (Fig. 5B & 5C). In fact, the phase-advance of VP mRNA expression in the present study fits nicely with the earlier arrest of SCN-VP release as observed in our previous microdialysis study using the same RF feeding paradigm (Kalsbeek *et al.*, 1998). Interestingly, the present results show that a 2-h restricted feeding regime affects the SCN activity by acting specifically on *Per2* and VP gene expression. Therefore, although the SCN is not supposed to be necessary for the anticipatory changes in behaviour and physiology accompanying the restricted feeding regimen (Inouye, 1982; Damiola *et al.*, 2000; Hara *et al.*, 2001; Wakamatsu *et al.*, 2001), it seems likely that feeding behaviour does feed back to the SCN. In the present study the food restriction caused an arrest of the body weight increase, in contrast to the study of Wakamatsu *et al.* (2001), but they allowed a 4-h feeding period. Apparently our animals are more food deprived, and therefore should be compared with rats under slight caloric restricted feeding in which SCN activity is also affected (Challet *et al.*, 2003). On the other hand, the present results are in agreement with other earlier studies that also provided indications for a feedback action of feeding behaviour on SCN activity (Inouye, 1982; Murakami & Takahashi, 1983; Kalsbeek *et al.*, 2000a). Moreover, Miki *et al.* (2003), recently demonstrated, using parenteral nutrition in rats, that feeding is able to affect *Per2* gene expression in the liver as well as in the SCN. At present it is not clear how the feedback to the SCN is effectuated. Recent results, however, indicate that this feedback might be mediated by other (cortical) brain areas that do show a phase-shift as a consequence of the RF (Wakamatsu *et al.*, 2001; Dudley *et al.*, 2003; Vansteensel *et al.*, 2003). Regarding the present results, showing a close relation between VP and *Per2* genes expression, it is likely that the dorsal part of the SCN is the most affected by feeding.

In conclusion, the present study provides indications that the dorsal and central subdivision of the SCN contain the neurons responsible for controlling the offset and onset of melatonin release, respectively. In addition, next to the close correlation between the expression of the *Per2* and VP gene, the present study also revealed a clear feedback action of feeding activity on the activity of SCN neurons. This feedback action of feeding activity nicely compares with the recently shown feedback action of sleep on SCN activity (Deboer *et al.*, 2003). Moreover the present results show that this feedback may target only specific parts of the molecular machinery of the circadian clock, i.e. VP and *Per2*, but not *Per1*.

Acknowledgements

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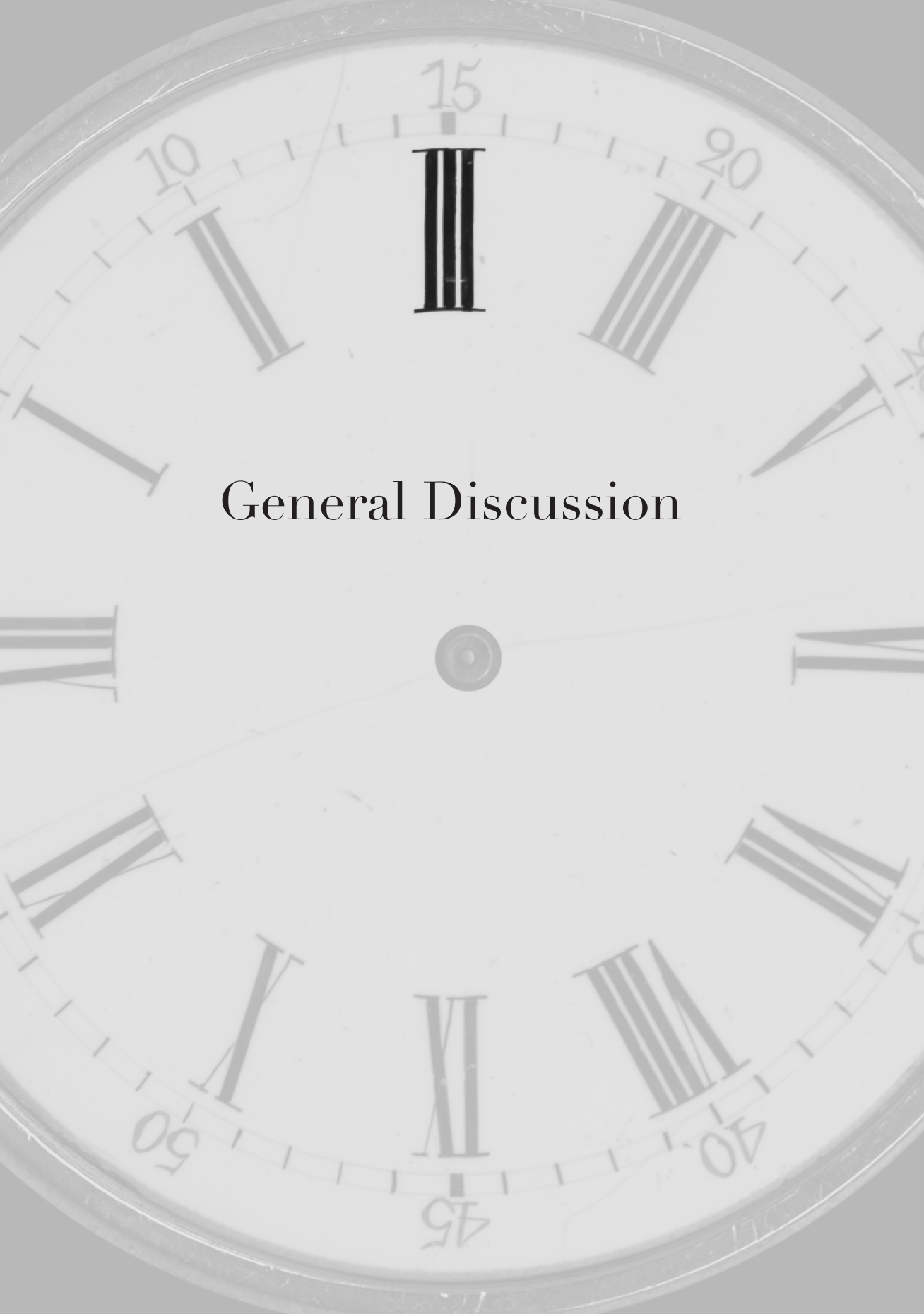

General Discussion

CHAPTER 6

General Discussion

Until a few years ago the biological clock was considered as a rather homogeneous structure sending one uniform time message to the rest of the brain. Yet, the results presented in this thesis, together with other recent publications, clearly reveal the necessity of a fundamental revision of this concept in order to make further progress in the field. Our study on the biological clock mechanisms that control pineal melatonin release uncovered several new aspects of the biological clock functioning.

First, the results presented in Chapters 2 & 3 provide clear evidence for the functionality of nocturnal SCN activity. The nocturnal electrical firing of SCN neurons, although less prominent as compared to the daytime activity of SCN neurons, and therefore probably often neglected, was proven to be a condition *sine qua non* for the nocturnal rise of pineal melatonin release. In addition, the results of these 2 chapters strongly suggest that without input from the biological clock the baseline activity of the pre-autonomic neurons controlling melatonin synthesis is rather low and that the SCN provides a continuous stimulation of the pre-autonomic neurons that are at the origin of the sympathetic activity. As will be discussed, this finding may have major implications for our view on how the biological clock via the hypothalamus affects the balance of life.

In Chapters 4 & 5 the control of the daily melatonin rhythm is further analysed. In Chapter 4 we show that further refinement of the GABA/glutamate hypothesis is necessary in order to explain the early morning decrease of melatonin. In Chapter 5 we revealed different subpopulations of SCN neurons that may be dedicated to the control of the daily melatonin rhythm. In addition, the results of this chapter suggest that expression of the *Per2* gene in the SCN might be more closely linked to SCN output (i.e. behaviour and VP release) than that of the *Per1* gene.

