Circadian rhythm dysfunction in the suprachiasmatic nucleus: Effects of *Trypanosoma brucei brucei* infection and inflammatory cytokines

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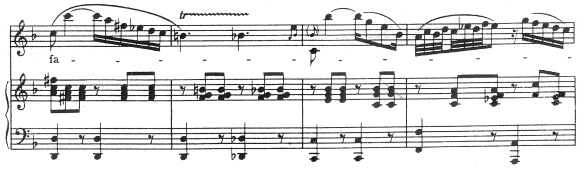
To my Parents



From the piano arrangement of 'Et incarnatus'
Aria for soprano and orchestra (Andante)
Mass in C-minor by Wolfgang Amadeus Mozart

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ABSTRACT

Alterations in the mammalian circadian pacemaker, the hypothalamic suprachiasmatic nuclei (SCN), were studied in an experimental rat model of African trypanosomiasis, or sleeping sickness, caused by infections with subspecies of *Trypanosoma brucei (T. b.)*. Characteristic signs of the disease are marked disturbances in circadian rhythms, such as a fragmentation of the sleep-wake cycle. Circadian rhythms are mastered from neurons in the SCN, which have an endogenous rhythm of spontaneous firing that is increased during the subjective day. The SCN rhythm can be recorded as spontaneous single unit activity in slice preparations containing the SCN. This activity was recorded in slices from controls and rats infected with *T. b. brucei.* The rhythm in spontaneous neuronal activity was markedly altered in trypanosome-infected rats, with a lower average frequency and a phase advance of the peak. No structural differences in the retinal afferents were detected.

The spontaneous postsynaptic activity was analyzed in slices by whole cell patch clamp recordings of SCN neurons. The inhibitory and excitatory postsynaptic events were identified as primarily γ-aminobutyric acid (GABA) $_A$ and α-amino-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor-dependent. The inhibitory and excitatory activity was compared between the subjective day and night in control rats. No significant difference was detected in amplitude or frequency of inhibitory synaptic events, but the frequency of excitatory events was significantly increased during the subjective day. In slices from rats infected with *T. b. brucei*, the frequency of excitatory events was significantly lower during the subjective day as compared with control rats. In addition, the protein expressions of AMPA glutamate receptor subunit 2 & 3, and N-methyl-D-aspartate receptor channel subunit ζ1 (NMDAR1), were decreased in trypanosome-infected rats.

Invasion of trypanosomes causes a substantial release of several cytokines as an immune response to the infection, for instance tumor necrosis factor (TNF)-α and interferon (IFN)-γ, which act in synergy. TNF-α and IFN-γ in combination with bacterial lipopolysaccharide were added to SCN slices and the spontaneous firing was examined. The cytokines altered the rhythm in firing frequency; i. e. caused an abolishment or shift of the peak.

The transcript and protein of the IFN-γ receptor (IFN-γR) was detected in the SCN. The molecular identity of the receptor transcript was determined, confirming that the receptor molecule was identical to the IFN-γR in the immune system. The expression of the receptor protein showed daily variations with a peak of expression during the early subjective night. The cyclic variation was abolished and the protein levels were increased in rats held in constant darkness. The postnatal development of the IFN-γR protein was studied. At postnatal day (P) 1 the protein was distributed throughout the entire nuclei, but relocated to the ventrolateral retinorecipient subdivisions of the SCN between P11 and P20.

In conclusion, the present findings demonstrate that *T. b. brucei* dysregulates the endogenous rhythm in SCN activity, which probably alters the circadian output and may be manifested as a fragmentation of the sleep-wake cycle. Further, the SCN contain glutamatergic synapses that display an increase in activity during the subjective day *in vitro*. This activity is decreased in slices from trypanosome-infected rats, possibly explaining the observed alteration in spontaneous firing. Cytokines released during trypanosome-infections, such as IFN-γ, may affect protein expression of glutamate receptors and glutamatergic postsynaptic transmission via its receptor, which is located in the ventralateral regions of the SCN.

Keywords: suprachiasmatic nucleus, slice, *Trypanosoma brucei*, sleeping sickness, spontaneous firing, rhythm, synaptic activity, GABA, glutamate, AMPA, cytokines, sleep regulation, interferon-gamma, tumor necrosis factor-alpha

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ORIGINAL ARTICLES

This thesis is based on the following original articles that are referred to in the text by their Roman numerals.

I. **Lundkvist G. B**., Christenson J., ElTayeb R. A. K., Peng Z-C., Grillner P., Mhlanga J., Bentivoglio M. and Kristensson K. (1998) Altered neuronal activity rhythm and glutamate receptor expression in the suprachiasmatic nuclei of *Trypanosoma brucei*- infected rats. *J. Neuropath. Exp. Neurol*., 57:21-29. *(Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.)*

II. **Lundkvist G. B**., Kristensson K. and Hill R. H. The suprachiasmatic nucleus exhibits diurnal variations in spontaneous excitatory postsynaptic activity. *Submitted for publication.*

III. **Lundkvist G. B**., Hill R. H. and Kristensson K. *Trypanosoma brucei brucei* infection and pro-inflammatory cytokines alter synaptic activity and firing frequency in the rat suprachiasmatic nuclei. *Manuscript*.

IV. **Lundkvist G. B**., Robertson B., Mhlanga J. D. M., Rottenberg M. E. and Kristensson K. (1998) Expression of an oscillating interferon-γ receptor in the suprachiasmatic nuclei. *NeuroReport,* 9:1059-1063. *(Reprinted from Neuroreport,9, Lundkvist G. B., Robertson B., Mhlanga, J. D. M., Rottenberg M. E. and Kristensson K. Expression of an oscillating interferon-γreceptor in the suprachiasmatic nuclei., 1059-1063, © 1999 with permission from Lippincott Williams & Wilkins.)*

V. **Lundkvist G. B**., Andersson A., Robertson B., Rottenberg M. E. and Kristensson K. (1999) Light-dependent regulation and postnatal development of the interferon-γ receptor in the suprachiasmatic nuclei. *Brain Res*., 849:231-234. (*Reprinted from Brain Research, 849, Lundkvist G. B., Andersson A., Robertson B., Rottenberg M. E. and Kristensson K. Light-dependent regulation and postnatal development of the interferon-γreceptor in the suprachiasmatic nuclei, 231-234, © 1999 with permission from Elsevier Science.)*

LIST OF ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	Analysis of variance
AP-5	D-2-amino-5-phosphonopentanoic acid
BBB	Blood-brain barrier
cDNA	Complementary deoxyribonucleic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CD	Clusters of differentiation
CNS	Central nervous system
EEG	Electroencephalographic
EPSC	Excitatory postsynaptic current
FITC	Fluorescent isothyocyanate
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
GluR1	Glutamate AMPA receptor subunit 1
GluR2/3	Glutamate AMPA receptor subunit 2 and 3
HAT	Human African trypanosomiasis
HRP	Horse radish peroxidase
IFN-γ	Interferon-γ
IFN-γR	Interferon-γ receptor
IL	Interleukin
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory postsynaptic potential
JAK	Janus kinase
LD	Light:dark
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
NF-κB	Transcription factor:nuclear factor-κB
NK cells	Natural killer cells
NMDAR1	N-methyl-D-aspartate receptor channel subunit ζ1
NO	Nitric oxide
NREM	Non-rapid eye movement
P	Postnatal day
p. i.	Post infection
per	*period*
REM	Rapid eye movement
RHT	Retino-hypothalamic tract
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCN	Suprachiasmatic nuclei
SEM	Standard Error of the Mean
STAT	Signal transducer and activator of transcription
SWS	Slow wave sleep
T. b.	*Trypanosoma brucei*
TNF-α	Tumor necrosis factor-α
TTX	Tetrodotoxin
ZT	*Zeitgeber* time

INTRODUCTION

The evolution of trypanosomiasis

Trypanosomiasis refers to a spectrum of diseases resulting from two related but distinctly different forms of the parasitic genus *Trypanosoma*. Trypanosomes are flagellated single-cell eukaryotes belonging to the family *Trypanosomatidae,* which reproduce asexually by longitudinal binary fission (Lim, 1989). These organisms are present in all vertebrate classes and are transmitted by arthropods or leech vectors. In Sub-Saharan Africa the extracellular form *Trypanosoma brucei (T. b.)* causes the lethal 'human African trypanosomiasis' (HAT; sleeping sickness) and *nagana* in domestic African animals. The closely related, but predominantly intracellular form, *T. cruzi*, is found in Central and South America where it causes Chaga's disease (American trypanosomiasis), discovered by Carlos Chagas in 1909 in the State of Minas Gerais in Brazil (for review, see Chimelli and Scaravilli, 1997). Although the geographical distribution, mode of transmission, pathogenesis, neurological involvement and clinical manifestations are distinct between the diseases caused by the two parasite forms, there is recent evidence that suggest a common ancestor of *T. b.* and *T. cruzi* approximately 100 million years ago (for review, see Stevens et al., 2001). At that time, Africa, South America and Australia together formed the super-continent Gondwana, from which Africa split off first. This event is reflected in the evolutionary tree of trypanosomes, because *T. b.* belongs to a branch separate from *T. cruzi*. In contrast, trypanosomes infecting kangaroos in Australia are more closely related to *T. cruzi* (for reviews, see Stevens et al., 2001; Zimmer, 2001).

Sleeping sickness is known to have been affecting humans for a long time during the history of human evolution, and African trypanosomes most likely co-evolved with human hosts for thousands of years (for review, see Stevens et al., 2001). Two subspecies causing HAT evolved: *T. b. rhodesiense*, which is highly virulent and causes a zoonotic, acute form of HAT in East Africa, and *T. b. gambiense*, which is less virulent and causes a chronic form in West and central Africa. *T. b. gambiense* was previously considered to be less virulent because of co-evolution with humans. However, molecular characterizations of different subspecies have revealed that the strain *T. b. gambiense* is greatly homogenous, while a substantial gene flow seems to occur between *T. b. rhodesiense* and *T. b. brucei*

(which affects livestock), resulting in a high heterogeneity and, thus, in higher virulence (for review, see Stevens et al., 2001).

