

From the Department of Neuroscience,  
Karolinska Institutet, Stockholm, Sweden

**EFFECTS OF AGING AND INFLAMMATORY  
MOLECULES ON THE SUPRACHIASMATIC  
CIRCADIAN CLOCK**

Mikael Nygård



**Karolinska  
Institutet**

Stockholm 2007

Cover: The flower clock (*Horologium Florae*), by Carl von Linné (1707-1778) in his *Philosophia Botanica* (1751). An early reference of circadian rhythms in plants. Linné made careful observations of the opening and closing of the leaves of different plant species, and noted that some plants open their leaves only for a short period each day. His suggested arrangement of the plants in a specific order enables the observer to tell time with half an hour's accuracy.

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

© Mikael Nygård, 2007  
ISBN 978-91-7357-394-8

*To my family*



## ABSTRACT

The suprachiasmatic nucleus (SCN) is considered the master circadian pacemaker in the mammalian brain and is vital for generating physiological and behavioral circadian rhythms. During aging and inflammatory conditions the circadian system is frequently affected, but relatively little is known about the mechanisms underlying such effects.

We compared electrophysiological properties of the young and aged SCN in slice preparations *in vitro* and found that aged mice had an overall reduced GABAergic synaptic transmission and an increased proportion of silent cells, i.e. cells that do not fire spontaneous action potentials. The *in vivo* response of SCN neurons to pro-inflammatory cytokines was also compared between young and aged mice by measuring the neuronal induction of the protein encoded by the immediate early gene *Fos*. Firstly, the response to a mixture of the pro-inflammatory cytokines tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  showed a clear day/night variation in both age groups with higher induction of Fos protein during the subjective night. This variation was paralleled by variations in the mRNA expression of suppressor of cytokine signaling (SOCS) molecules that are important negative regulators of cytokine signaling pathways; when SOCS molecule expression was low, TNF- $\alpha$ /IFN- $\gamma$  had the strongest effect, indicating that SOCS molecules may regulate the responses to cytokines in the SCN. Secondly, the Fos response during early subjective night was lower in aged as compared to young animals. There was no difference between the age groups in the mRNA expression of SOCS or the TNF- $\alpha$  receptor, whereas IFN- $\gamma$  receptor mRNA expression was lower in aged mice during subjective night, which may underlie the blunted responses in aged mice at this time point.

Neurophysiological effects of TNF- $\alpha$  on the SCN were also investigated. Spontaneous firing as well as synaptic transmission, was affected by the cytokine and the observed effects mostly persisted throughout the recording period. The effect on spontaneous firing could be blocked by the nitric oxide synthase (NOS) inhibitor L-NAME. TNF- $\alpha$  may therefore affect the SCN neuronal activity via induction of NO, to alter circadian output signals from the SCN.

The functional role of SOCS molecules in neurons of the central nervous system was verified in primary hippocampal neuronal cultures. In these cultures IFN- $\gamma$  induced SOCS1 and SOCS3 mRNA expression. Using neurons from *Socs1*<sup>-/-</sup> mice, SOCS1 was found to negatively regulate the response to IFN- $\gamma$  in terms of transcriptional responses and antigen presentation.

In summary, this thesis shows that during aging the SCN synaptic network and spontaneous neuronal activity is compromised. TNF- $\alpha$ , the levels of which often are increased in aged individuals, affects SCN neuronal firing and synaptic activity and may thereby contribute to circadian rhythm disturbances during aging. SCN neurons in aged mice also responded differently to IFN- $\gamma$ /TNF- $\alpha$  exposure as compared to young mice. Furthermore, SOCS molecules, which are expressed in the SCN with a day/night rhythm, can regulate the responses of brain neurons to cytokine exposure, and may therefore play a role to regulate the sensitivity of the SCN to pro-inflammatory cytokines.

**Keywords:** circadian, suprachiasmatic, synaptic, inflammation, cytokine, SOCS, interferon, tumor necrosis factor, neuron, firing.



## LIST OF PUBLICATIONS

This thesis is based on the following publications which will be referred to in the text by their roman numerals:

- I. **Nygård M**, Hill RH, Wikström MA, Kristensson K. Age-related changes in electrophysiological properties of the mouse suprachiasmatic nucleus *in vitro*. *Brain Res Bull.* 2005 Mar 15;65(2):149-54.
- II. Sadki A., Bentivoglio M., Kristensson K., **Nygård M**. Suppressors, receptors and effects of cytokines on the aging mouse biological clock. *Neurobiol. Aging.* 2006 Feb;28(2):296-305.
- III. **Nygård M**, Hill RH, Lundkvist GB, Kristensson K. Tumor necrosis factor- $\alpha$  modulates spontaneous firing of mouse suprachiasmatic neurons via a nitric oxide-mediated mechanism. *Submitted manuscript.*
- IV. **Nygård M**, Rottenberg M, Chen M, Kristensson K. Regulation of expression and role of SOCS1 in response to interferon- $\gamma$  in central neural cells. *Submitted manuscript.*

## RELATED PUBLICATIONS:

Bentivoglio M, Deng XH, Nygård M, Sadki A, Kristensson K. The aging suprachiasmatic nucleus and cytokines: functional, molecular, and cellular changes in rodents. *Chronobiol Int.* 2006;23(1-2):437-49.

Nygård M, Palomba M. The GABAergic network in the suprachiasmatic nucleus as a key regulator of the biological clock: does it change during senescence? *Chronobiol Int.* 2006;23(1-2):427-35.

Bentivoglio M, Nygård M, Palomba M, Kristensson K. The Biological Clock in Flame and Sleep Switch Alterations. In: *Neuroimmunology of Sleep*. Part II (87-96), 2007. Springer US

Palomba M, Nygård M, Florenzano F, Kristensson K, Bentivoglio M. Age-related alterations of the presynaptic network in the mouse suprachiasmatic nucleus. *Journal of Biological Rhythms*. *Under revision*.

