CHAPTER 19

Rhythms of inhibitory and excitatory output from the circadian timing system as revealed by in vivo microdialysis

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Introduction

Now more than 20 years ago, guided by the efferent pathways of the retina, tracing experiments indicated the suprachiasmatic nuclei (SCN) in the anterior hypothalamus as a possible target for retinal information and thus a putative location for the circadian pacemaker (Hendrickson et al., 1972; Moore and Lenn, 1972). Subsequent studies using specific lesions (Moore and Eichler, 1972; Stephan and Zucker, 1972; Dunn et al., 1977; Ibuka et al., 1977; Stephan and Nunez, 1977; Abe et al., 1979) or, more recently, transplantation of embryonic SCN tissue (Drucker-Colin et al., 1984; Sawaki et al., 1984; Ralph et al., 1990) established the SCN as the seat of the mammalian biological clock. In the following years eight different afferent projections to the SCN have been described (see Moore, Ch. 8) characterized, among others, by glutamate (Castel et al., 1993; De Vries et al., 1993), NPY (Harrington et al., 1985; Ibata et al., 1988; Morin and Blanchard, 1995), serotonin (Ajika and Ochi, 1978; Azmitia and Segal, 1978; Van de Kar and Lorens, 1979) and acetylcholine (Van Der Zee et al., 1991; Bina et al., 1993). Important progress, especially by using in vitro slice preparations containing the SCN (Smith et al., 1992; chapter Gilette), has been made in gaining an understanding of how these different afferent inputs entrain the endogenous rhythm, generated by the circadian pacemaker, to the changing conditions of the outside world. Although tracing experiments have also disclosed the efferent connections of the SCN in great detail (Watts and Swanson, 1987; Watts et al., 1987; Buijs et al., 1993b; Kalsbeek et al., 1993b; Orpen and Steiner, 1994; Morin et al., 1994; Vrang et al., 1995b), we are still far from understanding how the pacemaker conveys its temporal information to the rest of the organism. A major obstacle is that the output of the clock can only be studied in vivo in the intact organism (for a possible exception see the Hermes chapter).

The first transmitter to be identified in SCN neurons was the nonapeptide vasopressin (VP; Swaab et al., 1975; Vandesande et al., 1975). Subsequent lesion experiments showed that the efferent projections of the SCN also contained VP (Hoorneman and Buijs, 1982; Kalsbeek et al., 1993b). The discovery of a diurnal rhythm of VP levels in the cerebrospinal fluid (CSF), as first described by Reppert et al. (1981a) in the cat, indicated that VP was probably released from these SCN terminals in a circadian fashion. This

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important finding was followed by reports on VP rhythms in the CSF of a number of other species, including monkey, rat, guinea pig, goat, sheep and rabbit (Günther et al., 1984; Reppert et al., 1987; Seckl and Lightman, 1987; Stark and Daniel, 1989; Forsling, 1993; Robinson and Coomes, 1993). In addition, rhythms have been described for (i) the VP peptide and mRNA content of SCN tissue (Inouye, Chapter), (ii) in vitro release of VP from extirpated SCN tissue (Earnest and Sladek, 1987; Gillette and Reppert, 1987; Murakami et al., 1991), and (iii) extracellular levels of VP within the SCN (Kalsbeek et al., 1995). In addition to VP, other transmitters have also been identified in efferent projections of the SCN, most importantly VIP (Card et al., 1981; Watts and Swanson, 1987; Kalsbeek et al., 1993b), GRP (Mikkelsen et al., 1991; Kalsbeek et al., 1993b) and GABA (Buijs et al., 1994). Like VP, these transmitters show rhythmic fluctuations in tissue content and in the amount of their mRNA (Inouye chapter).

Recently, a circadian release pattern has also been reported for VIP in vitro (Tominaa et al., 1994; Shinohara et al., 1995). So far, however, a clearly rhythmic in vivo secretion pattern has only been described for VP. In order to investigate the neural mechanisms responsible for the transmission of circadian rhythms from the SCN to the rest of the organism, we put forward the following hypothesis: 'the rhythmic release of SCN transmitters within its target areas is responsible for the expression of circadian rhythms in locomotor activity, body temperature, autonomic functions, and hormone secretion'.

Methodology

Strategy

To test the above hypothesis we concentrated on the SCN projection to the neuroendocrine centre of the hypothalamus, i.e. the paraventricular and dorsomedial nuclei of the hypothalamus (PVN/DMH), and the possible implication of this projection for the circadian control of hormonal rhythms. Our first experiments aimed at unravelling the enigma of the transfer of circadian information were guided by two observations: (1) the proximity of efferent SCN fibres and CRF-containing motoneurons for the release of corticosterone in the PVN/DMH area (Buijs et al., 1993b; Vrang et al., 1995b); and (2) the reverse patterns of circulating corticosteroid levels on the one hand and the secretory activity of the VP-containing SCN neurons on the other (Fig. 7). Initially, the specific effects of SCN transmitters on the release pattern of corticosterone were investigated by localized microinfusions of VP and VIP in the PVN/DMH area and the concomitant collection of blood samples. Since in the SCN-intact animal the release of endogenous SCN transmitters might interfere with the effect of the infused transmitter, SCN lesioned as well as SCN-intact animals were used (Kalsbeek and Buijs, 1992; Kalsbeek et al., 1992).