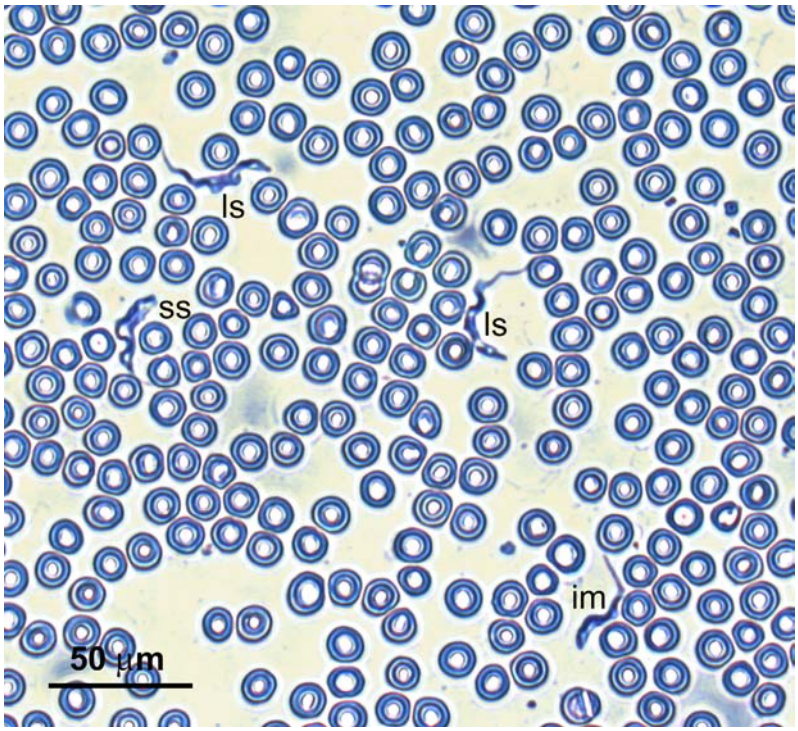

Figure 1*: Subspecies of the extracellular haemoflagellate Trypanosoma brucei (T. b.) cause sleeping sickness. The photograph shows a staining of T. b. brucei in different stages in a blood smear from mouse by the method of May Grünwald-Giemsa. Ls, long slender form; ss, short stumpy form; im, intermediate form. Only the long slender forms divide. By Jama D. Mhlanga.*

Historical perspective of African trypanosomiasis

T. b. and the role of tsetse flies (*Glossina*) as vectors were identified in game animals in Zululand and first described in 1895 by Sir David Bruce (1855-1931). Forde and Dutton detected morphologically identical trypanosomes in 1902 in human blood in a case of 'Gambian fever' (see Mbulamberi, 2001). In 1903, during the epidemic in Africa's tropical regions, the Italian bacteriologist Aldo Castellani (1874-1971) observed trypanosomes in the cerebrospinal fluid (CSF) from subjects afflicted by sleeping sickness, while working in Uganda (for review, see Bentivoglio et al., 1994b). The chronic progressive nature of human infection in West and Central Africa was subsequently designated as *Gambian trypanosomiasis* caused by *Trypanosoma gambiense*, later called *T. b. gambiense*. In 1908, a rapid form of trypanosome infection was identified in Luangwa valley, Zambia, which was described as *Rhodesian trypanosomiasis* due to *Trypanosoma rhodesiense* (later *T. b. rhodesiense*).

The strain in livestock that was non-infective to humans was called *Trypanosoma brucei* (*T. b. brucei*; see Mbulamberi, 2001).

Vector transmission

The large wild animals in Africa are normally trypanosome-resistant and act as reservoirs for the parasites (for review, see Mhlanga, 1996). *Glossina palpalis* and *tachynoides* are vectors for *T. b. gambiense* while *Glossina morsitans, swynnertoni* and *pallidipes* transmit *T. b. rhodesiense*. The geographical distribution of HAT is directly correlated to the residence and/or spreading of the tse-tse fly, which is confined to the tropical belt of the African continent where it nests in forests and savannas. When pleomorphic trypanosomes have entered the fly during a blood meal, they are transferred via the oesophagus and cardia proventriculus to the midgut. Here the stumpy forms transform to procyclics, which divide and reach the salivary glands. They attach and reproduce as epimastigotes and are finally transformed to the non-dividing metacyclic forms (for review, see Mhlanga, 1996), which make the fly infectious during the whole of its life span (for review, see Dumas and Girard, 1978).

Clinical picture

After an insect bite a swelling, or chancre, may form. The metacyclics transform to dividing long slender bloodstream forms (for review, see Mhlanga, 1996), which multiply outside the cells in the subcutaneous tissue at the site of inoculation. The signs of disease are polymorph and may vary between affected humans. In the first, or hemolymphatic stage, the trypanosomes spread rapidly to the lymph nodes and the blood stream. In general, the early systemic signs include irregular fever, headache, tachycardia, exhaustion, extreme thirst, anorexia, skin changes and pruritus. The trypanosomes then invade the central nervous system (CNS). This subsequent meningoencephalitic stage is often prolonged, characterized by severe sleep-wake disturbances, deep sensory disturbances resulting in hyperpathia, extrapyramidal signs (for instance tremor and abnormal movements) and endocrine dysfunctions leading to impotence and amenorrhea. Psychiatric and mental problems are common and include character instability associated with aggression, apathy, depression, memory disturbances, confusion, manic episodes, hallucinations, catatonia and disturbances in consciousness. Without chemotherapeutic treatment, which may cause severe side-effects and sometimes results in lethal encephalopathy (Haller et al., 1986), the course

of disease reaches terminal stages that are hallmarked by dementia, increased disturbances in consciousness and epileptic seizures (Dumas and Bisser, 1999). The histopathology is characterized mainly by a marked inflammatory cell infiltration in the white matter of the brain and the occurrence of Mott's morular cells, which probably are acidophilic over-stimulated plasma cells that contain immunoglobulins of the IgM type. No significant structural changes of neurons have been observed (Kristensson and Bentivoglio, 1999).

Disturbances in sleep and circadian rhythms in African trypanosomiasis

A most characteristic symptom is the severe disturbance in the sleep-wake rhythm with typical diurnal somnolence and in many cases nocturnal insomnia, which also has given the disease its name (sleeping sickness). These disturbances are reflected in specific and characteristic changes in the electroencephalographic (EEG) pattern, which are polymorph and appear both in awakening and sleep tracing. *Awakening*: EEG recordings may include bursts of delta waves (of 50 μV amplitude) and theta waves that appear in intervals. In advanced stages of trypanosomiasis the alpha rhythm disappears, the delta bursts become even more periodic and epileptic stages may appear (for review, see Dumas and Girard, 1978). *Sleep:* The EEG pattern during nocturnal sleep is disturbed and can be correlated to the severity of clinical symptoms. These changes are marked and after introducing pharmacological therapy to the patient they are the last to disappear (for review, see Dumas and Girard, 1978). Schwartz and Escande performed EEG recordings on one subject with sleeping sickness and found that rapid eye movement (REM) sleep stage was normal; whereas non-rapid eye movement (NREM) sleep stages I-IV (slow wave sleep; SWS) was pathological with abnormal bursts of frontal delta waves (Schwartz and Escande, 1970). In general, sleep is light and frequently interrupted. Stages I-III (light and middle) of SWS sleep are indistinct and transient stages are abolished, which makes it difficult to interpret different sleep stages. Stage IV (deep SWS) is discontinuous and markedly reduced. Even if the REM sleep seems to be normal, intermediate stages of SWS with REM have been described from patients affected by sleeping sickness (for review, see Dumas and Girard, 1978). *24 h sleep cycle:* The 24 h rhythm of the sleep pattern in patients with African trypanosomiasis can be summarized as being completely disorganized in the final stage of disease as compared to a normal sleep-

wake pattern. The distribution of awakening and sleep stages is equal (Schwartz and Escande, 1970) and, in contrast to what the designation 'sleeping sickness' indicates, the total duration of sleep is not increased (for review, see Dumas and Girard, 1978). This was further demonstrated by Buguet et al who performed 24 h polysomnographic recordings (EEG, electrooculogram, electromyogram, electrocardiogram, and nasal, buccal, and thoracic respiratory traces) from nine patients affected by trypanosomiasis in different stages of the disease (Buguet et al., 1989; Buguet et al., 1993). The results showed that hypersomnia did not occur. Instead, the sleep periods were severely disorganized and interrupted. In addition, the severity of the disease correlated to the degree of sleep disturbances (Buguet et al., 1993).

In addition to changes in sleep-wake rhythms, alterations of rhythms in hormonal secretion have been reported. Profound disruptions in the circadian fluctuations of cortisol, prolactin (Radomski et al., 1994), and growth hormone secretion (Radomski et al., 1996) as well as plasma renin activity (Brandenberger et al., 1996), have been observed. The rhythm in secretion of the pineal hormone melatonin was maintained in plasma from patients suffering from sleeping sickness; however, the rhythm was phase advanced by approximately 2 h (Claustrat et al., 1998). Taken together, these data indicate that major alterations of circadian rhythms occur in human subjects who develop African trypanosomiasis.

An experimental model of trypanosomiasis: alterations in circadian rhythms

The subspecies *T. b. brucei* is non-pathogenic to humans and is used in experimental models of trypanosome infections in rats. Infected rats display clear disturbances in the sleep-wake cycle. Montmayeur and Buguet (1994) performed continuous EEG recordings on rats during an acute infection with *T. b. brucei* and found that the sleep was markedly fragmented. Sleep alterations were also reported in rabbits inoculated with *T. b. brucei*, which showed a decrease in bout length and amount of SWS (Toth et al., 1994).

We use an experimental rat model with a more chronic course of disease, which reaches a terminal stage after 55-60 days. Disturbances in behavioral and physiological rhythms have been reported. Rats are nocturnal animals and under 12:12 h light:dark (LD) conditions the locomotor activity and body temperature reach a peak during the early dark phase of the day. In our chronic model the rhythm in locomotor activity is markedly blunted and the fluctuation in rectal temperature is

altered, with a 3 h phase advance of the peak rectal temperature (Grassi-Zucconi et al., 1995). A phase advance of the rhythm in binding of melatonin receptors has also been reported (Kristensson et al., 1998). Because the sleep pattern in rats typically consists of many short cycles of SWS and desynchronized REM sleep during the light hours, EEG recordings have been performed during 4 h intervals of spontaneous sleep. The rats display frequent awakenings during the SWS stages, which resembles the SWS fragmentation in humans, and a reduced REM sleep latency, resulting in a marked fragmentation of the sleep structure (Grassi-Zucconi et al., 1995). The sleep-wake pattern can be restored with exogenous administration of melatonin (Grassi-Zucconi et al., 1996).

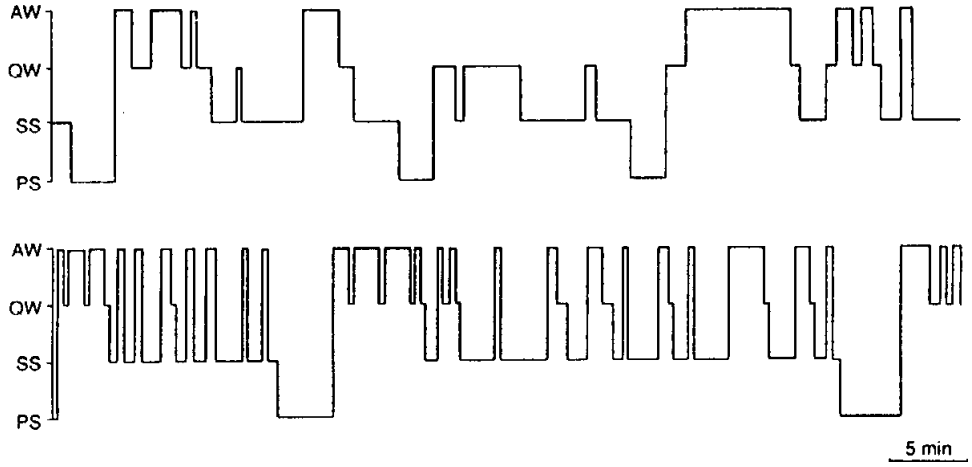

***Figure 2**: Hypnograms of a control and trypanosome-infected rat. AW, active wake; QW, quiet wake; SS, synchronized sleep; PS, paradoxical sleep (Grassi-Zucconi et al., 1995; Reprinted from Brain Research Bulletin, 37, Grassi-Zucconi G., Harris J. A., Mohammed A. H., Ambrosini M. V., Kristensson K. and Bentivoglio M. Sleep fragmentation, and changes in locomotor activity and body temperature in trypanosome-infected rats, 123-129, © 1995 with permission from Elsevier Science.)*

Taken together, these results indicate an alteration in the control of endogenous rhythms in rats infected with *T. b. brucei*.