# CONTENTS

1	Introduction.....	1
1.1	Circadian rhythms.....	1
1.2	The suprachiasmatic nucleus.....	2
1.2.1	SCN afferents, input and anatomy.....	3
1.2.2	Clock genes and rhythm generation.....	4
1.2.3	Coupling and synchronization of SCN neurons.....	7
1.2.4	SCN efferents and output.....	9
1.3	Circadian rhythm disturbances during aging.....	11
1.4	The nervous and immune system interplay – effects on circadian rhythms.....	14
1.4.1	Cytokines can modulate circadian rhythms.....	15
1.4.2	Control of intracellular responses to cytokine signaling.....	16
1.4.3	Aging and neuroinflammation.....	18
2	Aims.....	21
3	Methodological considerations.....	23
3.1	Animals.....	23
3.2	The SCN slice preparation.....	24
3.3	Electrophysiology.....	25
3.4	PER2::LUCIFERASE recordings.....	26
3.5	Immunohistochemistry.....	26
3.6	Real-time PCR.....	27
3.7	Cell culture.....	29
4	Results and discussion.....	30
4.1	Synaptic properties of the SCN in young and aged mice (Paper I).....	30
4.2	Pro-inflammatory cytokines – effects on the young and aged SCN (Paper II) ...	33
4.3	Effects of TNF- $\alpha$ on the SCN (Paper III).....	34
4.4	SOCS-mediated regulation of cytokine responses in neurons (Paper IV).....	37
5	Concluding remarks.....	40
6	Acknowledgements.....	43
7	References.....	45

# LIST OF ABBREVIATIONS

aCSF	- artificial cerebrospinal fluid
CCG	- clock controlled gene
CNS	- central nervous system
Cry	- Cryptochrome
CT	- circadian time
DMH	- dorsomedial nucleus of the hypothalamus
DNA	- deoxyribonucleic acid
EPSC	- excitatory postsynaptic current
GABA	- $\gamma$ -amino-butyric acid
i.c.v.	- intracerebroventricular
IFN- $\gamma$	- interferon- $\gamma$
IL	- interleukin
IP-10	- interferon- $\gamma$ inducible protein
IPSC	- inhibitory postsynaptic currents
JAK	- janus kinase
LMP	- low molecular weight protein
LPS	- lipopolysaccharide
L-NAME	- N <sup>G</sup> -nitro-L-arginine methyl ester
MHC	- major histocompatibility complex
mRNA	- messenger RNA
NO	- nitric oxide
NOS	- NO synthase
OVA	- ovalbumine
PCR	- polymerase chain reaction
PER2:: <i>LUC</i>	- PERIOD2:: <i>LUCIFERASE</i>
Per	- Period
PIAS	- protein inhibitor of activated STAT
PVN	- paraventricular nucleus
RHT	- retino-hypothalamic tract
RNA	- ribonucleic acid
SCN	- suprachiasmatic nucleus
SH2	- Src homology 2
SHP	- SH2-containing phosphatase
SOCS	- suppressor of cytokine signaling
SPZ	- subparaventricular zone
STAT	- signal transducer and activator of transcription
VIP	- vasoactive intestinal polypeptide
VP	- vasopressin
WT	- wildtype
TNF- $\alpha$	- tumor necrosis factor- $\alpha$
TTX	- tetrodotoxin
ZT	- <i>Zeitgeber</i> time
NREM	- non-rapid eye movement

# 1 INTRODUCTION

The circadian timing system prepares the body for the changes associated with the daily rise and fall of the sun by regulating physiological and behavioral parameters, e.g. the sleep-wake cycle, core body temperature and hormone levels. Disruptions of circadian rhythms can have negative influence on the health of the individual, with increased risks of cancer, and metabolic and psychiatric diseases (Lewy *et al.*, 2006; Maywood *et al.*, 2006a; Partonen *et al.*, 2007). This thesis analyzes the impact of aging and inflammatory molecules on the suprachiasmatic nucleus (SCN), the master circadian pacemaker in the mammalian brain.

## 1.1 CIRCADIAN RHYTHMS

Everyday life is to a great extent affected by the 24 h cycle of day and night. Such cyclic changes with a period of around 24 h are termed circadian from latin “*circa diem*” meaning “about a day”. Circadian rhythms have evolved in most species, ranging from single cell organisms to plants and animals, as a way to adapt to and prepare for the changes that occur throughout a day, mainly due to the rise and fall of the sun and the environmental changes that this brings about. Disturbances in circadian rhythms may compromise the ability to cope with and take advantage of such cyclic changes in the environment. This can have devastating effects on the survival or well-being of an individual, and hence negatively affect the evolution of a species. The fact that circadian rhythms are not merely passive responses to the environmental changes associated with day and night becomes evident when rhythms persist in subjects deprived of all external cues. This phenomenon was described already in the 1700s by the French scientist Jean-Jacques d’Ortous de Mairan, who observed that the daily rising and lowering of the leaves of a plant was maintained also when the plant was put in a room with no exposure to sunlight (d’Ortous de Mairan, 1729). This implies that there is an internal timekeeping system, or “biological clock”, which can sustain circadian rhythms in the absence of external cues. Circadian rhythms that are expressed in the absence of external rhythmic cues are said to be “free-running”, meaning that they are not affected by cyclic changes in the environment. The free-running rhythms often deviate somewhat from 24 h, and external synchronizing signals, so called *Zeitgebers*, are therefore necessary to align the rhythm to the environment, a phenomenon known as entrainment. The major entraining signal, or *Zeitgeber*, for most

animals is the light-dark cycle, but also other factors such as social interactions and food intake can modulate circadian rhythms.

Different species have developed different biological systems of varying complexity in order to maintain circadian rhythms in the body, and adjust these rhythms to keep them synchronized to the solar day (Herzog, 2007). In the marine snail *Bulla gouldiana*, each of the basal retinal neurons in the eye acts as a pacemaker to regulate locomotor activity rhythms (Michel *et al.*, 1993), whereas in the fruit fly *Drosophila melanogaster*, populations of neurons in the brain form a more complex timekeeping system (Herzog, 2007). In several non-mammalian vertebrates, such as the sparrow, extra-retinal photoreceptors within the brain can entrain the circadian system (Menaker, 1968). Although the circadian time-keeping systems differ substantially between species, there are also certain components of these systems that are remarkably conserved phylogenetically, e.g. the genes encoding the essential components of the molecular circadian clock (Panda *et al.*, 2002b).