Problems related to intracerebral microinfusions

In order to study the relevance of the effects observed in our initial short-term infusion experiments (i.e. a VP-mediated inhibition of corticosterone release (Kalsbeek et al., 1992)) for the circadian regulation of hormonal release, it was necessary to prolong the infusion period. Increasing the intracerebral infusion period from 15 min to 5 h, however, revealed a major drawback of the microinfusion technique. Long-term infusions augmented the corticosterone stimulating effect of short-term infusions even further and caused a prolonged and sustained increase of circulating corticosterone levels, exceeding the normal basal release several times (Fig. 1). The major cause of the problems with the traditional intracerebral infusion technique is that the introduction of a certain volume of fluid into the brain is unavoidable, and that the mere movement or pressure of the volume of the infused substance may affect the activity of neurons and glia in the infused brain areas in several ways: (1) by mechanical stimulation causing deformation of the cell membrane (Charles et al., 1991); (2) by producing the release of local hormones from membrane phos-
pholipids, so-called autocoids, that stimulate the HPA-axis (Whitnall, 1993); or (3) by inducing the release of excitatory amino acids (Zivin and Choi, 1991). Thus, in the case of infusions in the PVN/DMH area, CRH-containing motoneurons will inevitably be activated, thereby causing increased plasma corticosterone levels (Kalsbeek et al., 1992; Rowe et al., 1995). Similar problems have earlier been encountered with infusions in the PVN and preoptic area of the hypothalamus. Saline infusions in the PVN have been reported to induce grooming behaviour (Van Erp et al., 1993) and control injections in the preoptic area almost invariably induced changes in body temperature (Raible and Knickerbocker, 1993; Sellami and De Beaurepaire, 1993). Therefore, we replaced the microinfusion technique by a more sophisticated and less stressful method for intracerebral administration of transmitters, i.e. microdialysis.

**Microdialysis**

Microdialysis was originally designed to allow recovery of endogenous chemical substances from brain tissue by the inward diffusion through a semi-permeable membrane (Benveniste and Hüttemeier, 1990; Westerink, 1995). However, the same technique can also be used to introduce substances into the brain. The advantage of this method as compared to the microinfusion techniques is that there is no significant net loss or gain of fluid into the neural tissue surrounding the probe tip, thus reducing the risk of neural damage. The large majority of microdialysis experiments are performed on the first 2 days after implantation of the dialysis membrane, due to a considerable decline in the recovery within the first 2 days after implantation (Landgraf et al., 1994; Westerink, 1995). Other studies, however, have shown that after invasive surgery (such as the intracerebral implantation of a dialysis probe) a recuperation time of at least 1 week is necessary to normalize circadian rhythms of body temperature, locomotor activity and feeding behavior again (Farr et al., 1988; Drijfhout et al., 1995). This also holds for the plasma corticosterone rhythm which was shown to regain its normal circadian pattern only 1 week after implantation of an intracerebral dialysis probe (Kalsbeek et al., 1996). However, a post-operative recuperation time of about 1 week, after implantation of the microdialysis probe, could diminish the recovery of the transmitters of interest probably even further. A series of pilot experiments was therefore, started to test the applicability of the microdialysis technique for our studies.

Using noradrenaline-induced feeding as a behavioral test we could show that the microdialysis probes were functional for at least 2 weeks after implantation (Fig. 2). Angiotensin-induced drinking behaviour showed that (small) peptides could also be delivered effectively into the brain for at least 10 days after implantation (Fig. 2), allowing sufficient time for post-operative recovery. These pilot experiments thus indicated that the rapid decline in recovery can be prevented by postponing the first perfusion of the probe (Fig. 2), at least as far as the outward transport of transmitters is concerned. This finding is in line with other studies showing that tissue reactions to the mere

![Figure 1](image-url)  
**Fig. 1.** Effect of either short (15 min; *n* = 13) or long (5 h; *n* = 7) microinfusions of Ringer in the PVN/DMH area of rats on plasma corticosterone levels. Data from the 15-min infusion and control data (○--○; *n* = 10) were re-drawn from Kalsbeek et al. (1992) and (1996b), respectively.
presence of the probe, i.e. without perfusion, were minimal (Woodroffe et al., 1991; Landgraf et al., 1994; Osborne, 1995; De Lange et al., 1995). Subsequent microdialysis experiments in animals provided with both a dialysis probe and a jugular vein catheter, revealed that in these animals

![Graph](image)

Fig. 2. Effect of post-operative recovery time on the efficiency of U-shaped microdialysis probes for the administration of noradrenaline (NA) and angiotensin (ANG) to the paraventricular nucleus of the hypothalamus (PVN). Efficiency was determined by measuring food or water intake in response to a fixed dosage of NA (6.4 mg/ml) and ANG (50 ng/ml), respectively. NA was applied for the first (●) or second (○) time with one or more non-infusion days in between.

![Graph](image)

Fig. 3. Effect of microdialysis mediated Ringer administration into the PVN/DMH area of rats at two different times of the L/D-cycle (i.e. CT6.5–CT7.5 (N = 6) and CT8–CT12 (N = 8)) on plasma corticosterone levels. Control data (○—○) are from animals with only a jugular vein catheter and no intracerebral equipment. Data redrawn from Kalsbeek et al. (1996a).