A. CIRCADIAN CONTROL OF MELATONIN SYNTHESIS

Combination of inhibitory and stimulatory clock outputs

As already presented in the Introduction, the biological clock is a time-dealer. In order to orchestrate diurnal rhythmicity in behavioural and endocrine functions, the biological clock is using diverse means to distribute the message of time. Initial electrophysiological and metabolic studies revealed the circadian rhythmicity of SCN activity peaking during daytime (see *Intermezzo*). In view of the fact that melatonin is a hormone with an extremely robust and stable circadian rhythm in a variety of species, it was quite logical to suppose a main inhibitory control of the melatonin rhythm by the SCN. However, low levels of pineal activity observed earlier in SCN-lesion animals (Klein & Moore, 1979) seemed to be in clear contradiction with such a pronounced inhibitory role. Nevertheless, it was not until 1996 that Moore (Moore, 1996) noticed the apparent contradiction between the nocturnal silence of SCN neurons and the stimulation of melatonin synthesis. Consequently, we had to check our hypothesis that the biological clock could control the melatonin rhythm by imposing a strong but "simple" daytime inhibition of pineal activity, as proposed previously (Kalsbeek *et al.*, 2000c). However, the results obtained in the first study (Chapter 2) already revealed that the melatonin rhythm is controlled in a more complex manner. Indeed, when one compares the effect of lesioning either the SCN, the PVN or the SCG on melatonin synthesis, it is clear that the SCN has an active role in both inhibition and stimulation of melatonin synthesis, whereas the SCG and the PVN are just simple relay stations of SCN outputs to the pineal gland. In view of these results, it became essential for us to elucidate this issue and to check *in vivo* whether the low SCN neuronal activity during the night was actually sufficient to sustain such a stimulatory action on melatonin synthesis. The results obtained (Chapter 3) clearly showed that, indeed, nocturnal neuronal activity of the SCN has important physiological implications. SCN nocturnal neuronal activity proved to be necessary to stimulate melatonin synthesis and to inhibit corticosterone at the same time. At the same time this study was the first to show an actual function for the nocturnal activity of the SCN. Therefore, contrary to the expectations at the start of this thesis, the SCN also uses multiple outputs for the control of melatonin synthesis, as it was shown previously for the control of corticosterone by the SCN (Kalsbeek & Buijs, 1996).

While the SCN derived factor involved in the daytime inhibition of melatonin synthesis had been shown previously to be GABA (Kalsbeek *et al.*, 1999; Kalsbeek *et al.*, 2000c), the nature of its stimulatory factor still needed to be determined. Considering that both GABA and glutamate may be used, respectively, as inhibitory and stimulatory SCN inputs to regions of the preoptic area involved in the control of sleep/wake

* ***Intermezzo***

Until recently the SCN peak of electrical activity has been reported exclusively during daytime. Nevertheless, the absence of evidence for nocturnal SCN neuronal activity might only be due to technical limitation. In fact, the initial in vivo *electrophysiological study that showed the circadian rhythmicity of neuronal activity in the SCN with a peak during day-time has been realised using the multiple-unit recording technique, a collective measure of the electrical discharge rate of up to 1000 neurons (Inouye & Kawamura, 1979). Later on, several* in vitro *studies, realised on hypothalamic slices or on cultured SCN, using single-unit recordings (Green & Gillette, 1982; Groos & Hendriks, 1982; Shibata* et al.*, 1982; Bos & Mirmiran, 1990), confirmed the existence of circadian rhythms of electrical activity, peaking during the light period in single SCN neurons. However, other* in vitro *electrophysiological studies also indicate that only 50-75% of SCN neurons show a circadian rhythm in their firing pattern (Bos & Mirmiran, 1990; Welsh* et al.*, 1995; Herzog* et al.*, 1997; Honma* et al.*, 1998; Liu & Reppert, 2000). In addition, a recent study has confirmed that* in vivo *about 2% of SCN cells show regular firing activity (Saeb-Parsy & Dyball, 2003a). Therefore, not all SCN cells do express an endogenously build circadian message. Besides, Welsh* et al. *(1995) revealed that, in cultures of dispersed rat SCN cells, SCN neurons express independently phased circadian firing rhythms even if connected together. Moreover, using prolonged single-unit recordings from mouse SCN explants cultured on a multi-electrode plate, Herzog* et al. *(1997) also found daily rhythms in firing rate that were clearly out of phase with each other. This observation has been confirmed in a slice culture of neonatal rat SCN (Nakamura* et al.*, 2001). Only a few studies have so far used single-unit recordings* in vivo. *For instance, light responsiveness of the SCN along the L/D cycle has been studied in anaesthetised (Meijer* et al.*, 1986) or freely-moving rats (Meijer* et al.*, 1996; Meijer* et al.*, 1998). In one of these studies, Meijer* et al. *(1998) noticed that, for all the 7 SCN neurons they recorded on a long term for at least 48h, baseline discharge patterns of single units showed increased activity during (subjective) day and decreased activity during (subjective) night. However, a recent* in vivo *study using single-unit recordings in anaesthetised rats showed a specific daily variation of the neuronal discharge of single SCN cells depending on their projection targets (Saeb-Parsy & Dyball, 2003b). For instance, cells that project to the arcuate nucleus or the supraoptic nucleus show two peaks of activity, one near the light/dark and the other near the dark-light transitions. In this* in vivo *study, although the overall mean of neuronal firing was still peaking during the light period, several cells showed a high firing rate (around 10 Hz) during the dark period. Moreover, during their long-term* in vivo *recordings Saeb-Parsy and Dyball (2003b) noticed that 2 out of their 30 "silent" cells finally increased their firing rate, one for a period of ~ 3h and one for a period of 8h. In addition, Schaap* et al. *(2003) have shown recently by extracting single unit activity from multiunit recordings on rat SCN slices, the strong heterogeneity of neuronal activity of individual SCN neurons. They actually reported peak times of neuronal activity during both subjective day and night. So far, the presence of a specific subpopulation of SCN neurons active during the dark period (i.e. out of phase with the majority of SCN neurons) still needs to be demonstrated* in vivo. *However, the existence of subpopulations of SCN cells that maintain different phase relationships becomes more and more evident.* ☽

rhythm (Sun *et al.*, 2000; Sun *et al.*, 2001) and that more and more evidence was accumulating for a specific glutamate release from SCN terminals onto (preautonomic) PVN neurons (Hermes *et al.*, 1996; Csaki *et al.*, 2000; Cui *et al.*, 2001), in the second part of Chapter 3, we investigated a possible contribution of glutamate release within the PVN to the nocturnal release of melatonin. The results of this experiment showed that indeed glutamatergic transmission within the PVN, and most likely derived from SCN projections, is a key player in the stimulation of melatonin release at night.

Tonic stimulatory output ?

Interestingly, unlike the blockade of GABA-ergic transmission within the PVN (Kalsbeek *et al.*, 2000c), silencing the inhibitory output of the SCN by intra-nuclear infusions of TTX during the light period did not induce any increase of melatonin levels. Apparently, during the light period, SCN control towards the melatonin rhythm is not only inhibitory, but also stimulatory. Consequently, we proposed that, next to its nocturnal stimulatory output, the SCN also sustains a stimulatory output to the PVN during the light period, the final effect of which, in normal conditions, is overwhelmed by the simultaneous activity of the inhibitory GABA-ergic output to the PVN. During our initial studies we supposed that the stimulatory input towards the melatonin-rhythm-generating-system could be derived from the intrinsic activity of pre-autonomic PVN neurons. Results of SCN-lesions and TTX infusions (within the SCN) clearly showed that this intrinsic or spontaneous activity of pre-autonomic PVN neurons is minimal, and that SCN neuronal activity is also required for a daytime increase of melatonin synthesis.

Initially the possibility for the existence of a nocturnal stimulatory output of the SCN seemed very unlikely, due to the well-know daytime activity of SCN neurons. The *Intermezzo*, however, illustrates that more and more evidence is accumulating for nocturnal activity of SCN neurons. The evidence for non-rhythmic cells within the SCN, nicely fits with our hypothesis of a tonic SCN stimulatory output originating from the same population of cells throughout the 24-h period. On the other hand, these recent electrophysiological data also support the possibility of nighttime and daytime stimulatory outputs originating from two distinct subpopulations of SCN neurons with an opposite phase-relation. Adoption of this second option would imply a trough of stimulatory SCN output during the L/D and the D/L transition. The depth of this trough (i.e. the amount of stimulatory SCN output remaining) will depend on the overlap in the activity periods of these two subpopulations of SCN neurons. We tried to address this issue in Chapter 4 by investigating the SCN control of the melatonin offset (D/L transition). Knowing that the blockade of GABA-ergic transmission within the PVN increased melatonin synthesis in the middle of the day, we were actu-

ally curious about its effect on melatonin offset, occurring just before the beginning of the light period in rat. Several mechanisms (including intrapineal ones) can be imagined to explain the arrest of noradrenalin stimulation through the sympathetic fibres that is responsible for the offset of melatonin synthesis. First, considering the hypothesis of a tonic stimulatory SCN output overwhelmed by a circadian GABA-ergic inhibitory signal during the light period in the PVN, the offset of noradrenalin release in the early morning would be a consequence of the increasing strength of the GABA-ergic signal, and the blocking of GABA-ergic transmission during this period of the light/dark-cycle would consequently prevent this offset. Indeed, the results obtained in Chapter 4 confirm that GABA-ergic signalling within the PVN inhibits melatonin release at the end of the dark period. But, these results also clearly show that, just before the transition from dark to light, the decline of melatonin can not be completely prevented by the administration of BIC. Therefore, next to an increased GABA-ergic inhibition these results also indicate a discontinuation of the stimulatory input at that time. In our view the arrest of the stimulatory input can be accomplished in two ways (Fig. 1). First, it could fit with the idea of two populations of stimulatory SCN neurons with opposite phasing and a trough of stimulation during the transition from one population to the other. Secondly, it could mean that during a short period (i.e. the dark/light transition) the stimulatory SCN neurons are inhibited, probably by another subpopulation of SCN neurons. However, both mechanisms indicate that at least 3 populations of SCN neurons are involved in the control of the melatonin rhythm.