## **1.2 THE SUPRACHIASMATIC NUCLEUS**

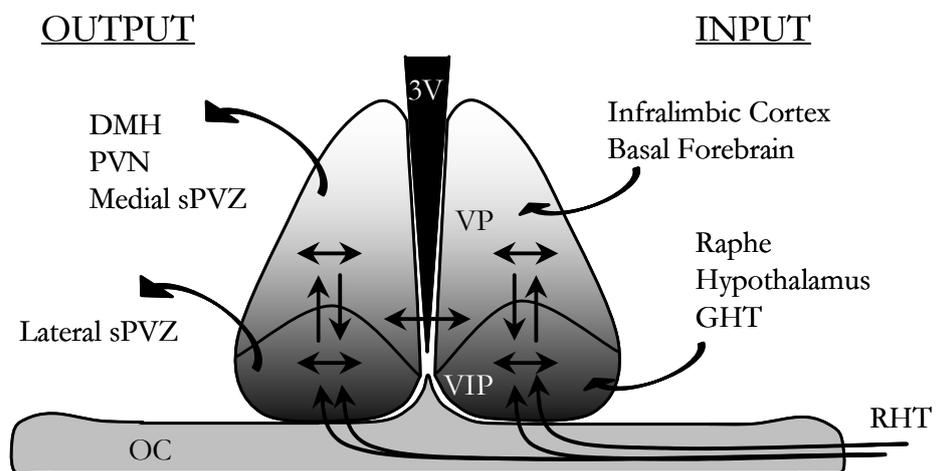
In mammals, the master circadian pacemaker in the brain resides in the hypothalamic suprachiasmatic nucleus (SCN). Lesions made specifically to this brain area cause a loss of circadian rhythms of e.g. corticosterone release, drinking behavior and locomotor activity (Moore & Eichler, 1972; Stephan & Zucker, 1972). When fetal SCN tissue is transplanted to such lesioned animals, the locomotor activity rhythm is restored (Lehman *et al.*, 1987). Later experiments demonstrated that the rhythm in transplanted animals is in fact derived from the donor and not the host (Ralph *et al.*, 1990). These experiments clearly demonstrate the importance of the SCN in determining circadian rhythms.

Although the SCN is considered the principal pacemaker that acts as a clock and drives circadian rhythms, also many other tissues show rhythmic activity (see for example Yamazaki *et al.*, 2002; Yoo *et al.*, 2004). Similar to the SCN, the retina and olfactory bulb can express self-sustained circadian rhythms without external input and can therefore be considered autonomous oscillators. Semi-autonomous oscillators, e.g. the lateral habenula and the arcuate nucleus, have oscillatory capacity but require rhythmic input to synchronize individual cells, and lastly there are slave oscillators, e.g. the amygdala, that respond to rhythmic input signals but are otherwise arrhythmic (for review, see Guilding & Piggins, 2007). The oscillatory capacities of extra-SCN tissues indicate that the circadian timing system, rather than being a strictly hierarchical

system, may constitute a network of interacting oscillating tissues. An important task for the SCN may be the coordination of separate oscillators to maintain circadian phase relationships between organs and tissues. In the following sections the anatomy, afferent and efferent connections and properties of SCN neurons will be reviewed.

### 1.2.1 SCN afferents, input and anatomy

The SCN consists of two bilateral nuclei situated above the optic chiasm adjacent to the third ventricle. There are widespread inputs from other brain areas terminating in the SCN (Fig. 1). Retinal ganglion cells (RGCs) send glutamatergic projections to the ventral SCN via the retino-hypothalamic tract (RHT) (Moore & Lenn, 1972). Most SCN-projecting RGCs express the photopigment melanopsin which makes them light sensitive, and thus able to transmit light information to the SCN (Melyan *et al.*, 2005; Panda *et al.*, 2005; Qiu *et al.*, 2005). Two other major input pathways are neuropeptide Y-containing fibers, which project from the intergeniculate leaflet via the geniculohypothalamic tract (GHT), and serotonergic fibers from the raphe nuclei. Both pathways project mainly to the ventral SCN (Moga & Moore, 1997; van Esseveldt *et al.*, 2000). The dorsal SCN receives input mainly from the infralimbic cortex and basal forebrain (Moga & Moore, 1997). In addition several minor projections to the SCN are found, e.g. from the hypothalamic tuberomammillary, arcuate and supraoptic



**Figure 1. SCN input and output pathways.**

The major input and output pathways to, from and within the SCN are depicted. The ventral SCN containing VIP neurons, and dorsal SCN containing VP neurons are outlined. DMH (dorsomedial hypothalamus), PVN (paraventricular nucleus), sPVZ (subparaventricular zone), RHT (retino-hypothalamic tract), 3V (third ventricle), OC (optic chiasm), GHT (geniculohypothalamic tract).

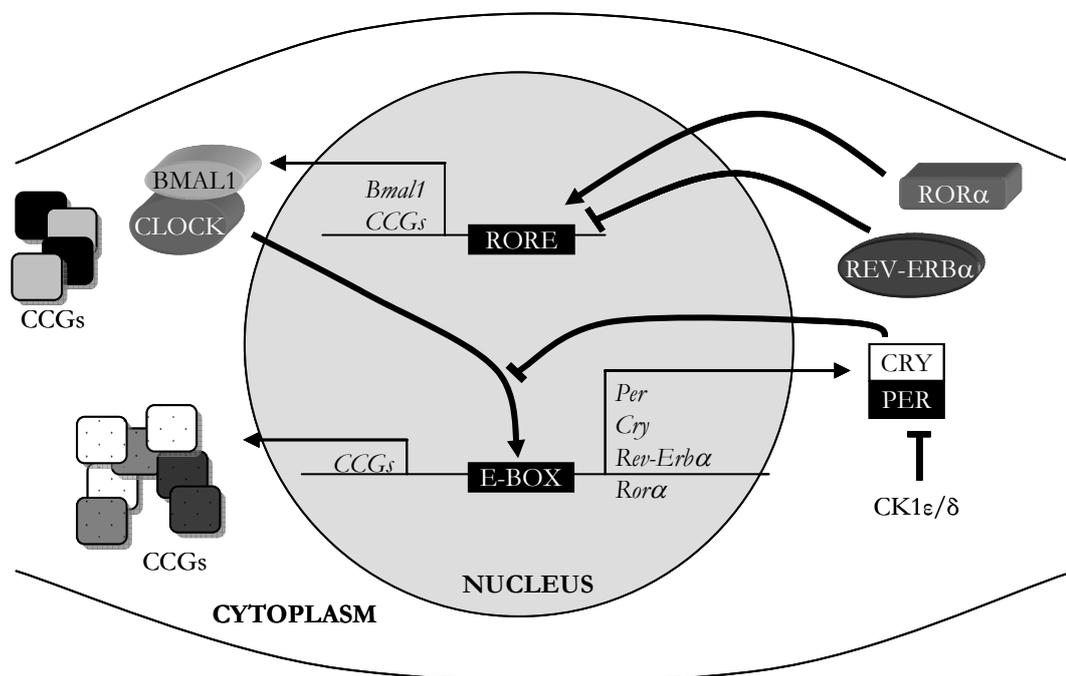
nuclei (Saeb-Parsy *et al.*, 2000; Morin & Blanchard, 2001; Krout *et al.*, 2002).