![Graph](image)

Fig. 4. Effect of repeated perfusions of the microdialysis probe on the efficacy of drug delivery to the brain. The stimulating effect of a hypothalamic administration of VP-antagonist on corticosterone release was significantly diminished during a second perfusion of the microdialysis probe. Plasma corticosterone levels during repeated Ringer perfusions of the microdialysis probe, however, were not different from those during non-perfusion sessions (●). Open symbols — first perfusion; closed symbols — second perfusion.

Ringer administration to the PVN/DMH area had no effect whatsoever on circulating corticosterone levels (Figs. 3 and 4). However, hypothalamic administration of VP-antagonist to stimulate the release of corticosterone showed that the efficacy of drug delivery decreases with subsequent perfusions of the probe (Fig. 4).

The present chapter describes a number of experiments designed to study how circadian information influences corticosterone or melatonin release by using microdialysis mediated delivery of SCN transmitters or their (ant)agonists into SCN target areas. In addition, in vivo microdialysis techniques were used to monitor extracellular levels of VP within the SCN, pineal release of melatonin, and free corticosterone levels in dialysates from brain tissue.

The circadian rhythm in corticosterone levels

**Inhibitory effect of vasopressin**

Circadian fluctuations in circulating glucocorticoid levels have been reported for many species.
In nocturnal animals such as the laboratory rat, plasma glucocorticoid levels are high at the onset of darkness and then decline, reaching a nadir in the morning. Our initial experiments using microinfusions in the PVN/DMH area showed a strong inhibitory effect of exogenous VP, but not VIP, on the release of corticosterone (Kalsbeek et al., 1992). The inhibitory effect of VP was only evident in SCN-lesioned animals, but not in intact animals. These results suggested an inhibitory effect of VP released by SCN terminals in the PVN/DMH area on the activity of the hypothalamo-pituitary-adrenal (HPA)-axis. The inhibitory effect of endogenously released VP on HPA-axis activity was confirmed by infusion of the VP V1-antagonist d-(CH₂)Tyr(Me)-AVP (i.e. Manning compound; Manning et al., 1993) in the PVN/DMH area of intact animals. Infusions were performed during the middle of the light period, i.e. when the secretory activity of VP-containing SCN neurons is maximal and corticosterone levels are low. Infusion of the VP-antagonist, but not the Ringer vehicle, caused an immediate and pronounced increase of circulating corticosterone levels (Kalsbeek et al., 1992). In SCN-lesioned animals there was also an infusion induced increase of corticosterone secretion, but here Ringer and the V1-antagonist were equally effective.

The above results indicated an inhibitory effect on the HPA-axis of endogenous VP, released from SCN terminals at the level of the PVN/DMH-area, during the middle of the light period. These initial data were further substantiated by microdialysis mediated administration of VP and its V1-antagonist using different dosages. Stress-free infusion of the VP V1-antagonist in the dorsomedial hypothalamus of freely moving, undisturbed animals during the middle of the light period (i.e. the trough of the corticosterone rhythm), caused an immediate dose-dependent increase of circulating plasma corticosterone. On the other hand, similar infusions of VP at the end of the light period completely prevented the diurnal rise in plasma corticosterone (Kalsbeek et al., 1996b). However, in all of the above studies only single time points were investigated. Therefore, to further specify the nature of the endogenous VP signal, intracerebral infusions of VP-antagonist were performed at different times of the day/night cycle. One hour infusions of the VP-antagonist were performed using in vivo microdialysis at CT2, 6, 10, 14 and 21. Perfusion of the microdialysis probe was initiated 4 h before the start of the VP-antagonist infusion and continued for 3 h more after the 1-h administration of the VP-antagonist. Microdialysis samples were collected in a vial on top of the swivel and used for measurement of free corticosterone levels in extracellular fluid. Free corticosterone levels in dialysates collected during Ringer infusions revealed a basal pattern of corticosterone release that was very similar to the one previously reported for the plasma levels of this adrenal hormone (Fig. 5). Infusion of the VP-antagonist at CT10 caused a premature increase of free corticosterone levels, preceding the endogenous peak by 1 h. Contrary to the plasma data (Kalsbeek et al., 1996c) the increase of free corticosterone levels at CT10 was less pronounced than at CT6 and comparable to the endogenous peak. This diminished response of free corticosterone levels at CT10 as compared to (total) plasma corticos-

![Fig. 5. Diurnal pattern of free corticosterone levels (○○○○; mean ± SEM, n = 8-10, pg/ml) in dialysate samples during Ringer perfusion of the DMH as compared to previously established plasma corticosterone values (shaded area; mean ± SEM, n = 10, ng/ml) in control animals without an intracerebral probe (Kalsbeek et al., 1996b).]
terone levels might be caused by a buffering effect of the corticosterone binding globulin (CBG), which is also increased at this time of day (Hsu and Kuhn, 1988).