The light-entrained suprachiasmatic nuclei – a master pacemaker for circadian rhythms

The control of endogenous rhythms, such as the daily sleep-wake cycle, is in mammals regulated by bilateral nuclei in the anterior hypothalamus, the *suprachiasmatic nuclei* (SCN), which thus function as a 'biological clock'. Located

immediately above the optic chiasm and lateral to the third ventricle, the SCN are easily distinguished to the naked eye as two oval-shaped regions. Each nucleus is approximately 950 μm long rostrocaudally, has a width of 425 μm and a height of 400 μm. One nucleus is estimated to contain approximately 8,000 neurons with an average diameter of 7.8 ± 0.9 μm in the dorsomedial region and 9.6 ± 1.5 μm for ventrolateral neurons (for review, see Van den pol, 1991). By injecting radioactive amino acids that were transported in an anterograde direction, Robert Moore showed in the early 1970s that a part of the retino-hypothalamic tract (RHT) terminates in the ventrolateral regions of the rat SCN (Moore and Lenn, 1972), and Hendrickson et al. (1972) demonstrated retinal projections to the SCN in rat, guinea pig, rabbit, cat and monkey. Bilateral lesions of the SCN have been shown to abolish diurnal rhythms in secretion of adrenal corticosterone (Moore and Eichler, 1972), drinking behavior and locomotor activity (Stephan and Zucker, 1972). Thus, in mammals the SCN were defined as a pacemaker for *circadian rhythms*, which are daily endogenous rhythms in physiological functions, hormonal release and social behavior with a 24 h cycle. Circadian periodicity has been demonstrated in many organisms, from algae to mammals (for review, see Buijs and Kalsbeek, 2001), and is essential for an organism's ability to adjust its behavior to the solar cycle, where the LD cycle is the primary '*Zeitgeber'* or 'time giver' (for review, see Aschoff, 1984). Light signals synchronize, or *entrain,* the phase of the SCN rhythm to the external LD cycle via the RHT, where glutamate is considered to be the main neurotransmitter (for review, see Meijer et al., 1996). Glutamate-immunoreactivity has been detected in presynaptic boutons (van den Pol, 1991), of which some are retinal terminals (Castel et al., 1993; de Vries et al., 1993), and glutamate receptors have been demonstrated in the SCN (Gannon and Rea, 1993; Mikkelsen et al., 1993; Ishida et al., 1994). Moreover, optic nerve stimulation causes an increase in extracellular glutamate and aspartate concentrations in slices containing the SCN (Liou et al., 1986). The SCN are not the only circadian clock in mammals. For instance, the cultured retina has a circadian rhythm in melatonin production (Tosini and Menaker, 1996) and explant tissue from the liver has a rhythm that is entrained by feeding cycles independent of the SCN (Stokkan et al., 2001). The SCN, however, are still considered to be the master pacemaker in mammals, which coordinate endogenous circadian rhythms in the body to the external environment.

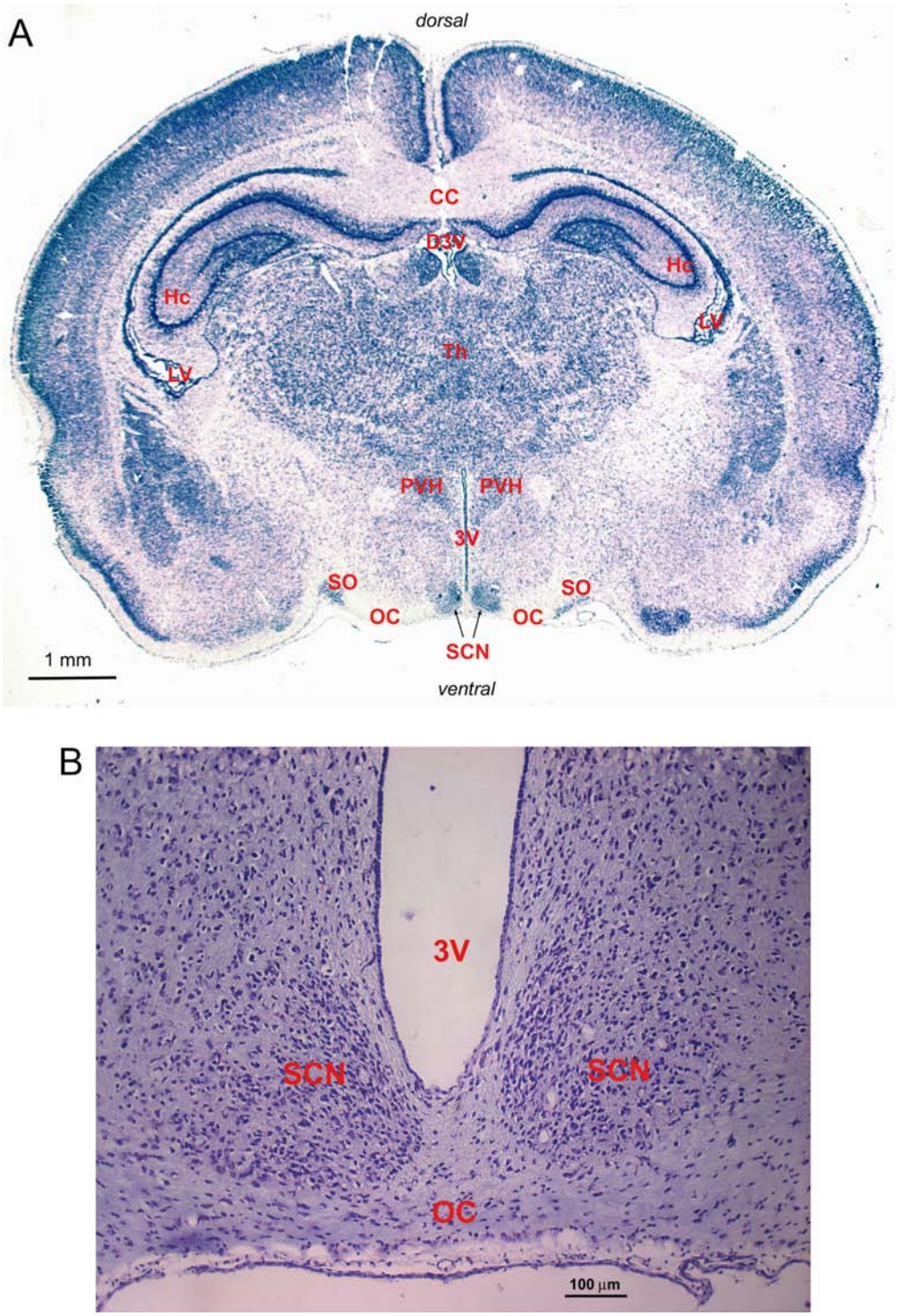

***Figure 3**: Cresyl-violet stainings of coronal sections from rat brains. **A)** A section from a brain sampled from a rat pup at postnatal day (P) 4. The suprachiasmatic nuclei (SCN) are located above the optic chiasm (OC) on the ventral side. **B)** A section from a brain sampled from an adult rat at P50, showing the oval-shaped bilateral nucleus located above the optic chiasm near the third ventricle (3V). D3V, dorsal third ventricle; LV, lateral ventricle; CC, corpus callosum; SO, supraoptic nucleus; Hc, hippocampus; Th, thalamus; PVH, paraventricular hypothalamic nucleus. Sections prepared by Margaretha Widing.*

The induction of light-responsive expression of Fos in the SCN is altered by infection with T. b. brucei

Light signals reaching the SCN via glutamate transmission in the RHT cause a rapid induction of the immediate-early gene *c-fos*, which encodes for the nuclear phosphoprotein Fos. Photic regulation of Fos is correlated with light-induced phase shifts of the circadian system and, thus, is a cellular marker of photic influence in the SCN (Takahashi, 1993). In the SCN of rats infected with *T. b. brucei*, cells immunopositive for Fos can not be detected during the light period (Bentivoglio et al., 1994c) and Fos induction in response to light pulses is markedly decreased (Peng et al., 1994). The decreased induction of Fos may cause a functional disturbance of photic entrainment in the SCN of trypanosome-infected rats, which in turn may alter circadian rhythms.

SCN afferents and efferents

The SCN have two functional subdivisions; a ventrolateral and a dorsomedial region. Entrainment seems to occur mostly in the ventrolateral region, which contains neurons immunoreactive to vasoactive intestinal peptide. The dorsomedial region can be distinguished by immunoreactivity to vasopressin (Moore, 1991). The SCN afferents and efferents have mainly been described in rat and hamster. The ventrolateral regions receive afferents from 1) the glutamatergic RHT, a portion of which projects directly to the SCN (Moore et al., 1995), 2) neurons from the intergeniculate leaflet containing neuropeptide Y (Moore and Card, 1994), 3) the pretectal area (Mikkelsen and Vrang, 1994) and 4) serotonergic neurons from the nucleus raphe (Moore et al., 1978; Steinbusch, 1981). 5) In addition, the paraventricular thalamic nucleus projects to the entire SCN (Moga et al., 1995).

The SCN project to 1) the hypothalamic subparaventricular zone dorsal to the SCN, 2) the preoptic area, retrochiasmatic area, dorsomedial nucleus and ventral tuberal area of the hypothalamus, 3) the paraventricular thalamic nucleus, 4) the lateral septal nucleus and 5) the lateral geniculate nucleus. However, a major portion of the neurons in the SCN project intrinsically within the SCN, both ipsi-and contralaterally (for reviews, see Watts, 1991; Buijs, 1996).

Neuronal circadian activity in the SCN

The SCN neurons exhibit a robust spontaneous activity, which in both nocturnal and diurnal animals is highest during the subjective light phase of the day peaking near ZT 7 (light onset at ZT 0, light offset at ZT 12), and low during the dark hours with a trough near ZT 19 (Gillette, 1991). This daily oscillation of the spontaneous firing frequency has been well established from *in vivo* (Inouye and Kawamura, 1979; Sato and Kawamura, 1984; Kurumiya and Kawamura, 1988) and *in vitro* extracellular recordings (Green and Gillette, 1982; Groos and Hendriks, 1982; Shibata et al., 1982; Bos and Mirmiran, 1990). The rhythmic firing is converted to a major output from the SCN to other brain regions (Inouye and Kawamura, 1979), and is translated into synchronization of the animal's metabolic, behavioral and hormonal activity to the external light-dark cycle (for reviews, see Hastings et al., 1998; van Esseveldt et al., 2000). Putative neurotransmitters and modulators, such as amino acids, peptides and nitric oxide (NO), have been shown to shift the phase of the SCN rhythm (which is primarily set by light inputs in the living animal) if applied *in vitro*. For instance, glutamate pulses administered to brain slices containing the SCN either phase advance or delay the phase of the SCN firing rhythm, dependent upon the time in the circadian phase at which they are applied (Ding et al., 1994). Recording the endogenous neuronal activity in slice preparations provides a powerful tool to examine the effects of different compounds on the circadian properties in the SCN.