The SCN constitutes a densely packed structure with each nucleus containing around 10,000 neurons in rodents (Abrahamson & Moore, 2001). The SCN neurons are small, ranging from 5 to 17  $\mu\text{m}$  in diameter (Pennartz *et al.*, 1998). Although  $\gamma$ -aminobutyric acid (GABA) is expressed in most SCN neurons (Okamura *et al.*, 1989; Moore & Speh, 1993; Morin & Blanchard, 2001), this structure is far from homogenous. The SCN is often divided into a ventrolateral ‘core’, and a dorsomedial ‘shell’ (Miller *et al.*, 1996; Tanaka *et al.*, 1997) based on the RHT input terminating mainly in the ventral SCN, and the expression of vasopressin (VP) (Vandesande *et al.*, 1975) and vasoactive intestinal polypeptide (VIP) (Card *et al.*, 1981) in neurons located in the dorsomedial and ventrolateral SCN, respectively (reviewed by Moore *et al.*, 2002). Also a number of other neuropeptides, including somatostatin, neurotensin, enkephalin, substance P, angiotensin II, calretinin and calbindin, are expressed within different areas of the SCN (Abrahamson & Moore, 2001). The complexity of the SCN anatomy becomes increasingly apparent as more detailed information becomes available, and there are significant species differences (Antle & Silver, 2005). The neuronal heterogeneity within the SCN is also reflected in electrical properties such as spontaneous firing rate patterns, resting membrane potential and phase relationships of firing rate rhythms (Pennartz *et al.*, 1998; Nakamura *et al.*, 2001; Schaap *et al.*, 2003) as well as in expression of the clock gene *Period1* (*Per1*, see below) (Quintero *et al.*, 2003). Since the chemoarchitecture varies throughout the rostral-caudal extent of the SCN, as does the temporal variation in the expression of different molecules, the usage of the terms ‘core’ and ‘shell’ has been questioned, and a more dynamic view of SCN anatomy has been called for, taking these mentioned factors into consideration (Morin *et al.*, 2006; Morin, 2007). In the following, common anatomical nomenclature as ventral/dorsal or ventrolateral/dorsomedial and the terms ‘core’ and ‘shell’, will be used for describing localization within the SCN, but the heterogeneity of the SCN will also be addressed when appropriate.

### 1.2.2 Clock genes and rhythm generation

Circadian rhythmicity is generated by autoregulatory transcription-translation feedback loops, including a set of genes that have been designated “clock genes”. The first clock gene, *Period* (*Per*), was identified by genetic screening in *Drosophila* flies (Konopka & Benzer, 1971), and the mammalian homologue was later identified (Sun *et al.*, 1997; Tei *et al.*, 1997). Other clock genes have subsequently been identified in both flies and

mammals using similar approaches (for details see Panda *et al.*, 2002b). Deletion or mutation of these genes results in behavioral arrhythmicity (Panda *et al.*, 2002b). In mammals, the basic helix–loop–helix transcription factors CLOCK and BMAL1 drive transcription of the clock genes *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) (Fig. 2). PER and CRY proteins form complexes that inhibit their own CLOCK:BMAL1-induced transcription. Post-translational modifications determine the turnover rate of PER and CRY, and thereby control the time delay after which the cycle can restart. This complex regulatory network forms two interlocked loops that mediate rhythms of gene expression with a period of about 24 h. These loops are stabilized by



**Figure 2. Simplified model of the mammalian molecular clock.**

Two interlocking positive and negative loops form the core of the molecular oscillatory system. During the circadian day, BMAL1 forms a heterodimeric complex with constitutively expressed CLOCK, that acts via E-BOX DNA regulatory sequences to drive the expression of *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. PER:CRY complexes then accumulate during circadian night and inhibit their own transcription, thus forming the negative loop. With the degradation of PER:CRY complexes during late night the cycle can start again. CLOCK and BMAL1 also drive transcription of other genes with E-BOX promoter sequences such as *Rev-Erbα* and *Ror*. REV-ERBα and RORα compete for binding ROR elements on the *Bmal1* promoter and act to stabilize the other loops. Casein kinase 1 epsilon (CK1ε) and delta (CK1δ) regulate the protein turnover of core circadian clock components. This simplified model is under constant revision and is likely to increase in complexity as more information accumulates.

auxillary loops that involve REV-ERB $\alpha$ , ROR $\alpha$ , Dec1 and Dec2 (for details see Hastings *et al.*, 2003; Ko & Takahashi, 2006). CLOCK:BMAL1, and also ROR $\alpha$ , do not only drive the expression of clock genes during specific time periods, but they also drive the expression of other so-called clock controlled genes (CCG's) (Ueda *et al.*, 2002; Hastings *et al.*, 2003). Microarray studies have shown that around 10 % of the transcriptome is expressed under circadian control in the SCN and also in other tissues. When comparing the sets of genes transcribed under circadian control, they differ substantially between tissues, and the CCG's often include critical factors involved in the physiology of a specific tissue (Akhtar *et al.*, 2002; Panda *et al.*, 2002a; Storch *et al.*, 2002).