The inhibitory control of the SCN-derived VPergic projection during the diurnal trough of adrenal activity is in agreement with the main inhibitory effect of the SCN on HPA activity as revealed by SCN lesions (Abe et al., 1979; Szafarczyk et al., 1979; Watanabe and Hiroshige, 1981; Buijs et al., 1993a). In this respect, there is a remarkable correlation between the age-related elevation of basal HPA secretory activity during the diurnal trough (Sapolsky, 1992) and the pronounced decline of VP activity in the SCN during aging (Roozendaal et al., 1987). A similar phenomenon is observed in aging humans, i.e. higher basal levels of cortisol (Dotl et al., 1994; Seeman and Robbins, 1994; Ferrari et al., 1995) together with a decreased presence of VP in the SCN (Swaab et al., 1985; Hofman and Swaab, 1994). In addition, daily and seasonal rhythms of VP activity in SCN neurons may disappear with increasing age (Hofman and Swaab, 1993), enhancing the deterioration of hormonal rhythms. Though correlative, these data strongly suggest that degeneration of the SCN (and more specifically its VP-containing population of neurons) with aging, is an important causal factor for elevated cortisol levels in elderly people.

**Stimulatory SCN input to the HPA-axis**

The differential effects of infusions of the V1-antagonist on plasma corticosterone levels depending on the time of the day (Fig. 6) provide further evidence for the rhythmic nature of the endogenous VP signal, with the peak release occurring during the light period. The variation in corticosterone responses upon application of the VP-antagonist, however, also illustrates that the VP-rhythm alone cannot explain the complete pattern of corticosterone responses. Therefore, in addition to the inhibitory control by VP, there is also a stimulatory SCN input to the HPA-axis, as discussed previously by us (Kalsbeek et al., 1992) and others (Cascio et al., 1987), which is active during the second half of the light period. Thus, the lack of a clear corticosterone response at CT2, notwithstanding the blockade of a VPergic inhibition, is caused by the simultaneous lack of a stimulatory input to the CRH neuron during the early morning. The decreasing levels of (free) corticosterone during the dark period, in spite of the absence of an inhibitory VP signal, are explained by the concomitant decline of the stimulatory input to the HPA system. Together, both rhythmic inputs from the SCN (i.e. the inhibitory and the stimulatory one) in the presently proposed phase relation (Fig. 7) are able to explain fully the observed rhythm in plasma corticosterone. The shape and height of the endogenous corticosterone peak is then defined by the internal synchronization of both rhythms. The importance of this 'tuning' of the different circadian rhythms is indicated by the study of Sparrow et al. (1993). This prospective study in humans shows that the height of the daily corticosterone peak is inversely related to the annual rate of decline of pulmonary function with aging (Sparrow et al., 1993). Furthermore, a broadening of the diurnal corticosterone peak in aged rats correlates with enhanced cognitive impairments (Issa et al., 1990).

In previous years, the apparent intact circadian rhythmicity of the VP-deficient Brattleboro rat
Fig. 7. Schematic presentation of the diurnal release pattern of SCN transmitters involved in the circadian control of corticosterone release. Vasopressin (i.e., inhibitory SCN signal) and corticosterone data are redrawn from Kalsbeek et al. (1995) and Fig. 5, respectively, whereas the release pattern of the SCN-transmitter stimulating the HPA-axis is extrapolated from the results of the timed VP-antagonist administrations (From Kalsbeek et al., 1996c).

has frequently been put forward as evidence against the notion that VP serves as an output signal of the circadian timing system (Ikonomov and Stoynev, 1994). The above scenario for the circadian control of basal corticosterone release explains why the Brattleboro rat may still show circadian behavior in general, and a circadian pattern of corticosterone release in particular. Due to the diurnal fluctuation of its stimulatory input to the HPA-axis, the SCN will still be able to create a circadian pattern of corticosterone release. However, without the inhibitory signal of VP, the timing of the peak will be different, viz. the onset of its daily rise will be earlier as compared to heterozygous animals. In fact, this premature rise of corticosterone in the Brattleboro rat was already observed by Ixart et al. (1982).

Feeding activity and the corticosterone rhythm

In the scenario outlined above, the inhibitory SCN input serves to prevent the rise of corticosterone during the light period, when the animal is not active and not feeding, and when sustained elevations of this highly catabolic hormone would be deleterious. The daily decline in VP secretion from SCN terminals at the end of the light period serves to set a specific time window for the increased corticosterone release, i.e. just before the period of increased locomotor and feeding activity. The stimulatory SCN input to the HPA-axis shows a rapid decline between CT10 and CT14, i.e. at the onset of the dark period (Fig. 7). The question can be put forward whether this decline is due to an endogenous rhythm or caused by an environmental factor. Two obvious factors that could be involved are the onset of darkness, and/or the increased feeding activity. As far as the effect of lighting conditions on the decline in plasma corticosterone values is concerned, previous results of a continued corticosterone rhythm in blinded animals (Takahashi et al., 1977; Kobayashi and Takahashi, 1979) and under DD conditions (Fischman et al., 1988) make it unlikely that the onset of darkness per se is responsible for the rapid decrease of the stimulatory SCN input. In order to investigate the effect of feeding activity on the decline in corticosterone levels, we subjected animals to overnight food deprivation and measured plasma corticosterone and insulin levels. Plasma insulin levels showed the expected increase in control animals upon onset of darkness and feeding activity. In the animals that had fasted overnight, circulating levels of insulin slowly declined (Fig. 8A), but the absence of food did not affect the circadian decline in plasma corticosterone levels (Fig. 8B). This result is comparable to that of Kobayashi and Takahashi (1979), who showed that even after 3 days of total food deprivation, the corticosterone rhythm is still intact. These results indicate that feeding activity does not induce the decrease in stimulatory SCN input to the HPA-axis either. On the other hand, Akana et al. (1994) recently reported a very strong increase of plasma corticosterone and ACTH levels during overnight fasting. The elevated levels of ACTH and corticosterone reported in that study, how-
ever, resemble those seen during non-rewarded lever pressing for food (De Boer et al., 1990) and suggest that a stress-component was also involved in the activation of the HPA-axis. Together, these results indicate that, similar to the inhibitory VP signal, the stimulatory SCN input to the HPA-axis