'Clock genes'

The mechanisms underlying the endogenous activity in SCN neurons are not clear. During recent years a plethora of genes that are crucial for maintaining circadian rhythmicity, i. e. 'clock genes', have been discovered and cloned in many organisms, such as prokaryotes, higher plants, fungi, mollusks, invertebrates, insects and vertebrates of different classes (for review, see van Esseveldt et al., 2000). The first was the *period (per)* gene found in Drosophila (Bargiello et al., 1984; Reddy et al., 1984). The *Clock* gene in mouse was the first identified circadian mutation detected by mutagenesis studies in mammals (Vitaterna et al., 1994). Characteristic for many of the proteins encoded by 'clock genes' is a particular protein-protein binding motif, which seems to be highly conserved (Kay, 1997). To be identified as a 'clock gene' the gene has to fulfill certain criteria: 1) when the gene is absent the

organism becomes arrhythmic, 2) the expression of the protein encoded by the gene has a circadian rhythm, 3) the rhythm in protein expression can be phase-shifted by *Zeitgebers*, 4) manipulation of the protein levels changes the phase of the rhythm (for review, see van Esseveldt et al., 2000). In mammals, 'clock gene' knockout mice have been used to study rhythmic properties of dispersed SCN neurons, which contain individual oscillators (Welsh et al., 1995; Liu et al., 1997; Herzog et al., 1998; Honma et al., 1998).

The synaptic network

Trafficking of membrane molecules dependent on circadian variations in expression of the clock gene products may be a mechanism underlying SCN rhythmicity (for review, see Hastings and Maywood, 2000). This could result in circadian variations in neuronal membrane properties and, consequently, affect the activity of individual neurons. In basal retinal cells of the marine mollusk *Bulla gouldiana*, variations in membrane conductance cause daily fluctuations in the membrane potential (Michel et al., 1993). In SCN neurons differences between the subjective day and night have been reported in membrane potential, input resistance (de Jeu et al., 1998) and holding current of SCN neurons (Jiang et al., 1997). Nevertheless, the role of synaptic interactions for generation of rhythms in SCN neurons remains to be clarified. The importance of synaptic activity in this context has been questioned, because circadian rhythms have been shown to be resistant to short-term treatment in explants and slices (Earnest et al., 1991; Shibata and Moore, 1993), and long-term treatment *in vivo* (Schwartz et al., 1987) with the sodium channel blocker tetrodotoxin (TTX;. However, two recent studies indicate that action potential-dependent synaptic communication is important for synchronizing circadian rhythms in individual SCN neurons. Shirakawa et al. (2000) reported that synchronized circadian rhythms of cultured SCN neurons could be demonstrated only when the neurons formed synaptic connections. In addition, Honma et al. (2000) found that long-term treatment with TTX desynchronized circadian firing rhythms in pairs of cultured SCN neurons.

The SCN, sleep and rhythm disorders

The American pilot Charles A. Lindbergh was the first person to cross the Atlantic Ocean in his airplane *The Spirit of St. Louis*. In his book with the same name

he described the 33,5 h long flight from New York to Paris in 1927. A great difficulty during the flight was to withstand the severe sleepiness, because Lindbergh had not slept the night before the departure. Still, he took off in his aircraft early in the morning May 20th from Roosevelt Field outside New York City. In the late afternoon, after he had been in the air for nine hours, he wrote: "Sleep is winning. My whole body argues dully that nothing, nothing life can attain, is quite so desirable as sleep. My mind is losing resolution and control". After struggling for many more hours to resist falling asleep, Lindbergh gradually became less tired and at dawn May 21st he reported: "I'm wide awake". At that time, he had been maneuvering *The Spirit of St. Louis* for more than 24 h (Lindberg, 1953). His story well illustrates that a strong homeostatic drive for sleep accumulates after sleep deprivation, but it also demonstrates that a circadian component is involved in the sleep-wake rhythm, which, in spite of being severely sleep deprived, made Lindbergh more awake again at dawn.

Recent studies have demonstrated that the circadian influence from the SCN provides a substantial, if not the major, contribution to the origin of sleep-wake rhythms (for review, see Lavie, 2001). Results from early experiments performed by Juergen Aschoff and Rütger Wever suggested that the average time period of daily endogenous cycles in humans are closer to 25 rather than 24 h when 'free-running' in an environment without time cues (for review, see Aschoff, 1984). A number of more recent studies of the circadian sleep-wake rhythm have shown that the periodicity may be very close to 24 h, with only a few minutes deviation (for review, see Lavie, 2001). For instance, Czeisler et al. (1999) used a 'forced desynchrony' experimental protocol on human subjects, which showed that the circadian rhythm was unaffected by the imposed short (20 h) or long (28 h) LD cycle. Moreover, unlike the results obtained by Aschoff and Wever that showed a 25 h long circadian cycle, Czeisler et al. (1999) found that the average periods of the rhythms in core temperature and secretion of cortisol as well as melatonin were 24.15 h ± 0.04 h for the 'short' day and 24.17 h ± 0.03 h for the 'long' day (Czeisler et al., 1999). Differences between the studies in the experimental conditions may explain the contradictory results. The studies by Aschoff and Wever were performed under constant indoor light conditions, whereas Czeisler et al. maintained constant low light levels during the scheduled wake episodes. The longer free running period may have been a result of light influence on the clock, since it has been demonstrated that light of low intensity (180 lux) can affect and phase shift human circadian rhythms (Boivin et al., 1994; Boivin et al., 1996). This

effect may be mediated by a change in the circadian release of the pineal hormone melatonin, which is inhibited by light via the SCN. Light of low intensity can suppress the release of melatonin (Arendt, 1993).

Surprisingly, most of the studies of the circadian sleep-wake rhythm consistently indicate that the propensity to sleep has a minimum during the early evening, before the opening of a 'sleep gate', which leads to a rapid accumulation of the sleep drive that peaks in the early morning (for review, see Lavie, 2001). The same phenomena was also observed in sleep deprivation studies performed by Åkerstedt et al. (1979), who found that the sleep pressure exhibited a 24 h rhythm with a peak of alertness during the afternoon and a peak in sleepiness during the night. In addition, Aeschbach et al. (1997) investigated the EEG activities during wakefulness and concluded that EEG power density, indicating sleep pressure, had a trough in the evening. This pattern may be linked to the circadian rhythm in body temperature. Isolation studies have demonstrated that the circadian pattern is highly correlated to the phase of the rectal temperature rhythm; i. e. the peak in body temperature is associated with a low sleep propensity (Åkerstedt and Folkard, 1993).

The circadian regulation of the sleep-wake rhythm is not clear. In one study, performed by Edgar et al. (1993), the circadian component of the sleep-wake regulation was investigated after SCN lesions in squirrel monkeys. The lesioned animals lost their circadian rhythms in sleep-wakefulness, NREM and REM sleep stages, drinking behavior and brain temperature. In addition, the time of wakefulness during the subjective day was markedly reduced. The investigators' interpretation was that the SCN activity promotes wakefulness during the subjective day (Edgar et al., 1993), thus opposing the homeostatic sleep propensity by generating a drive for wakefulness (for review, see Lavie, 2001). Why this type of regulation is beneficial to the organism remains unclear. However, Dijk and Czeisler (1994) proposed that by positioning the circadian peak of alertness right before time of sleep, and the peak for sleep propensity before awakening, the decrease in sleep propensity with accumulated sleep is counteracted, thus promoting an 8 h period of sleep during the night. Likewise, a 16 h period of wakefulness during the day is consolidated. Experiments have indeed demonstrated that when sleep is postponed from the evening to noon the following day, the time of sleeping is shortened (for review, see Åkerstedt, 1988). The early nocturnal release of melatonin may be closely linked to this regulation of the sleep-wake cycle by inhibiting the wakefulness-producing machinery generated by the

SCN, and by such a mechanism allow a smooth transition from wakefulness to sleep (Lavie, 1997).

Circadian rhythm disorders generally result in alterations of the homeostatic regulation of physiological and hormonal functions. This is a major problem for shift-workers who suffer from insomnia, fatigue, reduced concentration and performance capacity, irritability and gastrointestinal problems. These symptoms are also evident during so-called 'jet-lag', a condition that occurs when the internal circadian system is out of phase with the external daily cycle (for review, see Redfern et al., 1994). Because entrainment of the circadian clock to a new environmental cycle requires a few days of time, rapid travelling across time zones often causes 'jet-lag'. Sleep displacement experiments have shown that sleep inversion results in an increase of wakefulness, sleep fragmentation and a shift of REM sleep from the later to the early sleep period (for review, see Lavie, 2001). Disturbances in sleep-wake rhythms also occur in major depression (Brunello et al., 2000).

Immune responses and cytokines: effects on sleep regulation

Since trypanosomes do not invade the cells, molecules released as a result of interactions between the trypanosomes and the host's immune response to the infection may cause the dysfunctions in the nervous system. During the infection there is a marked release of pro-inflammatory cytokines such as interleukin (IL)-1, -2, -4 and -10, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), as well as NO (Quan et al., 1999; Rhind and Shek, 1999) and prostaglandins (Kubata et al., 2000). Radomski et al. (1994) found a positive correlation between plasma levels of cytokines, in particular IFN-γ, and the severity of the disease, in patients with trypanosomiasis. IFN-γ was first described as an anti-viral agent (for review, see Farrar and Schreiber, 1993), and has been demonstrated to operate in synergy with TNF-α (for review, see Paludan, 2000). TNF-α was first isolated from rabbits infected with trypanosomes under the name of cachectin and may inhibit growth of the parasite (Daulouede et al., 2001). Interestingly, IFN-γ may have an opposite, proliferative effect on *T. b. brucei* (Bakhiet et al., 1996), while it inhibits the growth of *T. b. rhodesiense* (Hertz et al., 1998).

Cytokines have been ascribed an important role in sleep induction. Certain cytokines, in particular IL-1, TNF-α and IFN-α, are involved in sleep responses to

infection and induce an ‘acute phase response’, which includes fever, loss of appetite, social withdrawal and excessive sleep. These responses are considered to be beneficial for host defenses (for review, see Krueger et al., 1995). TNF-α and IL-1 induce increases in SWS (Krueger et al., 1994), and IFN-α has been ascribed somnogenic properties in a number of studies (for review, see Krueger et al., 1995). Less is known about the effects of IFN-γ, but it was reported to induce sleep and cause tiredness and lethargy in patients with solid tumors treated with this cytokine (Sriskandan et al., 1986). Prostaglandin D_2 and E_2 are also major sleep promoting substances that induce SWS (Hayaishi and Matsumura, 1995), and NO may be involved in sleep regulation because inhibition of NO synthase inhibits sleep in rats (Kapas et al., 1994).

In contrast to the well-established sleep inducing effects, little is not known about the effects of cytokines on SCN function and circadian rhythms. The last decade’s research in the field of trypanosomiasis has provided data that strongly suggest that this parasitic disease is a disorder with marked disturbances in circadian rhythms. The present studies were undertaken in order to examine whether experimental trypanosome infection affects the neuronal and synaptic function in the SCN, and if trypanosome-related cytokines can alter this activity.

SPECIFIC AIMS

The general aim of this work was to study alterations in the mammalian circadian pacemaker, SCN, in rats affected with experimental African trypanosomiasis, or sleeping sickness. Specifically, we sought to:

1. Examine if the endogenous SCN rhythm in spontaneous firing is altered after trypanosome infection by extracellular recordings of the spontaneous activity of SCN neurons in slice preparations.

2. a) Study the spontaneous postsynaptic inhibitory and excitatory activity within the SCN in order to establish if there are daily variations in frequency or amplitude of synaptic events.

   b) To compare the spontaneous synaptic activity in slices of the SCN from brains of control rats with synaptic activity in slices from brains of rats infected with trypanosomes.

3. Explore the effects of pro-inflammatory cytokines that are involved in the immune responses to trypanosome infections, in particular IFN-γ, on regulation of SCN function.