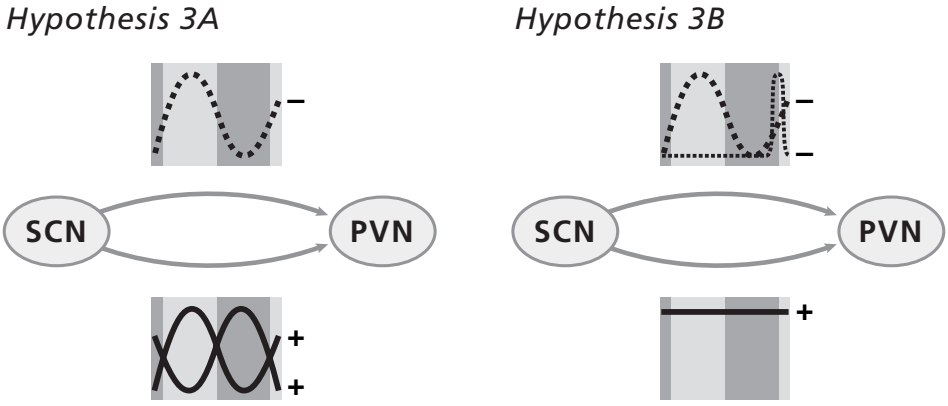

Figure 1 Schematic illustration of the two versions of the "final" hypothesis explaining the clock-controlled synthesis of melatonin. Version A illustrates the idea of two populations of stimulatory SCN neurons with opposite phasing, and version B illustrates the idea of a subpopulation of SCN neurons continuously firing throughout the 24-h of the L/D cycle but silenced by a non-GABA-ergic subpopulation of SCN neurons at the transition from dark to light.

In Chapter 5, we tried to identify the subpopulation(s) of SCN cells that could be responsible for the stimulatory action of the SCN on melatonin synthesis. This anatomical study did reveal the necessity for the absence of *Per* gene expression within the dorsal SCN to enable the expression of the melatonin rhythm, probably indicating the location of the SCN neurons that are responsible for the inhibition of melatonin during the (subjective) light period. Mitogen-activated protein kinase activity has been shown to display a circadian rhythm in the SCN (Obrietan *et al.*, 1998). Interestingly, a small subpopulation of these MAPK cells located in the central middle SCN showed a specific activity during the nocturnal period, for at least 8-h (Nakaya *et al.*, 2003). This central subpopulation of neurons could be responsible for the stimulation of melatonin synthesis at night. However, the peculiar recent finding that this rhythm seems to depend on an intact retinal input (Lee *et al.*, 2003) makes it an unlikely candidate for stimulating nocturnal melatonin release. Still, we propose a central position for the central region of the SCN, containing for instance distinct peptidergic cell groups such as the SS-positive neurons (Card *et al.*, 1988; Tanaka *et al.*, 1996), with regard to the localization of the stimulatory neurons. Further anatomical experiments will be necessary to localise specifically and definitely each subpopulation of the SCN responsible for the inhibition and the stimulation of melatonin synthesis. Once glutamatergic neurons will be tracable, by labelling of vesicular glutamatergic transporters for instance (Ziegler *et al.*, 2002), we can imagine to localise at least the glutamatergic neurons of the SCN that project to the PVN-pineal pathway with combined retrovirus tracing technique from the pineal gland.

General scheme

The present results demonstrate that the SCN is a heterogeneous structure, not only structurally but also not functionally. Taking these observations together we propose that the dorsal subdivision of the SCN contains neurons responsible for controlling the offset of melatonin release (most likely of GABA-ergic nature), while a central subdivision (most likely glutamatergic) controls the nocturnal stimulation of melatonin synthesis. In addition, we propose a third subpopulation of neurons. Either one that exerts a stimulation of pre-autonomic PVN neurons specifically during the (subjective) light period, or one that inhibits a population of continuously active stimulatory neurons during the dark/light transition. Moreover, we propose that light has an additional, direct (i.e. circumventing the central oscillator) inhibitory influence on melatonin release via activation of GABA neurons located in the ventral part of the SCN. This general view on the different subpopulations of SCN neurons involved in the control of the daily rhythm of melatonin synthesis is presented in figure 2.

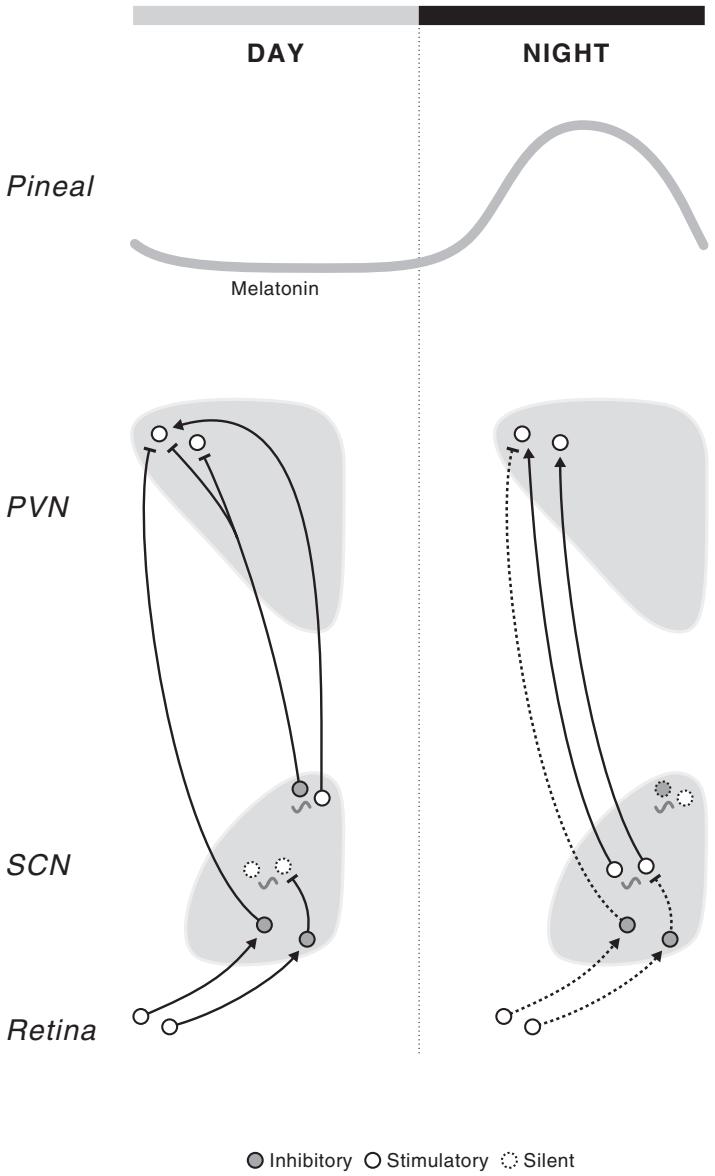

Figure 2 Schematic presentation of the different subpopulations of SCN neurons implicated in the circadian control of melatonin synthesis. Filled circles indicate inhibitory and open circles stimulatory neurons, respectively. Dashed circles indicate silent neurons and dashed lines indicate the network that can be activated by light during the night period. For simplicity reasons, I chose to show in the present illustration only the population of SCN stimluatory neurons in opposite phasing.

B. BIOLOGICAL CLOCK: FUNCTIONALLY SUBDIVIDED

Spatio-temporal compartmentalisation of the SCN

These last years more and more evidence has been provided for a functional compartmentalization of the SCN. It was first suggested that in hamsters a certain subpopulation of SCN neurons, containing a specific calcium calbinding protein, could control the locomotor activity rhythmicity (LeSauter & Silver, 1999). The same year, Yan *et al.* (1999) revealed the circadian profile and the compartment-specific response to light of the clock genes *Per1* and *Per2* in rats. The concept of different functions for different subdivisions of the SCN (i.e. a ventral subdivision used to synchronise the dorsal subdivision to the light environment) has been further confirmed later on by others. Indeed, Miyake *et al.* (2000) suggested that the transient induction of *Per1*, specifically, in ventrolateral SCN neurons was critical for resetting the biological clock to a new environmental L/D cycle. This idea has been confirmed by Hamada *et al.* (2001) who revealed the compartmentalisation of the SCN with one set of neurons showing endogenous rhythmicity in *Per1* and *Per2* mRNA expression, and another set showing no rhythmicity in mRNA expression but gating the light-induced *Per1* and *Per2* gene expression. Some studies have extended these findings by proposing gastrin-releasing peptide, localised in ventral SCN neurons, as the neurotransmitter responsible for the synchronisation of dorsal SCN neurons by the ventral subdivision (Aida *et al.*, 2002; Dardente *et al.*, 2002). In addition, a functional compartmentalisation of the SCN has also been revealed by the recent experiments of Buijs *et al.* (2003a), showing the existence of different subsets of SCN neurons dedicated to either the parasympathetic or sympathetic branch of the autonomic nervous system.