An important output of the molecular clock machinery in SCN neurons is the generation of an electrical activity rhythm. This rhythm shows high spike activity during the day and low activity at night, and has been demonstrated by both *in vivo* (Inouye & Kawamura, 1979) and *in vitro* recordings (Green & Gillette, 1982; Groos & Hendriks, 1982; Shibata *et al.*, 1982). Spontaneous firing rate in individual SCN neurons is correlated to *Per1* expression levels (Quintero *et al.*, 2003) and deficiencies in core clock components results in disrupted SCN firing rate rhythms (Liu *et al.*, 1997; Herzog *et al.*, 1998; Albus *et al.*, 2002), demonstrating that electrical rhythms are an output of the molecular clock. Several CCG's have been identified that contribute to rhythms in resting membrane potential and spontaneous firing. Molecules that are involved in rhythmic firing in the SCN include L-type voltage-dependent Ca<sup>2+</sup> channels (Pennartz *et al.*, 2002), fast-delayed rectifier (FDR) K<sup>+</sup> channels (Itri *et al.*, 2005), and Ca<sup>2+</sup>-activated K<sup>+</sup> channels (iberotoxin-sensitive BK channels) (Meredith *et al.*, 2006). Changes in basal membrane potential is an important component of the circadian firing rhythm and is mediated by changes in K<sup>+</sup>-conductance, but the specific channels that mediate this rhythm remain to be identified.

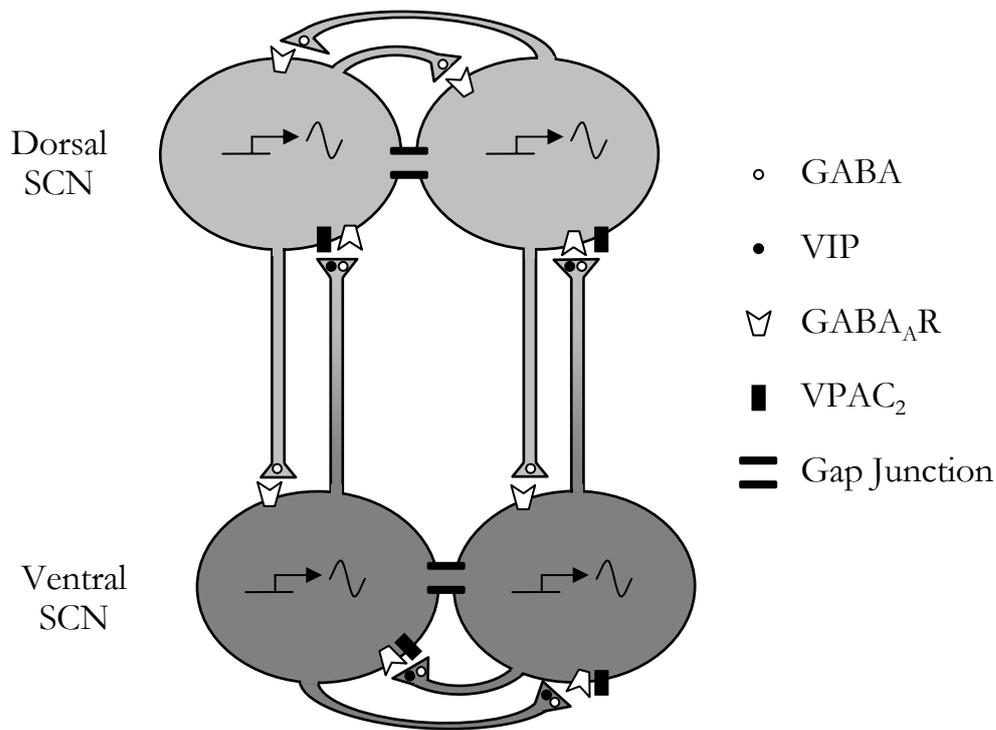
The electrical activity is not merely a passive output, but seems also to feedback on the molecular clock. For instance, in transgenic *Drosophila* flies expressing a mutant K<sup>+</sup>-channel that hyperpolarizes neurons, the molecular rhythms are abolished (Nitabach *et al.*, 2002). Hyperpolarization of SCN slices also abolishes rhythmic *Per1* and *PER2* expression (Lundkvist *et al.*, 2005), and neurons in SCN slices from VIP receptor subtype 2 (VPAC<sub>2</sub>) deficient mice, which presumably are hyperpolarized, are arrhythmic (see below), but show molecular rhythms when they are depolarized using medium with high K<sup>+</sup>-concentration (Maywood *et al.*, 2006b). Neuronal membrane

events may thus play an important role in molecular rhythm generation in the SCN and could present a pathway for incoming neuronal and humoral signals to modulate the core clock mechanism.

### 1.2.3 Coupling and synchronization of SCN neurons

Individual SCN neurons are able to sustain expression of rhythms *in vitro*, as has been demonstrated for electrical firing (Welsh *et al.*, 1995), cytosolic Ca<sup>2+</sup> concentration (Colwell, 2000) and gene expression (Yamaguchi *et al.*, 2003). In order for the SCN to form a coherent output, the individual neurons must be synchronized so that their phases of activity are aligned and do not drift apart. Desynchronization of SCN neurons occurs when mice are exposed to constant light, which causes loss of the SCN rhythm as well as behavioral rhythm (Ohta *et al.*, 2005). Similarly, when SCN slices are treated with tetrodotoxin (TTX) that blocks Na<sup>+</sup>-dependent action potentials (Yamaguchi *et al.*, 2003), or when dispersed SCN neurons are cultured at low density (Welsh *et al.*, 1995), the inter-cellular coupling is disrupted or weakened and the individual cells tend to drift out of phase, resulting in a dampened amplitude of the ensemble rhythm. When SCN neurons are dispersed at high density they are able to synchronize their activities (Aton *et al.*, 2005). Furthermore, from experiments where clock gene deficient mice were crossed with PER2::LUCIFERASE (PER2::LUC) mice (See Methodological considerations), it was found that SCN tissue slices from *Per1* or *Cry1* deficient mice remained rhythmic, whereas dispersed SCN neurons derived from *Per1* or *Cry1* deficient mice showed arrhythmic PER2::LUC expression (Liu *et al.*, 2007). It therefore seems that the coupling and synchronization of the approximately 20,000 neurons of the SCN is vital for appropriate functioning of these nuclei.