4. To locate and identify a receptor for IFN-γ in the SCN.

METHODOLOGICAL CONSIDERATIONS

General aspects of the methods used are discussed here with an emphasis on electrophysiological recording techniques applied on the brain slice preparation. Detailed descriptions of the procedures and materials used are provided in the respective papers.

Animals

All animal procedures were conducted under institutional guidelines and with ethical committee approval (Stockholms Norra djurförsöksetiska nämnd, application numbers N133/95, N295/95 and N152/98). Adult male Sprague-Dawley rats (150 g at the day of arrival) from B&K, Sollentuna, Sweden, were used in all studies. Also, for the studies in paper V, male pups were used at each of postnatal day (P) 1, 5, 11, 20 and 50. Adult rats and pregnant females were housed in the animal facilities in the Anatomy building at the Department of Neuroscience, Karolinska Institutet. It has been demonstrated that rats are synchronized to new environmental LD cycles in 5-7 days (Hillegaart and Ahlenius, 1994). In these studies, the animals were entrained in 12:12 h LD cycles at least three weeks before any experiments were performed. The rooms (temperature 22^{o}C, humidity 46-48%) were carefully sealed to prevent light leakage and double doors were used as a 'light lock' for personal entry. The animals had access to food and water *ad libitum*.

Infection with *T. b. brucei*

For experimental trypanosome infections, rats were infected by intraperitoneal injections with pleomorphic *T. b. brucei* (stabilates from the Laboratory of Serology, Institute of Tropical Medicine "Prince Leopold", Antwerp, Belgium). The trypanosome parasitemia in rats is expressed as a waveform with peaks of parasites every 3-4 days. The wave troughs are the result of defense mechanisms of the immune system that kills subpopulations of *T. b. brucei*. Because of the antigenic variation of the coat protein 'variant surface glycoprotein' on the trypanosome in some subpopulations, a proportion of the trypanosome population escapes the immune system actions, proliferates and another relapse of parasites can be observed in the blood (Pays, 1999). The parasitemia was verified by estimating the density of

trypanosomes in thick smears of blood samples obtained from tail bleeds. In the rat model this method is not sensitive enough to detect parasites during the two first waves; therefore parasitemia was determined around day 10 p. i. *T. b. brucei* were found in the blood of most rats in which the parasites had been injected. The rats typically did not display signs of disease during the first 30 days after infection. After 30-35 days p. i. the rats started to lose weight, and by day 40-45 p. i. weight loss was marked. Sprague-Dawley rats infected with the presently used stabilate of the parasites generally do not show severe signs of disease until they become moribund and rapidly die 55-60 days p. i. In the present studies, rats were sacrificed between 36-45 days p. i.

The SCN brain slice and electrophysiology

The brain slice technique enables studies of rhythmic properties that are endogenous to the pacemaker, i. e. functional properties generated in the SCN itself. Also, the isolation of the nuclei facilitates studies of the SCN largely without active afferent inputs from other regions in the brain. In 1979, Inouye and Kawamura isolated the SCN *in situ* in rats and implanted electrodes that measured the electrical activity both outside and within the SCN. They demonstrated that the SCN generate a circadian rhythm of electrical activity that was high during the subjective day and low during the subjective night. This activity was shown to affect the activity of other brain regions via efferent projections (Inouye and Kawamura, 1979). While using slightly different experimental designs, three laboratories independently reported results in 1982 obtained from extracellular single unit recordings from coronal rat brain slices containing the bilateral SCN. These studies showed that the SCN maintain a circadian oscillation of spontaneous neuronal activity *in vitro* that was similar to the rhythm *in vivo*, if plotted as average frequencies over time during a 24 h cycle. The average frequency reached a peak in the middle of the subjective day and a trough during the subjective night (Green and Gillette, 1982; Groos and Hendriks, 1982; Shibata et al., 1982). The same pattern of oscillation was subsequently demonstrated by recordings of neuronal multiple unit activity *in vivo* in the SCN from Siberian chipmunks and guinea pigs (Sato and Kawamura, 1984; Kurumiya and Kawamura, 1988), which are diurnal animals in contrast to the nocturnal rat. Extracellular recordings of the SCN brain slice has since been used in a number of studies in order to investigate the circadian phase of the slice donor, and to examine direct effects of

various neurotransmitters and other compounds on the rhythmic electrical SCN activity. In the present work, the slice preparation was used to examine if the rhythm in activity of SCN neurons was affected by trypanosome infections, and if cytokines can cause changes in this rhythm. Also, it was used for studies of the spontaneous synaptic activity of SCN neurons.

Slice preparation

If kept under optimized bath conditions, the slice can remain viable for several days. A quick and very gentle dissection procedure is essential for preparing a slice of high quality. After dissection, the slice has to be kept in a buffered and balanced salt solution containing glucose and oxygen (Suter et al., 1999).

Rats were sacrificed by decapitation under halothane anesthesia. The brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) solution saturated with oxygen. A small block was dissected from which horizontal slices (500 or 350 μm thick) of the hypothalamus, including parts of the optic nerve and chiasm, were cut with a vibratome. The level of the cut was standardized from the ventral surface of the optic chiasm. The dissection procedure was performed within 6 min from the time of decapitation. Slices were placed dorsal side up in a perfusion chamber and bathed in oxygenated ACSF solution. The size of the chamber was small (volume 500 μl), accomodating only one slice (approximately 0.5 x 0.4 cm), in order to maintain as even temperature as possible. The temperature of the ACSF in the chamber was held constant at 35-35.5^{o}C, and the ACSF had a flow rate of 2-3 ml/min. The slices were held down with nylon threads attached to a platinum ring and were left to stabilize for 1-2 h before recordings.

Extracellular single unit recordings

Extracellular recordings of SCN neurons *in vitro* were used to examine neuronal spiking behavior and determine slice viability, and to analyze the intrinsic SCN electrical rhythm. Spontaneous single unit activity was recorded blindly with a glass microelectrode (impedance 2-4 MΩ) containing 1% NaCl. The electrode was advanced with a hydraulic micromanipulator in the slice. The SCN were visually located in a light microscope as two oval-shaped regions immediately above the optic chiasm. Neuronal activity was identified by the sound of spiking from an audiomonitor. Only cells with a regular firing rate were recorded, each one for at least

3 min. Spikes were analyzed with a wave discriminator and the signals were amplified and digitally converted before storage and analysis with Spike2 software (Cambridge Electronic Design Limited, Cambridge, UK). The average frequency of firing from each neuron was calculated. The individual firing frequencies of the sampled neurons were averaged into 2 h intervals using a 1 h lag and plotted over circadian/*Zeitgeber* time in order to study the phase of activity.

Whole cell patch clamp recording

Intracellular recordings in the SCN are laborious because of the small size of the neurons (~8-10 μm in diameter). Traditionally, sharp electrodes have been used for recording intracellular events in neurons (Suter et al., 1999). One of the disadvantages with this technique is the high resistance of the electrodes, due to the small tip diameter, which typically introduces increased recording noise. Because of difficulties impaling SCN neurons in slices with sharp electrodes, and high noise levels due to the small currents recorded in these neurons, the patch clamp recording technique was chosen for studying intracellular synaptic events in the SCN. A substantial part of the present work was devoted to setting up the instrumentation and developing the techniques for implementation of this method on the SCN slice.

The gigaseal patch and whole cell technique (Hamill et al., 1981) was developed by Erwin Neher and Bert Sakmann. It was originally designed for recording currents passing through single ion channels in the cell membrane. To patch clamp a cell, a glass electrode with a tip diameter of approximately 1 μm is filled with intracellular solution and placed against the cell membrane. By applying suction inside the electrode, a seal is formed between the glass and the membrane having very high resistance, i. e in the giga-ohm range (cell-attached mode), whereby currents passing through single ion channels can be studied. By increasing the suction or giving an electrical ‘buzz’, the membrane is ruptured and molecules are exchanged between the electrode and the cell (whole-cell mode). The electrode solution is exchanged with the cytoplasm and the ion concentrations of the intracellular solution therefore match physiological ion concentrations inside the cell. Depending on the nature of the experiment, specific channel blockers may be added to the intracellular solution. The recording time is typically limited because of ‘run down’ of second messengers like adenosine triphosphate and calcium in the cell. However, in the presently used

protocol, patched cells were often recorded between one and two hours without noticeable ‘run down’ effects.

Recordings were performed in whole cell configuration or perforated patch mode. In the latter case, access to the whole cell is given by adding a pore-forming antibiotic (amphotericin B) in the tip of the electrode, instead of membrane rupture by suction. Spontaneous and evoked postsynaptic currents and potentials were recorded with an Axoclamp 2B amplifier (Axon Instruments, Inc., Foster City, CA, USA) in conjunction with a DC amplifier/filter (FLA-1, Cygnus Technology, Inc., Delaware, PA, USA). The traces were stored on a personal computer with a digital interface (Digidata 1200, Axon Instruments). Data acquisition and analysis were performed using pClamp7 & 8 software (Axon Instruments). The recording pipettes contained potassium gluconate instead of potassium chloride, which resulted in a more successful patching of SCN neurons. Also, the equilibrium potential for Cl^- ions became more negative, resulting in making inhibitory postsynaptic events more easily distinguishable from excitatory events. For perforated patch, the basic electrode solution was used to fill the pipette tips, and the rest was backfilled with amphotericin B (250 μg/ml) dissolved in electrode solution. Electrodes had impedances of 6-9 MΩ. The tissue was approached ‘blindly’ (i. e. cell bodies could not been seen by the naked eye). The electrodes were advanced from the dorsal side into the ventrolateral regions of the SCN close to the optic chiasm, with slightly positive internal pressure to avoid accidental occlusion of the tips. Cell approach and giga-seal formation were achieved in voltage clamp mode by monitoring the changes in current responses to voltage pulses. Membrane rupture was performed by syringe suction. With amphotericin B, perforation was achieved 5-45 min after sealing. The cells were allowed to stabilize for at least 4-5 min before voltage or current clamp recordings. Cells were held at –60 mV. Channel blockers, receptor antagonists and other drugs were solubilized in dimethylsulfoxide or water, mixed in ACSF solution and superfused at the same flow rate and temperature as the normal perfusion medium. Maximal effects of the drugs were observed within 10 minutes.

For analysis of spontaneous synaptic potentials and currents, software MiniAnalysis (demo version, Synaptosoft, Inc., Leonia, NJ, USA) was used as a tool to count the number and amplitudes of current or potential peaks of spontaneous activity. The software parameters were adjusted to choose synaptic events (for details,

see paper II). The program could be calibrated to set the noise level and the minimum detection limit of the amplitudes of excitatory and inhibitory postsynaptic events was defined as double the value of the noise level for a given recording. Each event was visually checked for conformance with the criteria for a synaptic event and then accepted or rejected. Events were chosen manually if missed by the program. For measuring amplitudes, each event was carefully checked so that the baselines and peak points were accurate. The mean frequencies and amplitudes of synaptic potentials and currents from recordings of each neuron were calculated during a one-minute interval. When comparing synaptic activity between subjective day and night, the records for analysis were selected blindly and randomly between the subjective day and night-group.

Stimulation technique

Electrical stimulation was used for stimulating fibers in the slice to study synaptic responses. To allow local stimulation, two thin platinum wires insulated with Teflon were used. The wires were placed 50-100 μm apart. The electrodes were connected to an optically isolated stimulator (A-M Systems, Carlsborg, WA, USA) and placed on the SCN prior to placement of the recording electrode. Current pulses were applied in pulses and increased in amplitude until reaching response threshold, which generally ranged between 1.2 and 1.6 μA. Timing for the stimulator, amplifier and digital interface was provided by a programmable digital pulse generator (Master-8, A.M.P.I., Jerusalem, Israel).