*Starting 2–4 weeks after sustaining an SCN-lesion all animals were scored behaviorally for the effectiveness of the SCN ablation. Drinking behavior and locomotor activity were monitored for at least 2 weeks. Only animals exhibiting a clearly arhythmic behavior were used for subsequent experiments.

Fig. 8. Plasma insulin (A) and corticosterone (B) values during light-dark transition in fed (●–●; n = 8) and overnight fasted rats (○—○; n = 8). Animals were habituated to a feeding regimen in which they received new food pellets (+20 g) every day at the onset of the dark period. The dashed line in B shows the plasma corticosterone data of a group of ad libitum fed control animals from a previous experiment (compare Fig. 5).

Fig. 9. Plasma melatonin values in SCN-lesioned animals (●) sampled in the middle of the dark period (either CT18 or CT20) as compared to melatonin levels (shaded area mean ± SEM) in intact control animals.

is an endogenous circadian rhythm not dependent on environmental lighting or feeding conditions.

The circadian rhythm of melatonin release

Effects of SCN lesions

Previous experiments with SCN-lesioned animals yielded equivocal results with respect to the abolishment of the circadian release of the pineal hormone melatonin (Reppert et al., 1981b; Lehman et al., 1984; Locatelli et al., 1994; Scott et al., 1995; Tessonneaud et al., 1995). We recently found that notwithstanding effective SCN-lesions* a considerable number of animals still showed increased levels of plasma melatonin during the dark period (Fig. 9). This observation seems to confirm previous observations that small parts of the SCN are insufficient to impose complex overt behavioral rhythms (Van Den Pol and Powley, 1979; Satinoff and Prosser, 1988), but apparently sufficient to impose a hormonal rhythm. Recently, a microdialysis technique was developed for measurement of melatonin directly upon its release from the pineal gland (Azekawa et al., 1991; Drijfhout et al., 1993). Obvious advantages of the in vivo microdialysis technique are: (1) melatonin levels in the dialysate are much higher than in plasma; (2) high frequency sam-
pling is possible without disturbances of plasma hemodynamics; and (3) melatonin levels measured directly in the pineal are not confounded by melatonin released from other melatonin producing organs such as the intestine, retina or hardarian gland. Therefore, we re-investigated the effect of SCN-lesions on the circadian release of melatonin by using transpineal in vivo microdialysis (Fig. 10).

Monitoring melatonin release by means of pineal dialysis during a 24-h period in control animals revealed a significant diurnal rhythm similar to previous results (Fig. 11A), the rhythm being clearly more pronounced than after plasma measurements (compare with Fig. 9). Measurement of melatonin release in SCN-lesioned animals during the final 2 h of the light period and the ensuing first half of the dark period revealed a significant increase of melatonin release upon onset of darkness in seven out of 18 behaviorally arhythmic animals (Fig. 11B). The increased nighttime release in these animals, although lower than in controls, is suggestive of an intact diurnal release pattern of melatonin in no less than 30% of the behaviorally arhythmic animals. The remaining 11 animals showed fairly constant extracellular levels of pineal melatonin with mean values intermediate between the day- and nighttime values of control animals (Fig. 11C). Closer examination, however, revealed that these well-lesioned animals had to be divided in two subgroups. Five out of the 11 animals exhibited a melatonin release pattern tonically elevated up to nighttime peak values of control animals. By contrast, the other half of the animals (six out of 11) displayed a constant release of melatonin just above, but significantly different from, basal daytime release in control animals (Fig. 11D). In a previous study using plasma samples to assess melatonin release, one out of ten SCN-lesioned animals still showed a diurnal pattern of melatonin release. From the remaining nine animals, two showed continuously elevated levels of plasma melatonin, whereas the remaining seven animals exhibited plasma melatonin levels just above normal daytime values (Kalsbeek et al., 1996a).
The results of the partially lesioned animals suggest that a small piece of VIP (probably colocalized with GABA (Buijs et al., 1995)) containing SCN tissue is sufficient to inhibit daytime melatonin release (Fig. 11B). A similar situation was noted previously with regard to the release of corticosterone (Buijs et al., 1993a). Probably the diurnal melatonin rhythm in these animals is induced by the L/D-cycle. Preliminary data show that, indeed, the diurnal variation in melatonin release disappears under D/D conditions in these animals. These data would implicate a third model of SCN functioning (see Moore chapter) in which the SCN neurons in the retino-recipient area are assumed not to contain a circadian pacemaker, but to solely transmit the presence of light, either to SCN target areas or to the pacemaker containing part of the SCN. On the other hand, there
may be too few SCN neurons left in these partially lesioned animals to create a coherent circadian rhythm.