In the SCN, not all neurons have exactly the same phase, but instead groups of neurons show similar phases in electrical activity and clock gene expression (Nakamura *et al.*, 2001; Quintero *et al.*, 2003; Schaap *et al.*, 2003; Yamaguchi *et al.*, 2003). It is not fully understood how synchronized activity between individual neurons and between SCN regions is achieved, but neurotransmission seems to play an important role (Aton & Herzog, 2005) (Fig. 3). Blocking action potentials with TTX disrupts the *in vitro* SCN *Per1* rhythm, that is restored when TTX is removed (Yamaguchi *et al.*, 2003). Similarly, when TTX is injected into the SCN *in vivo*, locomotor activity rhythms are lost but are restored upon washout (Schwartz *et al.*, 1987). Interestingly, separating the dorsal from the ventral SCN results in lost synchrony only between neurons in the dorsal SCN, which implies that a synchronizing factor is derived from



**Figure 3. Coupling and synchronization in the SCN.**

Subsets of SCN neurons in the ventral (core) SCN release VIP, necessary for synchronization of circadian periods among SCN neurons. Most neurons express GABA and daily GABA application can synchronize SCN neurons, and blockade of GABA<sub>A</sub> receptors interferes with rhythm coordination between the dorsal and ventral SCN. Gap junctions may also synchronize neighboring SCN neurons. Adapted from (Aton & Herzog, 2005) with permission from Elsevier.

the ventral SCN (Yamaguchi *et al.*, 2003). VIP, expressed in the ventral SCN, is one strong candidate molecule to play such a synchronizing role (Aton *et al.*, 2006). Mice deficient in the VPAC<sub>2</sub> receptor show a loss of rhythm in locomotor behaviour, SCN electrical firing rate and clock gene expression (Harmar *et al.*, 2002; Cutler *et al.*, 2003). Deficiency of either VIP or its receptor VPAC<sub>2</sub> results in desynchronized firing rate rhythms of individual SCN neurons (Aton *et al.*, 2005). Gastrin releasing peptide, which is also expressed in the ventral SCN, can promote SCN electrical and molecular rhythms in the absence of VIP-signaling, indicating a role also for this peptide in SCN coupling (Brown *et al.*, 2005; Maywood *et al.*, 2006b).

GABA is another candidate synchronizing factor that has been demonstrated to synchronize firing rate rhythms of cultured SCN neurons when applied daily (Liu & Reppert, 2000). However, since GABA is expressed also in dorsal SCN neurons that cannot sustain synchrony when separated from the ventral SCN (Yamaguchi *et al.*, 2003), GABAergic signaling within the dorsal SCN is apparently not sufficient to synchronize these neurons. Furthermore, blocking GABAergic signaling does not

desynchronize PER2::LUC rhythms in SCN slices, or firing rate rhythms in dispersed cells, but instead affects the amplitude of these rhythms (Aton *et al.*, 2006). Thus, GABA may not be crucial to synchronize individual SCN neurons, but could still play an important role in coupling the ventral and dorsal regions of the SCN (Albus *et al.*, 2005). VIP also modulates GABAergic synaptic transmission (Itri & Colwell, 2003) and the synchronizing effect of this neuropeptide may thus be mediated in part via modulation of GABAergic signaling in the dorsal SCN.

Other mechanisms may also contribute to the synchronization of SCN cells. Connexin36, which forms neuronal gap junctions, is expressed in neurons throughout the SCN, and most SCN neurons appear to have “miniature” gap junctions that may allow weak neuronal coupling (Jobst *et al.*, 2004; Rash *et al.*, 2007). Furthermore, mice lacking connexin36 show disrupted circadian behavioral rhythms (Long *et al.*, 2005), suggesting that gap junctional coupling may play an important role in synchronizing SCN neurons. Paracrine factors such as nitric oxide (NO) may also couple SCN neurons, which is supported by findings in the SCN derived cell line SCN2.2, where blockade of NO synthase (NOS) caused a loss of rhythm of glucose utilization (Menger *et al.*, 2007).

Taken together, there is strong support for chemical neurotransmission, and possibly also gap junction-mediated electrical neurotransmission, as means to synchronize SCN neurons. The candidate mechanisms discussed above are not mutually exclusive, and may work together in a complementary fashion to synchronize SCN neurons and regions.

#### 1.2.4 SCN efferents and output

A characteristic feature of SCN neurons is the expression of a cell autonomous rhythm in spontaneous action potential frequency. A close connection of behavioral rhythms and SCN electrical rhythm is evident from experiments with the *tau* mutant hamster (lacking the gene encoding CK1 $\epsilon$ , see Clock genes), which has a period of about 20 h in both spontaneous firing in the SCN and locomotor activity (Ralph & Menaker, 1988; Davies & Mason, 1994). Furthermore, blocking electrical activity with TTX causes behavioral arrhythmia (Schwartz *et al.*, 1987), demonstrating that electrical activity is a critical output of the SCN.

The main efferents from the SCN project to other closely situated hypothalamic areas, mainly the paraventricular nucleus (PVN), the subparaventricular zone (SPZ) and the dorsomedial nucleus of the hypothalamus (DMH) (Leak *et al.*, 1999) (Fig.1). These

areas act as relays and convey the circadian message to other downstream target brain areas, which are involved in the circadian regulation of specific body functions. Lesion studies have provided information on the involvement of various SCN target areas in the regulation of different rhythms (for review see Saper *et al.*, 2005). The ventral SPZ controls circadian rhythms of sleep–wakefulness, locomotor activity and feeding whereas the dorsal SPZ mediates circadian rhythms of body temperature (Lu *et al.*, 2001). The DMH plays a key role in sleep regulation, and lesions of the DMH cause altered circadian rhythms in sleep-wakefulness behavior, but also alters rhythms in feeding, locomotor activity and corticosteroid secretion (Chou *et al.*, 2003). In addition, the SCN has sparse direct projections to other brain areas, e.g. the tuberomammillary nucleus and the ventrolateral preoptic nucleus that are involved in regulating sleep-wakefulness (Abrahamson & Moore, 2001; Chou *et al.*, 2002) and the paraventricular nucleus of the thalamus, that in turn projects to the limbic system (Peng & Bentivoglio, 2004).