In addition to the spatial compartmentalisation of the SCN in *Per* gene expression, Yan & Okamura (2002) have revealed the existence of a temporal compartmentalisation of the SCN with different subgroups of SCN neurons expressing the *Per1* and *Per2* genes at different moments of the L/D cycle. More recently, Yamagushi *et al.* (2003) have confirmed such a spatiotemporal compartmentalisation in a real-time analysis of *Per1* gene expression in organotypic slice cultures of SCN transgenic mice carrying *mPer1*-promotor-driven luciferase reporter gene. In addition, the authors elegantly demonstrated, by culturing independently the dorsal and the ventral parts of the SCN, that although the dorsal part of the SCN is the first to express *Per1* along the light/dark cycle, it is not responsible for the synchronisation of *Per1* gene expression of the ventral part. The spatio-temporal compartmentalisation of *Per1* and *Per2* genes was also confirmed by the experiments presented in Chapter 5 of the present thesis.

The spatiotemporal organisation has been examined further following phase-shifts of the light-dark cycle (Yan & Silver, 2002; Nagano *et al.*, 2003; Yan *et al.*, 2003).

The phase-shift experiments revealed a differential effect of delaying and advancing phase-shifts on *Per* gene expression. Moreover, our results with animals on restricted feeding (Chapter 5), showing a different effect of the 8-h phase advance on the expression of the different *Per* genes, suggest an even more complex regulation mechanism. The results of the present thesis, showing the involvement of different SCN signals at different moments of the L/D cycle in the control of melatonin synthesis, reveal that SCN spatio-temporal compartmentalisation is also crucial even if only *one specific (hormonal) rhythm* is concerned.

Molecular compartmentalisation of the clock

In addition to the spatiotemporal organisation of the SCN, more and more evidence is provided for differentiated molecular clockwork functioning in SCN cells according to their position within the nucleus. Indeed, synchronizing factors (photic or non-photic) have different effects on the diverse components of the molecular clockwork (i.e. *Per1* and *Per2* genes) depending on their location within the SCN. For instance, delaying phase pulses (applied early at night) increase the expression of both *Per1* and *Per2* genes in the ventral part of the SCN, but in the dorsal part of the SCN only the expression of *Per2* is increased. On the other hand, an advancing light pulse (applied at late night) increases *Per1* gene expression in both subdivisions of the SCN, but has no effect on *Per2* gene expression (Yan & Silver, 2002).

Besides, although products of both *Per1* and *Per2* genes are active components of the molecular clockwork, recent studies strongly suggest a differentiation of function for these two genes. For instance, *mPer1* mutant mice show an inability to phase-advance, while *mPer2* mutant mice show an inability to phase-delay, indicating different function of these genes in the synchronisation to new environment cycle (Albrecht *et al.*, 2001). In addition, null mutant mice for the *Per1* gene still show circadian locomotor activity in constant darkness, whereas after a few days *Per2* gene null mutant mice become arrhythmic (Steinlechner *et al.*, 2002). Consequently, Yan *et al.* (2003) recently suggested that, in mice, the *Per2* gene might have a more important role in behavioral phase delays than *Per1*. Moreover, non-photic inputs, which probably also involve internal feedback signals to the clock, also seem to affect the two *Per* genes differently. Indeed, in Chapter 5, we have shown that an aberrant feeding rhythm more specifically affects the expression of the *Per2* gene. In view of the fact that light pulses have a specific effect on *Per1* expression only at late night, we can even imagine that the specific effect of feeding on *Per2*, as shown in Chapter 5, might depend on the time of feeding of the animals. In our experimental conditions, animals were fed from ZT4 to ZT6, a time-period that corresponds to the maximal and widest expression of *Per1* but not to the one of *Per2*. In addition, the results of Chapter 5 showed that especially

expression of the *Per2* gene is closely correlated with VP gene expression. Considering these two observations, and since VPergic SCN output is crucial in the control of the daily rhythm of corticosterone release for instance, we suggest that *Per2* gene is not only linked in a closer way than *Per1* gene is to behavioural output rhythms, but also to hormonal output rhythms. In order to further understand the respective link of *Per1* and *Per2* genes and endocrine clock outputs, it could be of great interest to measure the circadian expression of endocrine functions of *Per1 versus Per2* mutant mice. One could expect, for instance, a more disturbed corticosterone rhythm in *Per2* mutant mice than in *Per1* mutant mice.

Different timing of SCN outputs

The results of the present thesis clearly illustrate that the SCN uses multiple outputs to control the circadian rhythm of melatonin. The functional subdivision of the biological clock is also nicely illustrated by examining the timing pattern of the different SCN outputs we know to be involved in the control of both melatonin and corticosterone release (Fig. 3). Previous studies have shown that the daytime inhibitory influence of the SCN on corticosterone release is transmitted by a VP containing projection to the DMH (Kalsbeek *et al.*, 1996b; Kalsbeek *et al.*, 1996c), VP being released during the first half of the day (Kalsbeek *et al.*, 1995), while, at the end of the light period, a corticosterone-stimulating agent (CSA) needs to be released from SCN terminals to stimulate corticosterone release (Kalsbeek *et al.*, 1996c). Besides, as shown by the results of the present thesis and according to version B of the "final hypothesis", the daily rhythm of melatonin release is induced by a daytime GABA release and a continuous release of glutamate from SCN terminals, to, respectively, inhibit and stimulate melatonin synthesis. Moreover, we propose that, in the early morning, the early decline of melatonin can be explained by an additional SCN inhibitory transmitter, or melatonin-inhibiting factor (MIF). In addition, the results of Chapter 3 revealed that the SCN also actively contributes to the offset of the circadian corticosterone peak. Since, administration during the dark period of neither the VP- nor the glutamate-antagonist affected corticosterone release (Kalsbeek *et al.*, 1996c; and Chapter 3), these results indicate the existence of an additional corticosterone-inhibiting factor (CIF), during the nocturnal period. Clearly, despite the low nocturnal electrical activity of SCN neurons, next to glutamate also other SCN transmitters might be released during the dark period in order to stimulate or inhibit other circadian controlled functions.

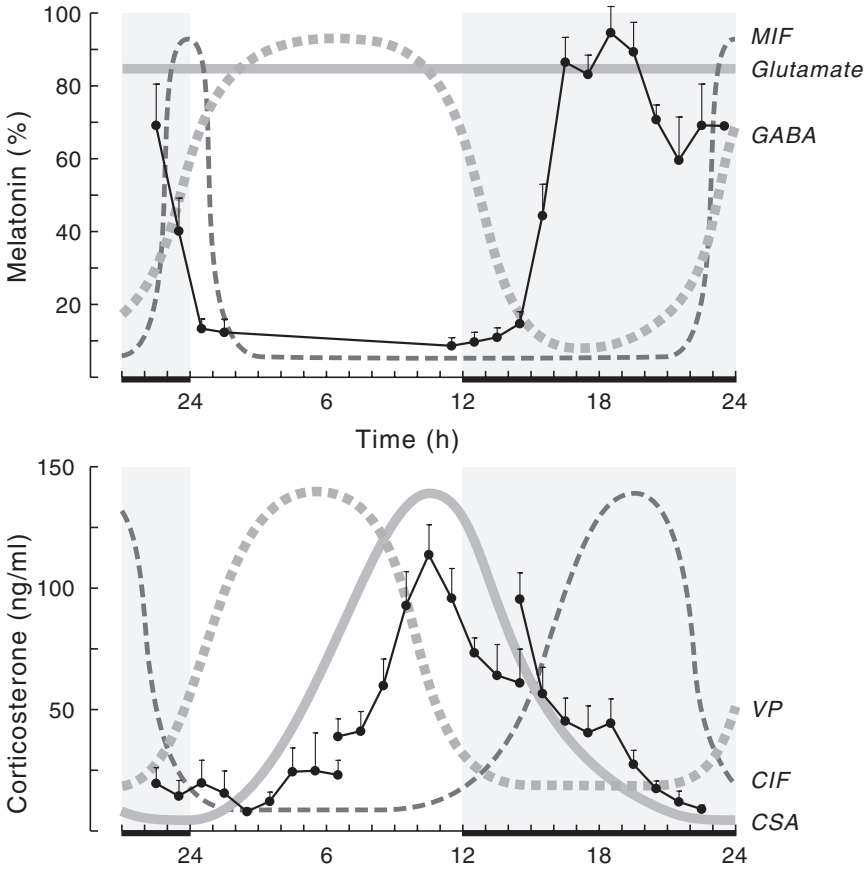

Figure 3 Multiplicity of SCN outputs with respect to the control of the melatonin and corticosterone rhythms, and their different circadian pattern of activity. Dashed and straight gray lines indicate the release pattern of inhibitory (GABA, MIF, VP, CIF) and stimulatory (glutamate, CSA) SCN outputs, respectively. For simplicity reasons, only the hypothesis of one continuously firing population of SCN neurons, in the control of melatonin synthesis, is indicated in the present illustration. Adapted from Buijs *et al.* (2003b).