**SCN inputs to the melatonin-rhythm-generating-system**

Similar to the circadian control of corticosterone secretion, the SCN may exert an inhibitory and excitatory influence on melatonin release as well. Our recent infusion experiments with a GABA-agonist and -antagonist (muscimol and bicuculline, respectively) indicated that the GABA containing fibers in the SCN projection to the dorsal hypothalamus may be an important mediator of the inhibitory SCN effect (Kalsbeek et al., 1996a). It is not clear yet how the SCN GABAergic neurons are implicated in the control of melatonin release; either (1) the GABAergic projection from the SCN solely serves to transmit the inhibitory effect of light to the melatonin-rhythm-generating-system in the dorsal hypothalamus; or (2) GABA transmits the circadian signal from the SCN to the dorsal hypothalamus. As far as the first possibility is concerned, it is possible that GABA release by SCN terminals is only provoked after retinal perception of light. In this respect, our experiments show a strong resemblance to the light induced inhibition of melatonin release (Kanematsu et al., 1994). Also, light-induced phase-shifts of the circadian pacemaker provide evidence for the involvement of GABAergic mechanisms. Phase-shifts induced by light pulses, can be blocked in a phase-dependent manner with intraperitoneal injections of the GABA antagonist bicuculline (Ralph and Menaker, 1989). The GABA agonists muscimol and baclofen can induce permanent phase-shifts in the circadian rhythm of activity onset of golden hamsters when injected into the region of the SCN (Smith et al., 1989, 1990). GABA-agonists can block the muscimol effect, but do not have an effect themselves (Smith et al., 1990). These results suggest that GABA is involved in a light-responsive pathway of the SCN, but that endogenous GABAergic pathways do not normally contribute to the temporal regulating mechanism of the circadian pacemaker. Thus, retinal perception of light may induce the release of GABA by SCN terminals both within the SCN itself (Bos and Mirmiran, 1993) and in its projection areas. It is intriguing that both the phase-shifting effect of light and its inhibition of melatonin release are dependent on the time of day (Illnerova and Vanicek, 1979; Honma et al., 1992; Owen and Arendt, 1992). With regard to the second possibility, i.e. transmission of a circadian signal, it is also conceivable that the GABA-containing SCN projection is largely inactive during the night but active during the (subjective) day. This would implicate an endogenous rhythm in GABA release by SCN terminals that is comparable to that of VP. This is in agreement with reported circadian fluctuations in the hypothalamic and SCN content of GABA or its turnover (Kanterewicz et al., 1993; Cattabeni et al., 1978), which seem to persist in constant darkness (Aguilar-Roblero et al., 1993). We are currently pursuing this line of research by investigating whether GABA-antagonists may prevent the light-induced decline of melatonin release.

The results of the partially lesioned animals (Fig. 11B) indicate that a certain amount of stimulatory input is also destroyed, since, compared to control animals, in a large proportion of lesioned animals peak melatonin values are reduced. The dual reaction of the well-lesioned animals too (i.e. a sustained secretion of melatonin either at a low or a high level), suggests that, depending on the amount or location of the SCN tissue destroyed, a stimulatory factor is, or is not, removed, respectively. So far, however, no obvious differences between both groups could be detected. Regarding the stimulatory SCN input into the hypothalamic melatonin-rhythm-generating-system, its identity is unknown at present. Previous results showed a stimulatory effect of both synthetic VP and VIP on plasma melatonin levels when infused in the PVN/DMH area (Kalsbeek et al., 1993a). However, the en-
dogenous stimulatory role of these SCN peptides could sofar not be confirmed.

**Vasopressinergic output of the circadian clock**

**Measurement of vasopressin release**

Until recently, in vivo measurement of intracerebral VP release was only possible with the CSF sampling technique. A drawback of monitoring the central release of VP via its CSF levels, however, is not only its low temporal resolution but also the uncertainty as to the origin of the peptide measured. Changes in CSF levels of VP are thought to primarily reflect secretory activity of VP terminals proximal to the ventricular ependyma. However, in addition to a large fraction of SCN projections (Hoorneman and Buijs, 1982; Kalsbeek et al., 1993b), projections from VP containing neurons in the bed nucleus of the stria terminalis to the medial part of the lateral habenula and the lateral septum, also terminate at a short distance from the ventricular system (De Vries and Buijs, 1983; De Vries et al., 1985). Therefore, additional lesion experiments were necessary to prove that the major portion of VP in the CSF is derived from SCN terminals (Schwartz and Reppert, 1985; Jolkkonen et al., 1986). Another method for in vivo measurement of central VP release is push-pull perfusion (PPP). The PPP-technique has been applied successfully for measurement of terminal VP release in brain areas ranging from the lateral septum and ventral septal area to the dorsal hippocampus and nucleus of the solitary tract (Ramirez et al., 1990; Landgraf, 1992; Roth et al., 1992; Watanobe and Takebe, 1994). But besides these projection areas of VP neurons contained in the PVN and BNST, no PPP studies are known to us regarding VP release in SCN projection areas. In addition, it is doubtful if long-term experiments necessary to establish a circadian release of VP would be feasible with PPP (Estupina et al., 1996). The continuous pumping of fluid into the brain and the associated problem of flow imbalance may be quite disruptive to the surrounding brain tissue (Gardner et al., 1993; Ixart et al., 1993).