In addition to classical neurotransmission, diffusible molecules can convey circadian signals. The rhythmic expression of such molecules may also depend on neuronal activity, as has been demonstrated for VP (Arima *et al.*, 2002). Transplantation of SCN tissue that is encapsulated to prevent outgrowth of neurites, to SCN lesioned animals, restores locomotor activity rhythm, demonstrating that some circadian signals are independent of neural projections (Silver *et al.*, 1996). Candidate molecules to act as diffusible circadian signals are transforming growth factor (TGF)- $\alpha$  (Kramer *et al.*, 2001), prokineticin-2 (Cheng *et al.*, 2002) and cardiotrophin-like cytokine (Kraves & Weitz, 2006), which are all rhythmically expressed in the SCN. They inhibit circadian rhythms of locomotor activity, possibly by acting on cells in the periventricular hypothalamus (Kramer *et al.*, 2001; Kraves & Weitz, 2006). Interestingly, TGF- $\alpha$  is produced by astrocytes in the SCN (Li *et al.*, 2002), thus implicating glial cells in diffusible outputs of the biological clock. SCN-derived astrocytes display clock gene oscillations *in vitro* (Prolo *et al.*, 2005) and respond to inflammatory signals (Leone *et al.*, 2006), but it is not known how the rhythmic properties of astrocytes may be affected by inflammatory molecules or how this in turn may affect SCN neuronal activity.

Notably, certain SCN mediated functions, including circadian rhythms in melatonin release and cortisol secretion as well as gonadal regression in the absence of light, are not restored by transplantation of encapsulated SCN tissue, indicating that they are dependent on SCN nerve fiber projections (Lehman *et al.*, 1987; Meyer-

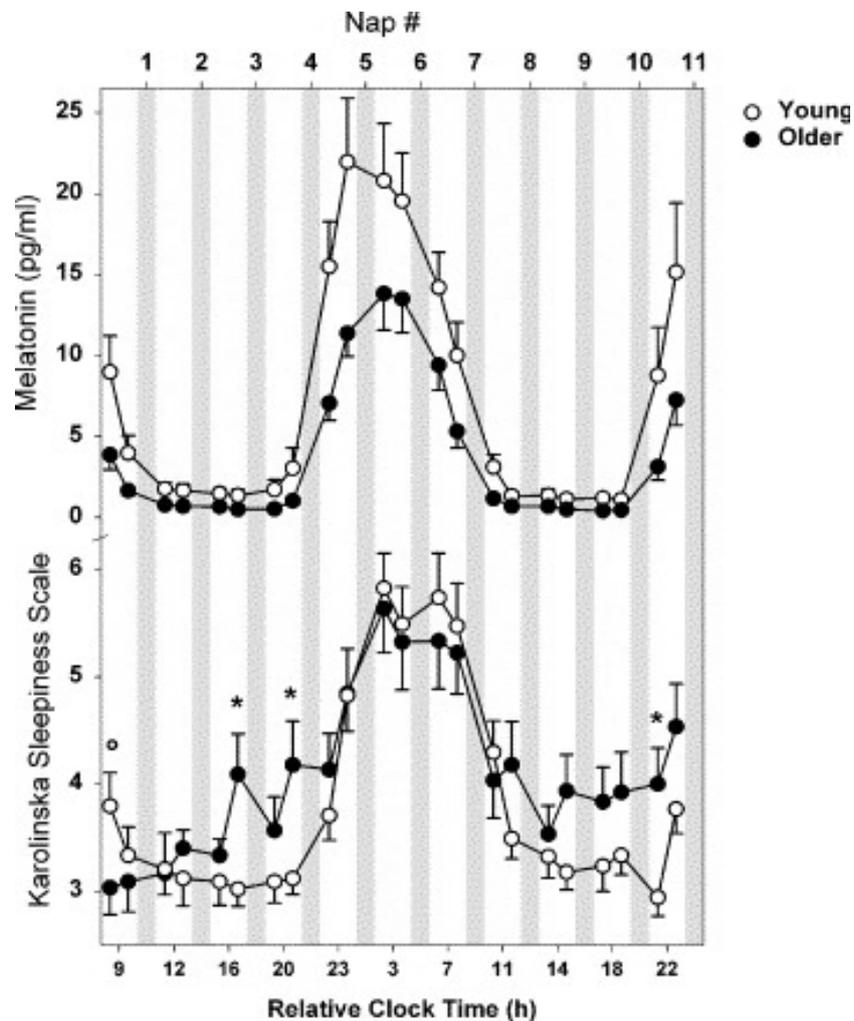
Bernstein *et al.*, 1999). Targets of SCN neurons, apart from interneurons in the medial hypothalamus as discussed above, include endocrine neurons that directly control hormonal secretion and autonomic sympathetic and parasympathetic neurons (Kalsbeek & Buijs, 2002). For example, circadian rhythm in corticosterone secretion is controlled indirectly by the SCN through corticotrophin-releasing hormone neurons in the PVN that control pituitary neurons. Similarly, melatonin release by the pineal gland is regulated by the SCN via a multisynaptic pathway connecting the PVN, preganglionic sympathetic neurons in the spinal cord and neurons in the superior cervical ganglion (Moore & Klein, 1974; Larsen *et al.*, 1998).

Altogether, both neuronal projections and diffusible factors serve as output signals from the SCN and are critical for maintaining circadian rhythms of different parameters. Diminished SCN output can be seen during disease conditions and is associated with dampened amplitude of downstream circadian rhythms (e.g. Morton *et al.*, 2005).

### **1.3 CIRCADIAN RHYTHM DISTURBANCES DURING AGING**

During aging the circadian timing system undergoes alterations that negatively affect the quality of life for the individual (for review, see Weinert, 2000; Hofman & Swaab, 2006). These changes include a decreased capacity to synchronize with external stimuli as well as fragmentation and decreased amplitude in the cycles of rest-activity, body temperature, melatonin secretion and sleep-wakefulness (see for example Czeisler *et al.*, 1992; Duffy *et al.*, 1998; Munch *et al.*, 2005) (Fig. 4). Normal aging is a complex and multifaceted process, and age-related disruption of circadian rhythms may be due to changes at several levels of the circadian system. Transmittance of the crystalline lens of the eye is reduced during aging (Weale, 1988), which may result in weakened light input to the SCN. Aging is also associated with changed oscillatory capacities of peripheral tissues that may contribute to altered behavioral rhythms (Yamazaki *et al.*, 2002). There is also evidence suggesting that changes within the SCN itself contribute to age-related circadian rhythm changes (Weinert, 2000). For instance, transplanting fetal SCN tissue to aged rats restores several rhythms, suggesting that properties of the SCN change during aging (Hurd *et al.*, 1995; Li & Satinoff, 1998).