C. MELATONIN: A MODEL FOR CLOCK CONTROLLED AUTONOMIC OUTPUTS

In the general Introduction we proposed that the SCN controls peripheral rhythms via a dual mechanism consisting of SCN connections with both endocrine and pre-autonomic neurons in the hypothalamus. In the SCN a circadian expression of clock gene mRNA and clock proteins is essential for the organization of the daily changes in SCN physiology. *Clock genes* have also been described in many other structures in the brain and in the periphery (Hastings & Maywood, 2000; Abe *et al.*, 2002; Balsalobre, 2002). Interestingly, rhythmicity of *clock gene* expression has also been shown in the pineal gland, but with a different relationship to each other than in the SCN (Namihira *et al.*, 1999; Fukuhara *et al.*, 2000; Takekida *et al.*, 2000). However, to our knowledge, so far no clear evidence has been presented for a role of clock genes in the circadian synthesis of melatonin. Although the *Per1* gene shows a circadian pattern of expression similar to that of *Aa-nat,* and noradrenergic stimulation induces pineal *Per1* as well as *Aa-nat* expression, *Per2* gene expression shows a completely different circadian pattern and is not induced by such noradrenergic stimulation (Fukuhara *et al.*, 2000; 2002). Therefore, if the master clock entrains the circadian expression of both *Per* genes in the pineal, it seems to use different pathways to do so. This idea was confirmed recently by the results of an extensive study on clock genes expression in the rat pineal gland that revealed differential daily rhythms and regulation of *Per1*, *Per3*, *Cry1* and *Cry2* genes (Simonneaux *et al.*, 2004). Besides, no clear link has been demonstrated so far for a possible implication of either clock gene in the chain of melatonin synthesis.

Apart from the sympathetic innervation, a parasympathetic innervation of the pineal gland has also been demonstrated (Moller *et al.*, 1999; Moller & Baeres, 2002). Although the effects of parasympathetic agents described so far were mainly inhibitory on melatonin synthesis (Romijn, 1976; Drijfhout *et al.*, 1996a), not much is known about the functionality of this parasympathetic innervation. Since our previous transneuronal tracing study revealing the SCN-pineal pathway did not show any labelling in the parasympathetic motornuclei in the brainstem (Teclemariam-Mesbah *et al.*, 1999), it seems that the parasympathetic innervation of the pineal gland has more of a modulator role in the synthesis of melatonin.

The present thesis revealed that melatonin synthesis is under complete and tight control of the biological clock, using signals of different nature for its inhibition and its stimulation. Although this clock control is certainly not ubiquitous for all physiological functions, we propose that the biological clock uses its multiple outputs to the autonomic nervous system to control the circadian rhythmicity of other functions,

such as corticosterone release (Kalsbeek *et al.*, 1996b,c), body temperature and the cardiovascular system (Scheer *et al.*, 2004a; Scheer *et al.*, 2003), as well. Despite the evidence for multiple autonomic connections with other glands and organs provided by several neuro-anatomical tracing studies, the functionality of these numerous outputs, however, still needs to be further investigated. So far, the best example is the circadian control of the corticosterone rhythm, for which the sympathetic innervation of the adrenal gland seems to be of major importance (Jasper & Engeland, 1994; Buijs *et al.*, 1999). In addition, it is likely that the autonomic nervous system is also involved in the circadian control of plasma glucose and plasma leptin concentrations (La Fleur *et al.*, 2000; Kalsbeek *et al.*, 2001; Kreier *et al.*, 2002). From the present studies, it is likely that the SCN will use GABA and glutamate to control the autonomic (or at least the sympathetic) outflow to other organs in order to control glucose homeostasis and leptin release. In addition, recently it has been shown that the blockade of endogenous GABA release in the PVN increases sympathetic activation and can cause heart failure in the rat (Zhang *et al.*, 2002). In our view, it is likely that endogenous GABA release from SCN terminals is involved in the inhibition of the sympathetic input to the heart as well (Scheer *et al.*, 2001; Scheer *et al.*, 2003).

There is, however, a non-negligible difference between the regulation of melatonin (and leptin) and most of the other functions controlled by the endogenous clock via the autonomic output. Sympathetic output to the pineal gland is, indeed, always stimulated during the night in both diurnal and nocturnal animals, whereas the timing of the stimulatory input of the autonomic nervous system to other physiological functions, is often linked to the activity state of the animal. Consequently, the "night physiology" clearly depends on the environmental niche of an animal, i.e. whether it is nocturnal or diurnal. This means that in nocturnal species, such as the rat, GABA-ergic projections from the SCN inhibit, during the light period, "pineal" and "cardiovascular" PVN neurons simultaneously. However, in diurnal species only the "pineal" PVN neurons need to be inhibited during the daytime, but the inhibition of the "cardiovascular" PVN neurons has to be shifted to the dark period. The comparison of diurnal and nocturnal species therefore illustrates two important characteristics of the control of the autonomic nervous system by the SCN. First, although the clock can use the same type of output signal, it is clear that the SCN has to target different sets of pre-autonomic neurons in the PVN. This idea is supported by the recent identification of two functionally different groups of spinal cord-projecting PVN neurons with sympathetic related activity (Chen & Toney, 2003). Secondly, the comparison of diurnal and nocturnal animals forces us to think about how the SCN can change the nature of the "cardiovascular" PVN neurons from diurnal to nocturnal. For instance, does this mean that the acrophase in activity of the GABA-ergic SCN neurons that innervate the "cardiovascular" PVN neurons, shifts from the light period to the dark

period, or does it indicate a shift from a GABA-ergic to a glutamatergic nature of the SCN neurons that innervate the "cardiovascular" PVN neurons?

Another specificity of the daily rhythm in melatonin synthesis resides in the fact that the SCN exerts total autonomic control over the melatonin synthesis, i.e. at present no other factors are known that can affect the melatonin rhythm in any substantial way (except for light, of course, but light also acts through the SCN). Such a total control by the clock explains the stability of the melatonin rhythm throughout different species and makes it very suitable to serve as a major hand of the clock, i.e. an endocrine signal of time (Pévet, 2000; Korf *et al.*, 2003). Although the SCN also uses the autonomic nervous system in the control of the daily rhythmicity of other endocrine functions, as presented in the Introduction, at present this property of a total control seems to be specific solely for melatonin. The differential effects of SCN lesions on melatonin, corticosterone and leptin levels nicely reveal the relative importance of autonomic innervation in the SCN control of these rhythms. Indeed, as illustrated in Figure 4, ablation of the SCN will cause low, intermediate and high levels of melatonin, corticosterone and leptin, respectively. The SCN stimulation of melatonin release is completely dependent on the sympathetic innervation of the pineal, whereas, with regard to the release of corticosterone, the sympathetic innervation seems to have only a modulatory effect, by changing the sensitivity of the adrenal gland for ACTH. Moreover, the input of the biological clock to the autonomic nervous system can have completely opposite effects. For instance, whereas the sympathetic input to the pineal gland stimulates melatonin synthesis, the sympathetic input to fat tissue inhibits the release of leptin (Sivitz *et al.*, 1999; Kalsbeek *et al.*, 2001). However, both hormones show a secretion peak during the night, which suggests that, during the day, the SCN both inhibits the sympathetic input to the pineal gland and stimulates the sympathetic input towards the fat tissue.

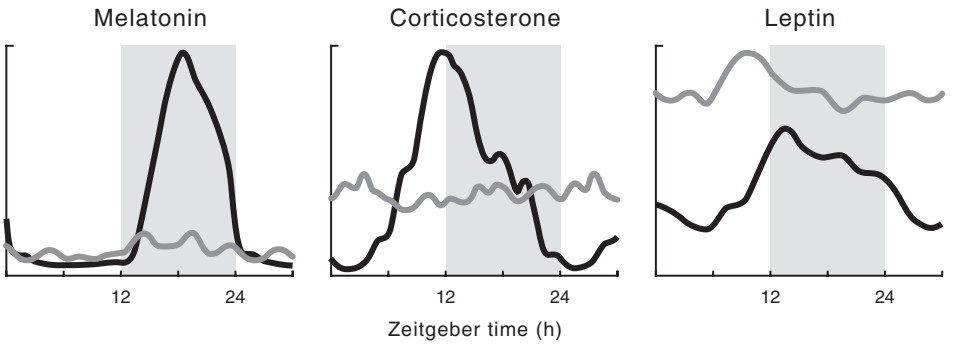

Figure 4 Schematic representation of dialysate levels of melatonin (A) and corticosterone (B), as well as plasmatic leptin (C) levels, in intact (black lines) *versus* SCN-lesioned (gray lines) rats. Graphics are based upon results of Chapter 2 and results by Kalsbeek *et al.* (2001).