For a few years now, measurement of VP release via microdialysis has been successfully applied during short-term experiments in brain areas containing VP producing neurons, e.g. the PVN and SON (Kimura et al., 1994; Landgraf et al., 1994; Moriguchi et al., 1994; Ota et al., 1994; Bealer and Abell, 1995). The advantages of the microdialysis technique are obvious: (1) it offers a high resolution in time (1-h samples are very well possible); (2) there is no tissue damage, and minimal disturbance of the interstitial fluid microenvironment during perfusion; and (3) it can be easily combined with behavioral tests. Although clearly advantageous as compared to other techniques, the dialysis membrane also has a serious disadvantage in that it provides a barrier to the recovery of the peptide. In vitro, recovery for VP is usually about 2% (Landgraf et al., 1995); own unpublished results), although higher values have also been reported (Ota et al., 1992). But in vivo, recovery may be even lower (e.g. 1 h after implantation, transfer of VP outside the dialysis probe has been calculated to be only 0.5% (Engelmann et al., 1992)). Thus, the technique can only be applied in brain sites with a relatively high extracellular concentration of endogenous VP in combination with a sensitive assay.

Despite this restriction, we explored the feasibility of measuring VP release in the SCN area with the microdialysis technique. Though tissue levels of VP and its mRNA in the SCN are very low as compared to the magnocellular PVN and SON (Noto et al., 1983; Uhl and Reppert, 1986; Burbach et al., 1989), the VP-containing neurons of the SCN provide a dense local innervation of the nucleus of origin (Van Den Pol and Tsujimoto, 1985; Castel et al., 1990; Kalsbeek et al., 1993b; Buijs et al., 1994). We succeeded in measuring the intranuclear VP release of the PVN, SON and SCN during 36 successive hours (Kalsbeek et al., 1995). Mean levels of VP in the SCN were about half of those in the PVN and SON. Furthermore, this experiment showed a sig-
nificant diurnal variation of VP release only in the SCN, with the highest levels occurring during the light period, similar to the previously published data on CSF levels of VP. These results thus indicate that the activity of separate VP projections to different SCN target areas may be synchronized with a peak secretory activity during the light period.

Circadian release of vasopressin

The diurnal VP rhythm seems to be a true circadian rhythm because it free-runs in vitro (Earnest and Sladek, 1987; Tominaga et al., 1994) and in vivo under constant lighting conditions (DD or LL), although the CSF VP rhythm diminishes after several days of constant light (Reppert et al., 1982; Schwartz et al., 1983; Schwartz and Reppert, 1985; Seckel and Lightman, 1987; Stark and Daniel, 1989; Tominaga et al., 1992). Entrainment of mammalian circadian rhythms to the ambient light-dark cycle is a consequence of the phase-specific resetting effects of environmental light on the activity of the circadian pacemaker. The resetting effect of light is defined by the phase-response curve to light pulses administered to rodents that were kept in DD (Honma et al., 1978; Summer et al., 1984; Bauer, 1992). Activity rhythms may take several days to reach a new steady state after a phase-shifting light stimulus. On the other hand, there are a few results indicating an immediate phase-resetting of the pacemaker (Takamura et al., 1991; Mead et al., 1992; Buijs et al., in preparation). We therefore investigated the instantaneous effect of an 1-hour light exposure during either the phase-delay or the phase-advance portion of the dark period (i.e. CT14 and CT20) on the intranuclear release of VP in the SCN. The time span of the L/D-cycle sampled in these experiments (i.e. 8 h) was too short for discerning a clear diurnal rhythm. On the second day of the experiment, no effect whatsoever of the 1-hour light exposure was found on the release of VP at either time of the night (Fig. 12). We ascertained patency, functionality and proximity to the SCN of the dialysis probes by means of perfusion of Ringer with a high [K+] on the third and last day of the experiment. In response to the depolarizing K+ stimulus all animals showed a clear increase in VP release, irrespective of the time of day (Fig. 12). Immediate phase resetting of the pacemaker by means of light exposure at night does therefore not seem to be accompanied by an immediate change in the release of VP. In accordance with this lack of change in VP release no expression of the protooncogene c-fos is found in VP-containing SCN neurons either (Romijn chapter).

Apart from its insensitivity to environmental lighting conditions, it was also shown that the diurnal rhythm of VP mRNA poly(A) tail length in the SCN occurs independent of a number of extrinsic neuroendocrine signals. Neither adrenalectomy, nor gonadectomy, serotonin-depletion, or treatment with melatonin or the benzodiazepine triazolam affected the basic VP rhythm (Carter and Murphy, 1989). On the other hand, some studies showed effects of adrenal
steroids on VP and/or VIP immunoreactivity and mRNA content in the SCN (Dierickx, 1980; Gozes et al., 1994; Larsen et al., 1994). Our own preliminary results show changes in the circadian pattern of intranuclear VP release of animals subjected to a restricted feeding paradigm (i.e. only a 2-h period of access to food during the light period). These feeding-induced changes may, however, also involve an action of adrenal steroids on the SCN, since previous studies have shown a profound effect of the restricted feeding paradigm on the daily pattern of plasma corticosterone release (Honma et al., 1992; Mitome et al., 1994).