An alternative to electrical stimulation is glutamate microdrop stimulation and photolysis of caged glutamate. In the latter technique, a flash of ultraviolet light is used to release photolabile glutamate molecules that in turn evoke postsynaptic responses. The light beam can be directed to very precise regions (Suter et al., 1999). However, the high specificity of the photolysis method was not required for the stimulation experiments performed in this study and was therefore not used.

Statistics

GraphPad Prism (GraphPad Software, San Diego, USA) and Excel (Microsoft Corp., Redmond, WA, USA) software were used for the statistical analyses. Significances of mean differences were determined with Student's *t* test, one-way

analysis of variance (ANOVA) and two-way ANOVA followed by Duncan or Bonferroni's post-hoc tests. Values were expressed as means ± Standard Error of the Mean (SEM) or proportions of means ± SEM or percent of control mean value. Time of peaks (acrophases) in mean firing rate of SCN neurons was determined with nonlinear regression analysis.

Immunotechniques

Detailed information of the different antibodies used for immunodetection is provided in the respective papers.

Immunohistochemistry

Immunohistochemical detection on SCN sections was used for location studies of proteins during different ZTs and postnatal days, and for densitometric analyses. Under chloral hydrate anesthesia, the rats were perfused with 4% buffered paraformaldehyde and the brains were postfixed and cryoprotected in 15-20% sucrose. Coronal sections with a thickness of 10-12 μm containing the SCN were cut. The sections were incubated with antibodies and mounted for immunofluorescence studies.

Western blotting

Most of the antibodies used for immunohistochemistry were also used for Western blotting, which allows quantitative analysis of protein expression. Explant dissections (the explants contained no other nuclei than the SCN) or punch-outs of the SCN were carried out under magnification on 500 μm thick vibratome-cut hypothalamic slices. Tissues were lysed, the amounts of proteins in lysates were estimated and the lysates were prepared for resolving proteins on sodium dodecyl sulphate or tris-glycine-polyacrylamide gels. The proteins were transferred to nitrocellulose or poly-vinyl difluoride membranes using a wet blot system and blocked with bovine serum albumin in combination with Tween 20. Blots were probed with antibodies and labeled proteins were detected using chemiluminescence (ECL^{TM} detection reagents, Amersham, Little Chalfont, UK).

Molecular biology

Reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot hybridization

The IFN-γ receptor has a ligand binding subunit, the α-chain, and a subunit required for intracellular signalling, the β-chain (Bach et al., 1995). RT-PCR was used to detect messenger ribonucleic acids (mRNAs) of the α-subunit of the IFN-γR in the SCN. To confirm the molecular identity of the amplified fragment, Southern blot hybridization was performed. The SCN were dissected as described for immunoblotting (see previous page). RNA was extracted with guanidium acid thiocyanate and phenol-chloroform, reverse transcribed and the resultant complementary deoxyribonucleic acids (cDNAs) were amplified in a PCR reaction mixture. The reaction parameters and specific primer sequences for β-actin (mouse), sense IFN-γRα (rat) and antisense IFN-γRα (rat) are described in papers IV and V. The PCR products were resolved by agarose gel electrophoresis and detected by ethidium bromide staining. The fragments were blotted over to Hydrobond N+ membranes (Amersham) overnight. The membranes were hybridized with a fluorescent isothyocyanate (FITC) labeled probe, washed and incubated with a horseradish peroxidase (HRP) labeled rabbit anti-FITC antibody (Amersham). Detection was performed using chemiluminescence (ECL^{TM} detection reagents, Amersham).

Cloning and sequencing the IFN-γR gene

To compare the IFN-γR in the SCN with that of the immune system, and to determine its identity, the IFN-γRα transcript was cloned and sequenced. IFN-γRα sequences were amplified by PCR, electrophoresed, purified using β-agarase digestion and ligated into a vector. *E. coli* XL1 blue competent cells were transformed with the ligation product and positive colonies selected. Plasmids were purified and the inserted fragments were amplified with single-strand PCR and sequenced in a reaction containing fluorescent-labeled nucleotides. The SCN cDNA sequence from the cloned transcript was compared to the rat IFN-γRα sequence (derived from GenBank, accession number U682272).

Tract tracing

To check the viability of the retinal afferents to the SCN after trypanosome infection, cholera toxin subunit B conjugated to HRP was used for tract tracing. The rats were injected in the vitreous body of the eye and perfused after 48 h with 1,25% glutaraldehyde and 1% paraformaldehyde. Sections (40 μm) through the optic nerve were cut out, incubated for HRP histochemistry, mounted and studied under bright-field illumination in the microscope.

RESULTS AND DISCUSSION

1. Effects of trypanosome infection on the endogenous SCN rhythm (paper I).

To answer the question whether intrinsic activity of SCN neurons is functionally disturbed after infection with trypanosomes, extracellular single unit activity was recorded in slice preparations containing the SCN. Neurons were highly active in slices from both control and infected rats. Thus, no obvious difference in the number of active neurons was detected in trypanosome slices. Recordings obtained during the day between ZT 5-12 showed a peak in firing activity at ZT 7.1 in slices from control rats, consistent with previous reports (Gillette, 1991). However, in none of the slices from infected rats was a peak of activity observed between ZT 5-12. To determine if the peak was abolished or phase shifted, continuous 24 h recordings of the extracellular activity were performed. These experiments revealed that the average peak of activity was phase advanced by approximately 3 h. The peak was small, resulting in a decreased total mean firing frequency of the neurons throughout the entire recording period. Still, the base line activity before the peak was the same in control and infected rats and the action potentials had a normal amplitude and duration, indicating that passive membrane properties and neuronal viability are preserved in trypanosome-infected rats.

The change in the firing rate demonstrates that a functional disturbance in the pacemaker system occurs in experimental trypanosomiasis, which probably alters the output from the SCN to other brain regions. Inouye and Kawamura (1979) demonstrated that brain activity in regions outside the hypothalamus show daily oscillations that are in antiphase with the SCN oscillation. These rhythms were abolished after isolating hypothalamic ‘islands’ containing the SCN from other brain regions by cuts with a Halasz microknife, but the rhythm remained intact in the hypothalamic ‘island’ near the SCN. Thus, the electrical signals from SCN efferents seem to carry timing information to other brain regions (Gillette, 1991). Because of the disturbance in the SCN rhythm, this information may be altered in trypanosome-infected rats. The abnormal firing rhythm indicates either a dysfunction within the SCN or a disturbance in the entraining pathways, in which the retinal afferents play a major role. Trypanosomes do not locate to the retina, but to the choroid, where they may cause release of factors impairing the signal

transduction from retinal ganglion cells. However, the RHT was examined with tracing studies that showed an intact innervation and transport of the tracer to the SCN. Thus, it does not seem likely that structural changes in the RHT can explain the observed alteration in the SCN rhythm.

The findings of a disturbed pattern of spontaneous firing activity show that infections with *T. b. brucei* in rats cause a disturbance of the functional rhythmic properties of SCN neurons, which may result in an alteration of the SCN output and consequently a dysregulation in the circadian control of physiological, hormonal and behavioral rhythms.

2. Spontaneous inhibitory and excitatory postsynaptic activity in the SCN and effects of trypanosome-infection (paper II and III).

To examine the mechanisms behind the observed alterations in the SCN rhythm after infection with *T. b. brucei*, the spontaneous postsynaptic activity was studied in the ventrolateral retino-recipient region of the SCN. Although both inhibitory γ-aminobutyric acid (GABA)-ergic and excitatory glutamatergic postsynaptic activity have been recorded within the SCN (Kim and Dudek, 1991; Kim and Dudek, 1992; Bouskila and Dudek, 1993; Jiang et al., 1997; Burgoon and Boulant, 1998), circadian fluctuations in spontaneous postsynaptic inhibitory or excitatory activity have not been previously investigated, which, in turn, may be correlated to the circadian variations in firing activity. Therefore synaptic inhibitory and excitatory events in control rats were analyzed and compared between the subjective day and night. For this purpose, patch clamp recordings were carried out in slice preparations.

The spontaneous postsynaptic activity in the SCN

With software designed for this purpose, the frequency and amplitude of the synaptic events were selected, analyzed and evaluated. Strecker et al. (1997) proposed that the intrinsic synaptic network in the SCN is mainly GABAergic with no or little glutamatergic activity, although daily variations in extracellular concentrations of glutamate have been reported in the SCN (Rea et al., 1993; Shinohara et al., 1998). The ventrolateral SCN neurons were found to exhibit a robust and intense spontaneous synaptic activity, with both substantial inhibitory

and excitatory activity. The postsynaptic inhibitory events were blocked with the $GABA_A$ receptor antagonist bicuculline. This finding is consistent with previous reports demonstrating the involvement of the $GABA_A$ receptor in SCN postsynaptic activity (Kim and Dudek, 1992; Strecker et al., 1997). The presence of the $GABA_A$ receptor was demonstrated by immunoblotting using an anti-$GABA_A$ antibody, which showed a protein band with a size of 55 kilo Dalton.

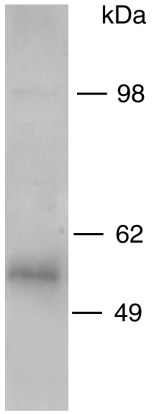

Figure 4*: Immunoblot of SCN whole extract probed with polyclonal rabbit anti-GABA receptor type A subunit α3 antibodies (1:100; Alomone labs, Jerusalem, Israel; not previously published).*

Most of the excitatory activity was blocked by the application of the glutamate α-amino-hydroxy-5-methylisoxazole-propionic acid (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), but not by the N-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonopentanoic acid (AP-5). Thus, the spontaneous excitatory activity seems to be mainly AMPA receptor-dependent. In order to remove the Mg^{2+} block that inactivates NMDA receptors at –60 mV, excitatory spontaneous synaptic currents (EPSCs) were recorded in Mg^{2+} -free ACSF. No significant difference in the amplitude or the frequency of EPSCs could be observed, lending further support to the involvement of AMPA receptors in spontaneous postsynaptic activity.

Slices sampled during the subjective night seemed at first glance to display more inhibitory activity than slices sampled during the subjective day. Surprisingly, the analysis did not reveal any significant differences in either frequency or amplitude of inhibitory events between day and night. Most neurons

in the SCN have been reported to utilize GABA as a neurotransmitter (Okamura et al., 1989; Moore and Speh, 1993; Buijs et al., 1995), and it has been proposed that GABA may play an important role in synchronizing rhythms in SCN neurons (Liu and Reppert, 2000; Shirakawa et al., 2000). In addition, the content of GABA (Aguilar-Roblero et al., 1993; Trachsel et al., 1996; Ikeda et al., 1997) and the expression of mRNA for its synthesizing enzyme, glutamate decarboxylase (GAD) may show daily variations. However, the timing of the peak expression of mRNA for GAD differs between reports (Cagampang et al., 1996; Huhman et al., 1996), and the rhythmic mRNA expression of GAD was not an intrinsic property of the neurons but dependent on light (Huhman et al., 1999). Because no significant difference in synaptic inhibitory activity was detected between the subjective day and night, the results from this study do not support a circadian release of GABA at synapses of the ventrolateral SCN.