Another notable difference between melatonin and other hormones is the strong feedback action of melatonin on the SCN, which has thus far not been described for other hormones. Despite the fact that hormones such as corticosterone and leptin show a pronounced circadian rhythm too, their (feedback) actions on the SCN still need to be demonstrated *in vivo*. When applied at the transition from dark to light, exogenous melatonin can entrain circadian rhythms such as other non-photic stimuli (Redman *et al.*, 1983; Cassone *et al.*, 1986; Pitrosky *et al.*, 1999), which strongly suggests that endogenous melatonin feeds back to the SCN to help its entrainment. As reported so far, the main effect of melatonin is to inhibit the electrical activity of the SCN neurons (Shibata *et al.*, 1989; Stehle *et al.*, 1989; Liu *et al.*, 1997; McArthur *et al.*, 1997; Van den Top *et al.*, 2001). Therefore, the most straightforward explanation for its entraining effect is that it enhances the "normal" nocturnal silence of the SCN. However, in both humans (Zaidan *et al.*, 1994) and rats (Bothorel *et al.*, 2002) it has been shown that exogenous melatonin stimulates the endogenous secretion of melatonin. Moreover, Bothorel *et al.* (2002) were able to show that, in rats, exogenous melatonin specifically acts on the SCN to increase the amplitude of endogenous melatonin release. The stimulatory effect of exogenous melatonin on endogenous melatonin release seems in contradiction with its inhibitory effect on the electrical activity of SCN neurons. Therefore, we hypothesise that melatonin specifically acts on the GABA-ergic SCN neurons. In addition, we think that silencing the GABA-ergic neurons increases the excitability of glutamatergic neurons involved in the stimulation of melatonin synthesis, i.e. an inhibition of GABA-ergic neurons might enable more glutamatergic SCN neurons to become active. This would explain the strong entraining capacity of melatonin on its own synthesis. Recently, Scheer *et al.* (2004b) have demonstrated that a longterm treatment of hypertensive patients with exogenous melatonin just before bedtime enhances or re-instates the nocturnal dip in the daily blood pressure rhythm. Keeping in mind our hypothesis, the entrainment effectiveness of melatonin, at least in humans, seems to be due not only to its strengthening of the daily rhythmicity but also to the effect it has on the "cardiovascular" PVN neurons that are responsible for the nocturnal decrease in blood pressure. It is to expect that in nocturnal rodents, such melatonin treatment would increase blood pressure.

The results as presented in Chapters 2 & 3 clearly showed that the intrinsic or spontaneous activity of sympathetic pre-autonomic PVN neurons is minimal. Although, the melatonin rhythm seems to be solely controlled via the sympathetic innervation of the pineal gland, we can imagine that the parasympathetic division of the autonomic nervous system is also dependent on a stimulatory input from the SCN. Indeed, viral tracing studies have previously shown that the SCN is connected to both branches of the autonomic nervous system. Moreover, recently we found that separate SCN neurons may be dedicated to either the sympathetic of parasympathetic branch

of the autonomic nervous system (Buijs *et al.*, 2003). Therefore, the "simple" finding of a stimulatory SCN input to pre-autonomic neurons might have major implications for the control of the autonomic nervous system in general, and for the SCN control of the balance of life in particular. It may be, therefore, of great interest to identify the specific subpopulation of SCN cells that send the stimulatory input to the pineal gland. Although the glutamatergic SCN output to pre-autonomic neurons in the PVN has been already described previously (Cui *et al.*, 2001), the results of Chapter 3 are the first to demonstrate the functional importance of the glutamatergic SCN output. In order to further localise the specific SCN cells responsible for the stimulation of melatonin at night, we could imagine a tracing study from the pineal gland combined with a specific glutamatergic marker (i.e. vesicular glutamate transporter mRNA in the hypothalamus (Ziegler *et al.*, 2002)).

In conclusion, the different results presented in this thesis further confirmed the complexity of one of the most interesting body mechanisms, i.e. the internal time keeping. Furthermore, new mechanisms were brought to light that will force us to think about the biological clock in a more heterogeneous way. In order to progress further in the understanding of the functioning of the circadian clock, it will be necessary to identify the different subgroups of SCN cells in relation to their target and functionality, and to establish the specific rhythmicity of their activity. However, the final and full identification of these outputs can be completed only when technical progress will allow us to measure the transmitter release of an identified neuron at the level of one specific target neuron. Therefore, although the present thesis has provided more insight in the circadian control of melatonin release, it has also uncovered a number of yet unsolved issues, such as the nature and relevance of the melatonin-stimulating factor released during daytime, or the location and the functional specificity of the SCN neurons active at night.

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Abbreviations

AA-NAT	arylalkylamine *N*-acetyltransferase
ACTH	adrenocorticotropin hormone
AVP	arginine vasopressin
BIC	bicuculline
CalB	calbindin D_{28K}
CRH	corticotropin-releasing hormone
CIF	corticosterone-inhibiting factor
CSA	corticosterone-stimulating agent
DMH	dorsomedial nucleus of the hypothalamus
GABA	γ-aminobutyric acid
GHT	geniculo-hypothalamic tract
GnRH	gonadotropin-releasing hormone
GRP	gastrin releasing peptide
HPA-axis	hypothalamo-pituitary-adrenal axis
HPG-axis	hypothalamo-pituitary-gonadal axis
HPT-axis	hypothalamo-pituitary-thyroid axis
ir	immuno-reactive
IGL	intergeniculate leaflet
ISO	isoproterenol
LH	luteinizing hormone
L/D	light/dark cycle
MIF	melatonin-inhibiting factor
MPOA	medial preoptic area
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NT	neurotensin
OT	oxytocin

Phe	phenylephrine
PHI	peptide histidine isoleucine
PVN	paraventricular nucleus of the hypothalamus
PRL	prolactin
RGCs	retinal ganglion cells
RHT	retino-hypothalamic tract
SCG	superior cervical ganglion
SCN	suprachiasmatic nucleus of the hypothalamus
SON	supraoptic nucleus of the hypothalamus
SP	substance P
sPVN	subparaventricular nucleus of the hypothalamus
SS	somatostatine
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
TTX	tetrodotoxin
VIP	vasoactive intestinal polypeptide
VLPO	ventrolateral preotic area
VMPO	ventromedial preoptic area
VP	vasopressin

Summary

In mammals, numerous physiological, behavioural and endocrine functions show an endogenous rhythmicity close to 24-h, but precisely synchronised at 24-h by the light/dark cycle of the environment. These so-called circadian (*circa* = about and *dies* = day) rhythms are under the control of a master biological clock, located in the suprachiasmatic nucleus of the hypothalamus (SCN). The SCN (1) generates and sustains an endogenous rhythm of neuronal activity of approximately 24-h, (2) perceives and integrates photic and non-photic information for an optimal synchronisation of this endogenous rhythm with the environment, and (3) distributes this integrated temporal message to the rest of the brain and the organism.

By coordinating the activity of such a vast amount of physiological functions, the SCN is a real bodyguard of temporal homeostasis within the body. However, the fine details of the distribution mechanisms that the SCN is using for this purpose are still to be defined. Indeed, the different physiological functions show a multitude of timings for their maximal activity, whereas the main activity of the SCN neurons has so far been reported to be restricted to the light period.

In order to further understand the mechanisms of time distribution by the SCN, in the present thesis the circadian control of the extremely stable daily rhythm of melatonin release was studied as a model of clock functioning. We aimed to determine the exact effect of the SCN on melatonin synthesis, as well as the nature of the transmitters involved in this control.

Our starting hypothesis stated that "The SCN controls the daily rhythm of melatonin synthesis by imposing an inhibitory signal, of GABA-ergic nature, onto the PVN→pineal pathway during (subjective) daytime", assuming that (without inhibition from the SCN) the PVN would provide a constant stimulatory input to the sympathetic system.

In the first study, presented in **Chapter 2**, we therefore compared the respective effects of lesioning or removing the SCN, the PVN or the SCG on *Aa-NAT* gene expression, which reflects melatonin synthesis the best. Interestingly, in contrast with our "inhibition" hypothesis, the results obtained revealed that both acrophase and nadir of the melatonin rhythm are driven by the SCN, and that PVN and SCG play a simple role of relay-station to the pineal gland. Consequently, we updated our hypothesis and proposed that: "The SCN uses a combination of both inhibitory (during daytime) and stimulatory signals towards the PVN→pineal pathway to control the daily rhythm of melatonin synthesis", suggesting, for the first time, a functional role for the nocturnal activity of the SCN. Since its main activity period (metabolic and electric) is situated in the (subjective) light period, the SCN had until then been commonly considered a

daytime functioning structure. Hence, it appeared necessary to check the concept of a nocturnal stimulatory SCN output in an acute and *in vivo* situation. In addition, it was of great interest to identify the (chemical) nature of this stimulatory signal coming from the SCN. These two points were successively addressed in the second study, presented in **Chapter 3**, using the multiple microdialysis technique. At first, we tested the effect of an acute and temporary shutdown of either PVN or SCN electrical activity on melatonin release. Thereby, we confirmed that the nocturnal electrical firing of SCN neurons was a *sine qua non* condition for the nocturnal rise of pineal melatonin release. In addition, we showed an important role of glutamatergic signalling from the SCN to the PVN-pineal pathway on the melatonin rhythm generating system.