Vasopressin as an output signal of the SCN

The conspicuous presence of VP fibres within the SCN itself has led to increasing speculation about its importance as circadian generator, next to its role as an output signal. Early immunocytochemical studies already indicated two major subdivisions of the rodent SCN: a dorsomedial part lodging the major population of VP-containing neurons, and a ventrolateral part consisting of VIP-containing neurons and the major afferent inputs (Moore chapter). However, this broad subdivision, based on the localization of identified somata, does not reflect the network organization of the neurons involved. Indeed, in addition to their efferent dorsal projections into the hypothalamus, the VP and VIP-containing neurons of the SCN form a dense network of axons and dendrites within both nuclei (Card et al., 1981; Van Den Pol and Gores, 1986; Castel et al., 1990; Kalsbeek et al., 1993b; Buijs et al., 1994). These extensive intranuclear VP- and VIP-projections make it tempting to speculate that these peptides not only serve to transmit the circadian rhythms to the rest of the brain, but may also serve as an entraining agent in the SCN itself. Microinjection of VP into the SCN, however, did not induce phase-shifts (Albers et al., 1984). On the other hand, our microdialysis experiments, showing rhythmic changes in the extracellular VP levels of the SCN in phase with the changing VP levels in the CSF, indicate that the diurnal release of VP may not only serve to transmit the message of the circadian oscillator to its target areas, but to synchronize and amplify the pacemaker output within the SCN as well.

The synchronized activity of the different VPergic projections from the SCN suggests that VP release from SCN terminals may also serve to inhibit other aspects of the animals' behavior and physiology. Indicative of a more widespread inhibitory effect of SCN-derived VP are previous results on the facilitatory effect of intracerebroventricular (ICV) applied VP-antagonist on female lordosis behavior, especially during the light period (Södersten et al., 1983; Södersten et al., 1985; Södersten et al., 1986). In line with our data on corticosterone secretion, also in the case of sexual behaviour, the effects of the VP-antagonist are similar to the disinhibiting effect of SCN-lesions (Hansen et al., 1979; Eskes, 1984). The VP innervation to the medial preoptic nucleus (MPN) is quite dense (Hoorneman and Buijs, 1982; Kalsbeek et al., 1993b) and may contact oestrogen-receptor containing neurons (Watson et al., 1995). Therefore, the VP projection to the MPN may serve to synchronize sexual activity and hormone secretion with the L/D-cycle (see also Van Der Beek chapter). Likewise, the inhibitory and stimulatory effect of ICV applied VP and VP-antagonist, respectively on the hyperglycaemic response to intracranial injections of 2-deoxy-d-glucose (Nagai chapter) are indicative of more widespread inhibitory effects of VP released by SCN terminals.

A number of other publications have appeared providing circumstantial evidence for a role of VP as an output signal of the circadian timing system. Intracerebral injection of VP-antagonist, but not VP, at the level of the SCN at the beginning of either the light or dark period causes a disruption of circadian feeding and drinking rhythms (Reghunandanan et al., 1987; Stoynev and Ikonomov, 1990; Reghunandanan et al., 1992). In addition, it has been shown in the house mouse and the common vole that interindividual differences in circadian patterns of locomotor activity are associated with differences in the number of VP-posi-
tive neurons in the SCN (Bult et al., 1992; Bult et al., 1993; Gerkema et al., 1994). Recently, it was shown that the circadian rhythms of body temperature and locomotor activity were severely disrupted in the VP-deficient Brattleboro rat, but not in its heterozygous littermate under restricted feeding conditions (Murphy et al., 1993).

Conclusions

A general issue in circadian biology still concerns the question how the pacemaker transfers temporal information to the rest of the organism. Using microdialysis as an important research tool, the experiments described in this chapter clearly show that the rhythmic release of SCN transmitters in their target areas is an important factor for the translation of endogenous SCN rhythms into hormonal (and probably behavioral) rhythms. The inhibitory effects of VP and GABA released from SCN terminals in the PVN/DMH area, form an important aspect in the circadian control of, respectively, corticosterone and melatonin release. In addition, the reviewed results make clear that the SCN control of both hormonal rhythms also contains a stimulatory signal. A circadian rhythm is then created by allowing a stimulatory input to the hormonal system during the secretional trough of the inhibitory transmitter. Using partial SCN-lesioned hamsters provided with fetal SCN transplants of tau-mutant hamsters, Vogelbaum and Menaker (1992) reached a similar conclusion regarding the control of locomotor activity, viz. ‘suggests the presence of both stimulatory and inhibitory inputs from the circadian system to the centres controlling locomotor behavior’. In conclusion, microdialysis has proven to be an important research tool for the exploration of circadian secrets, and, perhaps in the future, it may even be helpful in unraveling the (circadian) secrets of the human brain (During et al., 1995; Kanthan et al., 1995).

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References