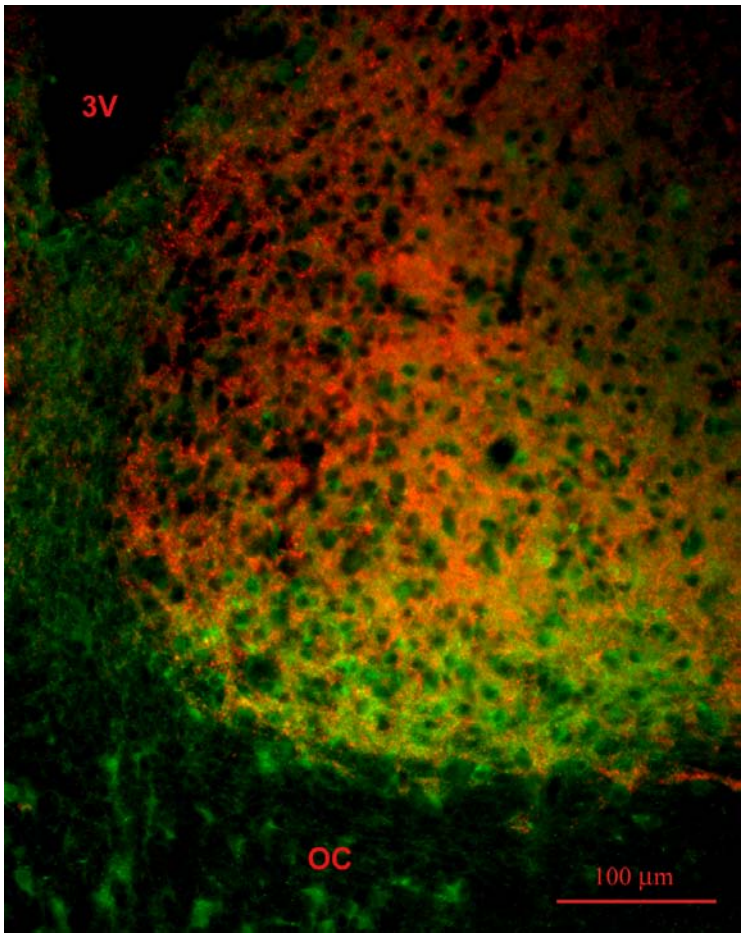

***Figure 4**: A photograph showing immunopositivity to glutamate decarboxylase (GAD) and glutamate α-amino-hydroxy-5-methylisoxazole-propionic acid (AMPA) receptor (GluR) subunits 2 and 3 in a coronal section of the SCN. The section is probed with polyclonal goat anti-GAD-65/67 (1:100; Santa Cruz Techonlogy, Santa Cruz, CA, USA) incubated with Cy™3-conjugated donkey anti-goat IgG (red; 1:500; Jackson ImmunoResearch, West Grove, PA, USA); and polyclonal rabbit anti-GluR subunit 2/3 (1:50; Chemicon International Inc., Temecula, CA, USA) incubated with Cy™2-conjugated donkey anti-rabbit IgG (green; 1:500; Jackson ImmunoResearch, West Grove, PA, USA). The photograph shows one SCN nucleus. The GAD protein is distributed throughout the entire nucleus, while the AMPA receptor subunit protein is located only in the ventrolateral region. OC, optic chiasm; 3V, third ventricle. (Not previously published).*

In contrast to the GABAergic synaptic release, the spontaneous glutamatergic synaptic activity differed between the subjective day and night. The frequency of the excitatory synaptic events was markedly higher during the subjective day as compared to the subjective night. Excitatory synaptic responses could be evoked by contralateral and ipsilateral focal stimulation of the SCN.

These data suggest that there is a substantial glutamatergic influence on the ventral region of the SCN, which may be either intrinsic or originate from regions outside the SCN. Although Strecker et al. (1997) could not evoke excitatory responses when stimulating the SCN by photolysis of caged glutamate, spontaneous excitatory activity has been recorded previously in the SCN (Kim and Dudek, 1991; Jiang et al., 1997). In addition, glutamatergic axon terminals have been identified in the ventrolateral SCN that were not of retinal origin (Castel et al., 1993; de Vries et al., 1993), and ultrastructural analyses have demonstrated that a substantial proportion of the SCN neurons are non-GABAergic (Castel and Morris, 2000). Daily variations in extracellular concentrations of glutamate in the SCN have been reported, although these concentrations increased during the subjective night (Rea et al., 1993; Shinohara et al., 1998), which contrasts with the results presented here. The reason for this is not clear, but the glutamatergic synaptic network observed in the ventrolateral SCN may be dissociated from excitatory networks in other SCN subdivisions. The finding that CNQX administered in the ACSF had an antagonistic effect on the inhibitory activity in the SCN strengthens this hypothesis. Because the spontaneous inhibitory activity did not show daily fluctuations, the CNQX effect may be exerted on a network separated from that in the ventrolateral SCN. Alternatively, the diurnal increase in excitatory synaptic activity in the ventral region may depend on increased protein expression of AMPA receptors. A diurnal fluctuation of the protein expression of the glutamate AMPA receptor (GluR) 2/3 subunit has been demonstrated in Syrian hamsters with an increase in expression during the subjective day (Chambille, 1999).

The role of the glutamate-mediated synaptic activity can at present only be speculated upon. An increased excitability of SCN neurons during the day would result in a higher frequency of neuronal activity, as observed in the SCN during the subjective day. Indeed, in this study we showed that increased strength of stimulation occasionally resulted in an action potential evoked from the excitatory postsynaptic potential. Somewhat paradoxically, an increase in excitability of SCN neurons during the subjective day was suggested in a previous report to be caused by GABA. Wagner et al. (1997) found that variations in the chloride equilibrium potential could shift an inhibitory effect of GABA during the night to an excitatory effect during the day in the SCN. This would result in a net increase of excitation and therefore the neurons would be more active during the day. However, other

investigators did not find any difference in the effect of GABA on SCN activity during the subjective day compared to subjective night (Gribkoff et al., 1999). A putative excitatory role of GABA therefore remains to be established.

The spontaneous postsynaptic activity in the SCN is altered by trypanosome infection

The information obtained on spontaneous postsynaptic activity in the SCN of control rats was used to design studies of spontaneous synaptic activity in SCN slices from rats infected with *T. b. brucei*. The spontaneous synaptic inhibitory activity in brain slices from infected rats did not significantly differ from controls, but a significant decrease in the frequency of excitatory events was found. A reduced activity in excitatory synapses may explain the decreased firing activity and smaller peaks observed in trypanosome-infected rats. The mechanism explaining this reduction may be postsynaptic in origin. Quantitative evaluations of three different glutamate receptor subunits, GluR1, GluR2/3 and N-methyl-D-aspartate receptor channel subunit ζ1 (NMDAR1), were made by immunostainings of SCN sections, densitometric analyses and Western blots. Immunopositivity to GluR2/3 and NMDAR1 was found in the ventrolateral region of the SCN, while the GluR1 protein was located also in other parts of the nuclei. After infection with *T. b. brucei* a significant decrease of the expression of the GluR2/3 and NMDAR1 proteins was detected, whereas the amount of the GluR1 protein did not significantly differ. These findings indicate that glutamate receptors are downregulated as a result of trypanosome-infection, which may affect neuronal signaling and, thus, be mirrored in a reduction of excitatory synaptic activity in the ventral SCN. Release of various factors induced by trypanosome infections may affect the expression of glutamate receptors. In cultures of hippocampal neurons, long-term exposure of IFN-γ causes a decrease in the protein expression of GluR1 (Vikman et al., 2001).

Taken together, these findings suggest that the SCN contain not only GABAergic neurons, but also a substantial glutamatergic network in the ventrolateral subdivisions, in which the activity increases during the subjective day. The daily fluctuations of the excitatory synaptic actions may underlie the rhythmic neuronal firing in the SCN. In rats infected with *T. b. brucei*, the frequency of spontaneous glutamatergic postsynaptic events was decreased and the

level of protein expression of GluR2/3 and NMDAR1 was significantly lower. These changes may underlie the dysregulation in the rhythmic SCN firing that occurs during trypanosome infection.

3. Effects of IFN-γ and TNF-α in combination with bacterial lipopolysaccharide (LPS) on the SCN firing rhythm (paper V).

Among the pro-inflammatory cytokines released during trypanosome infection are IFN-γ and TNF-α, which act in synergy and have effects that are amplified by LPS (Paludan, 2000). IFN-γ and TNF-α were applied in combination with LPS on SCN slices in order to explore their effects on rhythmic neuronal activity. Slices were prepared during the late subjective day, the cytokines were administered for approximately 1 h in the subjective evening and the rhythm in spontaneous firing of SCN neurons was recorded extracellularly between ZT 2 and 11 the following day. The rhythm in untreated control slices was also recorded the following day and a significant average peak of activity occurred around ZT 7. In three slices treated with IFN-γ, TNF-α and LPS, and in three slices treated with IFN-γ and LPS, no significant peaks of activity were detected during the recorded intervals, suggesting that these cytokines affect the SCN rhythm. However, as 24 h recordings were not performed it was not determined if the peaks of neuronal activity were ablated or phase shifted by the cytokines. The number of sampled neurons in treated slices was high and the average baseline activity did not differ from activity in control slices. A general toxic effect (i. e. causing neuronal death) is therefore not likely the reason for the observed changes.

Interactions between the immune and circadian system

The number of circulating immune cells and activation of immune functions are subject to circadian control; also light has an impact on immune responses to antigenic exposure (Roberts, 1995). For instance, the activity of murine natural killer (NK) cells reaches a peak during the early subjective dark hours, and the number of circulating T lymphocytes in humans is high during the night. In addition, the *in vitro* response to IFN-α of murine splenocyte NK cell activity is high during the early subjective day and mid-to late subjective night, and the density of clusters of

differentiation (CD) 3 molecules on human lymphocytes (i. e. immune response to antigen representation) is high during the early morning (for review, see Levi et al., 1991). However, not many studies have examined whether cytokines, released as a result of immune responses, affect the SCN. In a recent study, IFN-γ and IFN-α were proposed to alter the expression of 'clock genes'. When IFNs were administered subcutaneously at ZT 12 in mice, they caused a marked reduction of the mRNA levels of *per* in the SCN and peripheral organs. No effect was observed after administration at ZT 0 (Ohdo et al., 2001). A direct action in the brain was suggested, but it is not clear whether or not these cytokines cross the blood-brain barrier (BBB). It therefore remains to be clarified if, and how, systemically administered cytokines can influence the SCN and rhythmic output.

Cytokines and NO

TNF-α or LPS-activated transcription factor nuclear factor (NF)-κB acts in synergy with interferon regulatory factor-1 activated NF-κB to cause release of NO (Paludan, 2000), which has been demonstrated to alter the circadian firing pattern in the SCN *in vitro* (Ding et al., 1994). Pro-inflammatory cytokines and LPS may therefore alter the diurnal increase in spontaneous firing in the biological clock via an NO-mediated pathway. NO may also be involved in the pathogenesis of the sleep-wake disturbances in experimental trypanosomiasis. In primates infected with *T. b. brucei*, increased NO concentrations were found in serum and CSF (Sternberg et al., 1998). NO is also released by macrophages in trypanosome-infected mice (Millar et al., 1999) and may cause immunosuppression (Sternberg and McGuigan, 1992). In rats infected with *T. b. brucei*, mRNA for inducible NO synthase was found in the choroid plexus, meninges and brain parenchyma (Quan et al., 1999).