Together, the results of **Chapters 2 & 3** strongly suggest that without input from the biological clock the baseline activity of the pre-autonomic neurons controlling melatonin synthesis is rather low, and that the SCN provides a continuous stimulation of the pre-autonomic neurons which are at the origin of the sympathetic activity. According to that idea, the morning decline of melatonin would be due to an increasing release of GABA onto the pre-autonomic PVN neurons. In **Chapter 4**, we showed that a further refinement of the GABA/glutamate hypothesis is necessary in order to explain the early morning decrease of melatonin. As it turned out, a blockade of the GABA-ergic signalling within the PVN during the dark/light transition was unable to prevent the early morning decline of melatonin completely, suggesting either a withdrawal of the glutamatergic stimulatory input at that time or the existence of a second inhibitory SCN signal involved in the control of the daily melatonin rhythm and specifically released at the dark/light transition.

During the course of the above experiments some evidence appeared in the literature for multiple clock outputs as well as for a clear compartmentalisation of the SCN. Therefore, it became of great interest to couple the activation of specific subsets of SCN cells with the onset/offset of melatonin synthesis. This aim was pursued in **Chapter 5**. In this chapter, we investigated whether we could correlate *Per1* and *Per2* gene expression in SCN neurons with the reappearance of either the onset or offset of the melatonin peak after an 8h advance of the light/dark cycle. In this chapter, we revealed different subpopulations of SCN neurons that may be dedicated to the control of the daily melatonin rhythm. In addition, the results of this chapter suggest that expression of the *Per2* gene in the SCN might be more closely linked to SCN output (i.e. locomotor behaviour and VP release) than that of the *Per1* gene.

Altogether, the studies presented in this thesis reveal an updated concept of the control of the daily melatonin rhythm by the biological clock. More importantly, the new concept for the SCN control of the melatonin rhythm may help to understand how the SCN controls the autonomic nervous system. This is all the more interesting since recent tracing studies provided evidence for neuronal connections between

the SCN and various peripheral organs (i.e. liver, pancreas, thyroid, spleen, and fat tissue), suggesting that the autonomic nervous system may be an important system for the SCN to distribute its time-of-day message to many other physiological and hormonal rhythms.

Résumé

Chez les Mammifères, de nombreuses fonctions physiologiques, comportementales et endocrines, présentent une rythmicité endogène proche de 24-h, synchronisée précisément à 24-h par le cycle lumière/obscurité. Ces rythmes biologiques, dits circadiens, sont sous le contrôle d'une horloge interne située dans les Noyaux Suprachiasmatiques de l'hypothalamus (SCN). Les SCN (1) génèrent et soutiennent un rythme circadien d'activité, (2) reçoivent et intègrent les informations photiques ou non-photiques synchronisant ce rythme à l'environnement, et enfin (3) distribuent ce message temporel intégré au reste de l'organisme. En coordonnant l'activité des diverses fonctions physiologiques, les SCN font office de garde du corps de l'homéostasie temporelle interne. Si de nombreux progrès ont été réalisés au cours de ces 10 dernières années concernant le fonctionnement moléculaire de l'horloge interne, les mécanismes de distribution du message temps restent cependant à définir. Toutes les fonctions physiologiques rythmiques présentent des rythmes d'activité variés tout au long du cycle jour/nuit, alors que les SCN ont une activité globale maximale pendant le jour.

Le but de cette thèse a donc été d'accroître nos connaissances dans les mécanismes de distribution du message temporel circadien par les SCN. Pour se faire, nous avons étudié le contrôle par l'horloge du rythme journalier de sécrétion de mélatonine, dans le but de déterminer l'effet exact des SCN sur la synthèse de mélatonine, ainsi que les différents neurotransmetteurs impliqués. Selon notre hypothèse de départ, "les SCN contrôleraient le rythme journalier de mélatonine en utilisant un signal inhibiteur, de nature GABA-ergique, pour inhiber la voie polysynaptique reliant les noyaux paraventriculaires de l'hypoyhalamus (PVN) à la glande pinéale (siège principal de la synthèse de mélatonine) pendant la journée", impliquant une action simulatrice constante des PVN sur le système nerveux sympathique en l'absence de cette inhibition par l'horloge. Cette thèse a donc été initiée afin de tester cette hypothèse « d'horloge inhibitrice ».

Dans un premier temps, nous avons comparé l'activité jour/nuit de la glande pinéale après lésions spécifiques de chacune des structures impliquées dans la voie SCN-pinéale (SCN, PVN ou ganglions cervicaux supérieurs (SCG)). Étonnamment, les résultats de cette première étude, présentée en **Chapitre 2**, ont montré un rôle de simple relais de l'information pour les structures PVN et SCG. Par ailleurs, ces résultats ont révélé que si les SCN avaient une action inhibitrice sur la synthèse de mélatonine de jour, ils exerçaient également un rôle stimulateur prépondérant pendant la période nocturne. Étant donnée la faible activité neuronale nocturne des SCN, il est apparu nécessaire de confirmer ce rôle stimulateur nocturne *in vivo*, et de déterminer sa nature. C'est dans ce but que nous avons réalisé la deuxième étude,

présentée en **Chapitre 3**. Dans cette étude, la sécrétion de mélatonine a été suivie par microdialyse transpinéale, pendant, durant et après le blocage temporaire de l'activité neuronale des PVN ou des SCN (infusion de Tétrodotoxine par dialyse inverseé). Nous avons ainsi pu montrer que l'activité neuronale nocturne des SCN était cruciale pour la stimulation nocturne de la synthèse de mélatonine par la glande pinéale. Par ailleurs, nous avons également révélé que le glutamate jouait un rôle stimulateur très important pour la synthèse nocturne de mélatonine.

Ensemble, les résultats des **Chapitres 2 & 3** suggèrent qu'en absence de signal de l'horloge l'activité basale des neurones pré-autonomes contrôlant la synthèse de mélatonine soit assez faible, et que les SCN stimuleraient ces neurones de façon continue. Selon cette hypothèse, la chute matinale de mélatonine serait induite par une augmentation de sécrétion GABA-ergique par les SCN sur les neurones pré-autonomes des PVN. Les résultats du **Chapitre 4** ont cependant montré que cette hypothèse devrait être reconsidérée. En effet, à la transition nuit/jour, le blocage au sein des PVN de la signalisation GABA-ergique n'a pu prévenir totalement la chute de mélatonine. Ceci suggère la disparition du signal stimulateur à ce moment précis, ou bien l'existence d'un second signal inhibiteur issu des SCN spécifiquement libéré à la transition nuit/jour.

Au cours de cette thèse, plusieurs autres études sont apparues dans la littérature attestant de la multiplicité des sorties de l'horloge ainsi que de la compartimentalisation des SCN. Il nous est donc apparu d'un grand intérêt de pouvoir associer l'activation spécifique de sous-groupes de cellules des SCN au début et à la fin de la synthèse de mélatonine. Pour cela, nous avons étudié l'éventuelle corrélation entre l'expression neuronale des gènes *Per1* et *Per2* au sein des SCN et l'apparition du pic de mélatonine suite à une avance de 8-h du cycle jour/nuit (**Chapitre 5**). Dans cette étude, nous avons révélé différentes sous-populations de neurones des SCN qui pourraient être dévouées au contrôle du rythme journalier de mélatonine. De plus, les résultats de ce chapitre semblent confirmer l'idée que l'expression du gène *Per2* est en plus étroite relation avec les sorties de l'horloge (comme le comportement locomoteur et la sécrétion de VP) que ne l'est le gène *Per1*.

Ensemble, les différentes études réalisées au cours de cette thèse ont permis de remettre à jour le concept du contrôle du rythme journalier de mélatonine par l'horloge biologique en particulier. Mais de façon plus générale, ce nouveau concept permettra sûrement d'aider à comprendre le contrôle du système nerveux autonome par l'horloge. Au vu des récents travaux de traçage viraux ayant mis en évidence l'interconnexion entre les SCN et divers organes périphériques (comme le foie, le pancréas, la thyroïde, la rate ou le tissus adipeux) *via* le système nerveux autonome, l'horloge biologique semble, en effet, utiliser le système nerveux autonome comme moyen de distribution du message temps au reste de l'organisme.

Samenvatting

Niet alleen vele fysiologische, maar ook gedragsmatige en endocriene functies in zoogdieren vertonen een endogeen ritme van ongeveer 24 uur, maar zijn desondanks nauwkeurig gesynchroniseerd aan het 24-uurs ritme van de omgeving. Deze zogenaamde circadiane (*circa* = ongeveer en *dies* = dag in het Latijn) ritmen staan onder controle van de (hoofd) biologische klok, gelegen in de suprachiasmatische nucleus (SCN) in de hypothalamus. De SCN (1) genereert en onderhoudt een endogeen ritme van ongeveer 24 uur in neuronale activiteit, (2) ontvangt en integreert fotische en non-fotische informatie voor een optimale synchronisatie van dit circadiane ritme met de omgeving, en (3) distribueert dit geïntegreerde tijdssignaal naar de rest van het brein en het organisme. Door het coördineren van zo'n verscheidenheid aan fysiologische functies kan de SCN met recht een beschermheer van de (temporele) homeostase in het lichaam genoemd worden. De fijne details van het distributiemechanisme dat de SCN gebruikt voor dit doel zijn echter nog grotendeels onbekend. De verschillende fysiologische functies vertonen namelijk een grote verscheidenheid aan tijdstippen waarop ze maximaal actief zijn, ondanks het feit dat de SCN neuronen voornamelijk actief zijn tijdens de lichtperiode van de licht/donker-cyclus. Om het mechanisme van de SCN controle beter te begrijpen hebben we in dit proefschrift de circadiane regulatie van het uiterst stabiele dagelijkse ritme in melatonine afgifte door de pijnappelklier bestudeerd. Het doel was om aan te tonen hoe de SCN de afgifte van melatonine beïnvloedt en welke neurotransmitter(s) zij hiervoor gebruikt. Onze uitgangshypothese stelde: "De SCN reguleert het dagelijkse ritme in melatonine synthese via inhibitie (door de afgifte van GABA) van de PVN projectie naar de pijnappelklier tijdens de (subjectieve) lichtperiode". Hierbij veronderstelden wij dat er (zonder remming door de SCN) een continue stimulatie uitging van de PVN naar de sympatische innervatie van de pijnappelklier. Om deze hypothese te testen hebben we in de eerste experimenten, beschreven in **Hoofstuk 2**, de effecten vergeleken van een uitschakeling of verwijdering van respectievelijk de SCN, de PVN of de SCG, op de expressie van het Aa-NAT gen in de pijnappelklier. *Aa-NAT* is het snelheidsbepalende enzym in de synthese van melatonine. In tegenspraak met onze "inhibitie" hypothese vonden we echter dat zowel de piek als het dal van het melatonine ritme bepaald werden door de SCN, en dat de PVN en de SCG slechts dienden als een tussenstation voor de transmissie van SCN signalen naar de pijnappelklier. Bijgevolg moesten we onze hypothese bijstellen en luidde deze nu: "De SCN controleert de PVN projectie naar de pijnappelklier middels een combinatie van remmende (gedurende de lichtperiode) en stimulerende signalen". Deze hypothese kende ook voor het eerst een functionele betekenis toe aan de (beperkte) SCN activiteit tijdens de donkerperiode. Aangezien

de overgrote meerderheid van SCN neuronen alleen actief is gedurende de lichtperiode, werd de SCN beschouwd als voornamelijk actief (=functioneel) gedurende de lichtperiode. Dit maakte het noodzakelijk om het concept van een stimulerende activiteit van de SCN tijdens de donkerperiode zorgvuldig in een *in vivo* situatie te toetsen. Daarbij was het natuurlijk zeer interessant om ook de (chemische) identiteit van dit stimulerende SCN signaal te achterhalen. Beide vragen werden onderzocht in de 2de serie experimenten, die gebruik maakte van de meervoudige microdialyse techniek, en die is beschreven in **Hoofdstuk 3**. Eerst onderzochten we het effect van een tijdelijke stillegging van de neuronale activiteit in de SCN of de PVN op de afgifte van melatonine. Deze experimenten toonden aan dat de nachtelijke activiteit van SCN neuronen een conditie *sine qua non* is voor de afgifte van melatonine tijdens de donkerperiode. Tevens konden we een belangrijke rol aantonen voor de afgifte van glutamaat in de transmissie van het SCN-signaal naar de PVN en de generatie van het dagelijkse melatonine ritme. Gezamenlijk tonen de resultaten, zoals beschreven in de **Hoofdstukken 2 & 3**, duidelijk aan dat zonder input van de biologische klok de basale activiteit van de pre-autonome neuronen (in de PVN), die de synthese van melatonine reguleren, bijzonder laag is, en dat de SCN verantwoordelijk is voor een continue stimulatie van de pre-autonome neuronen die aan de basis staan van de sympathische activiteit. Volgens bovengenoemde hypotheses wordt het einde van de melatonine piek in de vroege morgen bewerkstelligd door een remming van de pre-autonome PVN neuronen middels een toegenomen afgifte van GABA. In **Hoofdstuk 4** wordt echter duidelijk dat een verdere precisering van de hypothese noodzakelijk is om het einde van de dagelijkse melatonine piek te kunnen begrijpen. Een blokkade van het GABA signaal in de vroege ochtend, middels toediening van een GABA-antagonist in de PVN, bleek namelijk niet afdoende om de daling van de melatonine piek te voorkomen. Dit resultaat suggereert dat: (1) de glutamaterge stimulatie van de pre-autonome PVN neuronen niet continu is maar even onderbroken wordt in de vroege ochtend, of (2) dat er nog een derde SCN signaal betrokken is bij de controle van het melatonine ritme, maar deze remmende (en niet GABAerge) neurotransmitter wordt dan alleen afgegeven in de vroege ochtend. Gedurende de jaren dat bovenbeschreven experimenten werden uitgevoerd werd er in de literatuur meer en meer bekend over meerdere klok signalen (i.t.t. één dag/nacht signaal) en een verdeling van de SCN in verschillende compartimenten. Het werd daarom interessant om te kijken of we de start en het einde van de melatonine piek konden koppelen aan de activiteit van een specifieke subset van SCN neuronen. In **Hoofstuk 5** hebben we onderzocht of de expressie van het *Per1* en *Per2* gen in SCN neuronen correleerde met het opnieuw verschijnen van de melatonine piek, nadat de dieren een 8-uur verschuiving van hun licht/donker-cyclus hadden ondergaan. De resultaten in dit hoofdstuk laten inderdaad verschillende subpopulaties van SCN neuronen zien die betrokken zouden

kunnen zijn bij de start danwel het einde van de dagelijkse melatonine piek. Tevens wordt in dit hoofdstuk duidelijk dat de expressie van het *Per2* gen in de SCN nauwer correleert met SCN output (zoals gedrag en vasopressine afgifte) dan de expressie van het *Per1* gen. Gezamenlijk hebben de verschillende experimenten zoals beschreven in dit proefschrift geleid tot een bijgestelde en veel nauwkeuriger hypothese voor de regulatie van het dagelijkse melatonine ritme door de biologische klok. Echter, nog belangrijker is het feit dat dit nieuwe concept voor de controle van het dagelijkse melatonine ritme ons ook meer inzicht geeft in hoe de biologische klok het autonome zenuwstelsel controleert. Dit is des te meer interessant daar recente tracing studies bewijs hebben geleverd voor een neuronale verbinding tussen de biologische klok en diverse perifere organen (zoals lever, pancreas, schildklier, milt en vetweefsel), hetgeen suggereert dat het autonome zenuwstelsel een belangrijke route is voor de biologische klok om vele andere fysiologische en endocriene ritmen te sturen.

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Curriculum Vitae

Stéphanie Perreau-Lenz was born in Dijon, France, on June 30, 1975. In 1993 she graduated from the *Lycée Louis Pasteur* of Strasbourg in France.

After a first year of medical school, she studied biology at the *University Louis Pasteur* of Strasbourg, specialised in Neurosciences and accomplished a Master's degree in Neurosciences in July 1999. During these studies, her first research training, on the juxtacellular neural labelling and electrophysiological recording technique, has been carried out at the laboratory *U-398 INSERM* in Strasbourg. Her second research training has been carried out at the Laboratoire de *Neurobiologie des Rythmes (UMR 7518 CNRS-ULP)* in Strasbourg. Under the supervision of Prof. Dr. Paul Pévet and Dr. Michel Saboureau she studied the effect of melatonin on its own secretion, in rats, using the microdialysis technique.

After her Master of Science, she took the opportunity to accomplish a co-supervised PhD between two world famous groups in the field of biological rhythms, the group of Prof. Dr. Paul Pévet in Strasbourg, and the group of Prof. Dr. Ruud Buijs in the *Institute for Brain Research* in Amsterdam (the Netherlands). During this PhD training period that started on September, 15th, 1999, she extended her expertise in biological clock functionning under the supervision of Dr. Andries Kalsbeek, Prof. Dr. Paul Pévet and Prof. Dr. Ruud Buijs. The results of this training period are presented in this thesis.

After her thesis promotion she will be appointed as a postdoctoral researcher in the group of Prof. Dr. Rainer Spanagel in the *Central Institute of Mental Health* in Mannheim (Germany), in order to study further the relationship between the biological rhythms and the addiction mechanisms.

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