The effects of IFN-γ and TNF-α in combination with LPS on the SCN rhythm suggest that cytokines can alter circadian rhythms *in vitro*. In experimental infections with *T. b. brucei*, release of cytokines may cause disturbances in circadian rhythms *in vivo*, possibly via an NO-mediated pathway.

4. Expression of the receptor of IFN-γ in the SCN (paper III and IV).

The role of IFN-γ

A well-established role of IFN-γ (type II interferon), which is produced by activated NK and T cells, is to control infection. For instance, it regulates the class II major histocompatibility complex, controls leukocyte-endothelium interactions and affects cell proliferation and apoptosis (for review, see Boehm et al., 1997). IFN-γ is released at the early stage in HAT and the plasma concentrations increase with the progress of the infection, being highest in patients who have lost their circadian rhythms (Radomski et al., 1994). IFN-γ may play a pivotal role in the interactions between the immune and nervous systems in experimental trypanosomiasis. To date it is not known to what extent IFN-γ can reach the brain from the circulation in healthy individuals. After trypanosome invasion, however, there is an infiltration of activated T cells of the $CD8^+$ phenotype in the choroid plexus and the circumventricular organs in the vicinity of the SCN (Schultzberg et al., 1988). The $CD8^+$ T cells are stimulated by a trypanosome-derived lymphocyte-triggering factor, which binds to the $CD8^+$ molecule and triggers production of IFN-γ (Olsson et al., 1993). Messenger RNA for IFN-γ has been detected in the brain parenchyma of trypanosome-infected rats (Quan et al., 1999).

The IFN-γ receptor

Expression of the IFN-γR is considered to be ubiquitous at various levels in different tissues, but information of its distribution in the nervous system is sparse (for review, see Farrar and Schreiber, 1993). In the rat and mouse spinal cord, immunopositivity to the IFN-γR is found mainly in lamina I and II of the dorsal horn, where the receptor has been suggested to be involved in eliciting neuropathic pain (Robertson et al., 1997). In the brain, IFN-γR immunopositivity has been demonstrated in the piriform and enthorinal cortex, midline thalamus, medial hypothalamus, brainstem nociceptive relays and the circumventricular organs (Robertson et al., 2000). In the present work, the IFN-γRα protein and transcript were detected in the SCN. IFN-γR immunopositive puncta were restricted to the ventrolateral retino-recipient part of the nucleus. In the same region a large number of cell bodies were immunostained for GluR2/3. The localization of the IFN-γR did not correspond to that of either astrocytes or macrophages/microglial cells, indicating that

the receptor appears on neuronal elements. To evaluate the occurrence and development of the receptor protein in the SCN, the localization of the receptor protein was examined at P1, P5, P11, P20 and P50. At P1 the receptor was distributed in the major part of the nucleus, but was gradually relocated to the ventrolateral region where it appeared as distinct puncta at P11. At P20, the IFN-γR immunopositivity had a similar appearance as at P50 and in adults. The postnatal period when the IFN-γR acquires adult characteristics corresponds to the period when the retinal afferents develop and retina-mediated photic entrainment is established in rat pups, i. e. between P10 and P20 (Reppert et al., 1984; Duncan et al., 1986; Takatsuji et al., 1995; Ban et al., 1997).

When rats were entrained in a 12:12 h LD cycle the receptor expression was low during the subjective day and increased during the early subjective night, with the highest expression at ZT 15 as compared with ZT 19, 23, 3, 7 and 11. The peak of IFN-γR protein expression coincided with increased expression of the tyrosine kinases janus kinase (JAK) 1 and JAK2 as well as the signal transducer and activator of transcription (STAT) 1. Cytokines, including IFN-γ, activate JAK molecules that phosphorylate STATs, which in turn form dimers and induce transcription in the cell nucleus (Briscoe et al., 1996). After exposing rats to a dark:dark cycle for three days, the expression of the IFN-γR protein lost diurnal variation and the amount of protein was constantly high at circadian time points corresponding to the above mentioned ZTs. These findings strongly indicate that the receptor expression is influenced by light and not by endogenous pacemaker mechanisms, similar to the intrinsic light-regulated vasoactive intestinal polypeptide and gastrin releasing peptide in the ventrolateral SCN (for review, see Inouye and Shibata, 1994).

Little information is available on the homology between cytokine receptors in the immune and nervous system (Hopkins and Rothwell, 1995). To establish the identity of the IFN-γR in the rat SCN, the receptor gene was cloned and sequenced. The SCN cDNA sequence from the cloned transcript was compared to the rat IFN-γRα sequence (Neumann et al., 1997). The analysis showed that the IFN-γR in the SCN and immune system are identical, which may imply that the receptor is involved in interactions between the immune and nervous system. NK cells, which reach a peak in activity during the early night (for review, see Levi et al., 1991), may release an IFN-γ ligand for the cycling IFN-γR. However, as discussed above it is not clear

whether a cytokine like IFN-γ can cross the BBB to reach the SCN. Ek et al. (2001) recently proposed that IL-1 may induce effects in the brain by stimulating the production of prostaglandin E_2, a small molecule that diffuses into the brain parenchyma, which triggers intrinsic production of cytokines in the brain. Of interest in this context is a molecule that cross-reacts with antibodies directed against different epitopes of IFN-γ. This neuronal 'IFN-γ-like' immunoreactive molecule is expressed in the rat brain in the histaminergic tuberomammillary nuclei of the posterior hypothalamus. Axon terminal-like structures are immunopositive to IFN-γ in the ventrolateral region of the SCN, i. e. the same region where the IFN-γR was found (Bentivoglio et al., 1994a). The tuberomammillary nuclei are considered to play a major role in arousal mechanisms (Schwartz et al., 1991).

The obtained data demonstrate that transcripts of the immune IFN-γR are present in the SCN. They also indicate the occurrence of a receptor protein for IFN-γ, which is redistributed in the ventrolateral region of the SCN around P11, i. e. during the same time period as when light entrainment of the SCN is established in the rat. In addition, the expression of the IFN-γR protein seems to follow a light responsive diurnal rhythm that is not controlled by endogenous mechanisms in the pacemaker.

CONCLUDING REMARKS

An experimental rat model of African trypanosomiasis, or sleeping sickness, was used in order to examine the mechanisms underlying functional alterations in circadian rhythms, which are characteristic signs of nervous system dysfunctions caused by this parasitic infection. The present study demonstrates that a marked dysregulation in the spontaneous intrinsic activity of SCN neurons occurs as a result of experimental trypanosome infection, and proposes that the SCN output is altered in rats infected with *T. b. brucei*.

This work also provides new data regarding the synaptic machinery in the SCN. Because single SCN neurons contain individual oscillators, the role of synaptic communication between SCN neurons for circadian time-keeping mechanisms has been questioned. The intrinsic neurotransmitter GABA has been ascribed the major pivotal role within the SCN, possibly by synhcronizing SCN neurons. However, we found that the ventrolateral SCN in addition contain a substantial glutamatergic synaptic network that acts mainly on AMPA receptors, in which there is a diurnal variation in synaptic activity with a marked increase during the subjective day. These variations may correspond to the rhythm in SCN neuronal activity, which is high during the subjective day. The activity of excitatory synapses in the SCN was significantly decreased in slices from rats infected with *T. b. brucei*, which raises the question whether trypanosome infection causes postsynaptic alteration of the expression of AMPA receptors. Immunohistochemistry revealed a down-regulation of AMPA receptor subunits in rats infected with trypanosomes. This alteration may be correlated to the dysregulation of the circadian firing pattern in the SCN *in vitro*.

Trypanosome invasion causes release of a vast number of immune response molecules, both systemically and within the CNS, which may affect the SCN function and output. For instance, increased levels of sleep inducing prostaglandins (Kubata et al., 2000) and the cytokines IL-1 and TNF-α (Kapas et al., 1994; Krueger and Majde, 1994) may alter the sleep-wake regulation and cause disturbances in the sleep-wake cycle in trypanosomiasis. The pro-inflammatory cytokine IFN-γ may also be involved in the cascade of released molecules that result from the interaction between the parasite and the immune system. IFN-γ is notable because the released levels are correlated to the severity of disease. The detection of an oscillating IFN-γR in the

SCN, which was shown to be identical with the IFN-γR in the immune system, suggests that IFN-γ released in the brain during infection acts directly on the pacemaker, which may trigger changes in the SCN function. The effects of IFN-γ on circadian rhythms *in vivo* remain to be clarified; however, it was here demonstrated that IFN-γ and TNF-α in combination with LPS altered the SCN firing rhythm *in vitro*. It is therefore suggested that these cytokines alter the SCN function and circadian output in rats infected with trypanosomes, possibly by inducing release of NO, which in turn can phase shift the neuronal activity rhythm in the SCN *in vitro* (Ding et al 1994).

In conclusion, the present results suggest that infections with *T. b. brucei* affect the biological clock and cause circadian sleep-wake disturbances. Based on the observations in this thesis, we propose one mechanism that may contribute to the dysregulation of the SCN function during trypanosome infection: the parasites interact with the immune system and large quantities of IFN-γ are released. IFN-γ affects the SCN and via its receptor causes a down-regulation of glutamatergic AMPA receptors, resulting in a decrease in the glutamatergic synaptic activity in the ventrolateral region of the SCN. The down-regulated glutamatergic synaptic communication reduces the excitability in SCN neurons, which results in a modification of the firing pattern and SCN output. The altered circadian signal from the SCN may cause a disturbance in the translation to rhythms in bodily functions, which is likely manifested as a fragmentation of the sleep-wake cycle. In order to verify such a hypothesis, future studies are required to reveal a functional role of the IFN-γR in the SCN. Of particular interest is to examine effects of IFN-γ on synaptic function, and explore the machinery underlying the alterations in excitatory synaptic activity following trypanosome infection.

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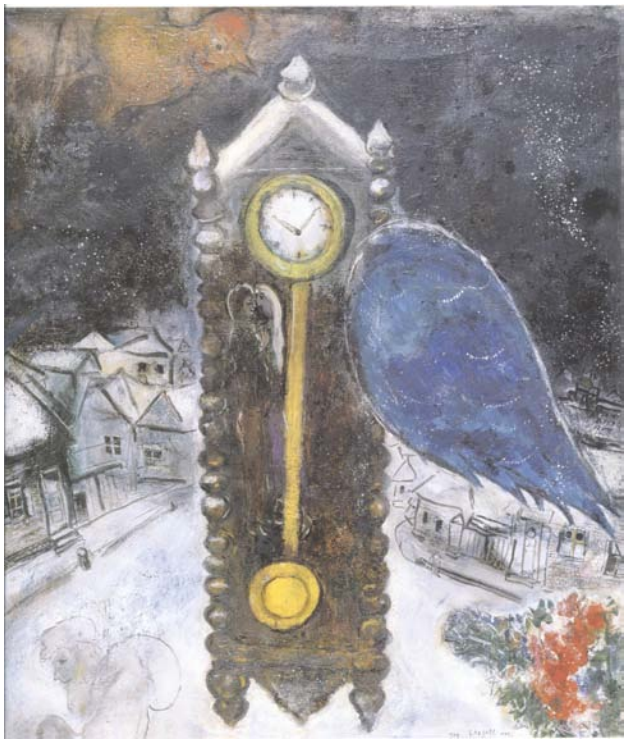

The pendulum with blue wings
By Marc Chagall