In aged humans the total number of neurons and the volume of the SCN decrease only after the age of 80 (Swaab *et al.*, 1985). Also the number of VP-expressing neurons decreases only after the age of 80, whereas the number of VIP-expressing neurons decreases already at middle age in males whereas no reduction occurs in



**Figure 4. Altered rhythms in aged individuals.**

Melatonin secretion and sleepiness ratings in young (20-31 years) and older (57-74 years) subjects during a 40 h protocol with scheduled 75/150 min sleep-wake cycles. Note that older individuals show lower melatonin secretion and increased subjective sleepiness in the afternoon and evening on both the first and second day of the study. From (Munch *et al.*, 2005) with permission from Elsevier.

females (Zhou *et al.*, 1995; Hofman *et al.*, 1996). In rodents, the total number of SCN neurons and the volume of the nucleus are unaffected in old compared to adult rodents (Madeira *et al.*, 1995; Tsukahara *et al.*, 2005). Regarding clock genes, the expression of *Per* and *Cry* are unaltered in old hamsters (Kolker *et al.*, 2003) and *Per* genes are normally expressed in aged rats (Asai *et al.*, 2001; Yamazaki *et al.*, 2002), although the free running period of *Per1* is shortened in aged rats (Yamazaki *et al.*, 2002). Expression of *Bmall* and *Clock* is on the other hand reduced in aged hamsters (Kolker *et al.*, 2003), and in aged mice the levels of *Per2* are lower at circadian time (CT) 7, which may reflect either a reduced amplitude of the molecular cycle or a shifted peak of its expression (Weinert *et al.*, 2001). In addition to these subtle changes in basal clock gene expression in the SCN, aged animals show diminished induction of *Per1* by

light pulses (Asai *et al.*, 2001; Kolker *et al.*, 2003) and after a phase shift, the *Per1* expression in the SCN of aged rats resynchronizes more slowly to the new phase (Davidson *et al.*, 2006b). Furthermore, in aged animals both faster and severely delayed *Per1* resynchronization occurs in other tissues as compared to young animals, indicating an internal organ-desynchrony in aged animals (Davidson *et al.*, 2006b). Old rodents also have a decreased sensitivity to the phase shifting ability of light on locomotor activity rhythms (Zhang *et al.*, 1996), and it takes longer time to re-entrain locomotor activity rhythms in old animals after a shifted light/dark (LD) schedule as compared to young animals (Valentinuzzi *et al.*, 1997). The compromised ability of aged mice to resynchronize after a phase shift becomes dramatically evident in a mouse model of chronic jet-lag in which phase advances are repeated once every week. This caused a high mortality rate in aged animals with only 47% survival after 8 weeks, which was not observed in young animals (Davidson *et al.*, 2006a). The underlying cause of the increased mortality is presently not known, but may involve sleep deprivation effects or disruption of the immune system (Davidson *et al.*, 2006a).

Electrophysiological studies demonstrate a reduced amplitude of the spontaneous firing rate rhythm both in SCN slices from aged hamsters and rats (Satinoff *et al.*, 1993; Watanabe *et al.*, 1995) and cultures of dispersed SCN neurons from middle-aged mice (Aujard *et al.*, 2001). This could reflect a reduced day/night oscillation of VIP in the aged SCN (Chee *et al.*, 1988; Kawakami *et al.*, 1997; Krajnak *et al.*, 1998; Duncan *et al.*, 2001; Kallo *et al.*, 2004), given that VPAC<sub>2</sub> receptor deficient SCN neurons are hyperpolarized (Pakhotin *et al.*, 2006) and that exogenous application of VIP modulates SCN spontaneous neural activity (Reed *et al.*, 2002). VIP also affects inhibitory synaptic transmission in the SCN (Itri & Colwell, 2003) that consequently may be affected by aging.

Gene-expression profiling in the human frontal cortex indicates that the expression of molecules involved in synaptic transmission is decreased during aging (Lu *et al.*, 2004), and a decreased density of synapses has been described in the aged monkey cerebral cortex (Peters *et al.*, 2001). This type of structural alterations can be found in the absence of major neuronal loss (Hof & Morrison, 2004; Burke & Barnes, 2006).

The impaired ability of aged animals to adapt to phase shifts and the reduced amplitude of several functional rhythms in the SCN indicates that the coupling of SCN neurons may be weakened. Coupling and synchronization in the SCN is to a large extent dependent on neurotransmission but it is not known if a loss of number and/or function of synapses occurs in the SCN during aging.

## 1.4 THE NERVOUS AND IMMUNE SYSTEM INTERPLAY – EFFECTS ON CIRCADIAN RHYTHMS

Age-related alterations in the nervous system are paralleled by marked changes in the immune system, and it is therefore of interest to study to what extent the two processes may be linked. The major function of the immune system is to protect the organism from infection. This is accomplished by the innate immune system, which responds rapidly but non-specifically to foreign antigens, and by the adaptive immune system, which is activated by the innate immune system and tunes the response to more efficiently target a certain pathogen. Cytokines are important signaling molecules that regulate the activity of immune cells in both the innate and adaptive immune system. Cells of the adaptive immune system include B and T cells. T cells recognize foreign antigens presented by major histocompatibility complex (MHC) molecules expressed on the surface of a target cell. The T cells are thereby activated to secrete interferon (IFN)- $\gamma$  and/or to exhibit cytotoxicity.

The brain is an immune privileged site, which is an important feature of this organ in order to protect non-replaceable neurons from deleterious damage by immune-mediated responses (Nieder Korn, 2006). The prevention of uncontrolled invasion of molecules and lymphocytes into the brain parenchyma by the blood-brain barrier, the high expression of transforming growth factor- $\beta$  and other immuno-suppressive factors in the brain and the insufficient neuronal expression of MHC class I molecules in terms of eliciting lysis by cytotoxic T cells, all contribute to avoid immune mediated damage of neurons (Joly *et al.*, 1991; Pachter *et al.*, 2003; Nieder Korn, 2006). The immune privilege is, however, not absolute. Expression of MHC class I molecules on neurons can be induced by IFN- $\gamma$ , and this induction is enhanced by TTX treatment (Neumann *et al.*, 1995; Medana *et al.*, 2000), rendering neurons sensitive to lysis by cytotoxic T cells (Medana *et al.*, 2000).

A bi-directional communication exists between the brain and the immune system, in which the brain has widespread effects on the immune system, and conversely, the immune system signals to, and affects functions of the brain (Wrona, 2006). Cytokines, which are potent, pleiotropic molecules involved in intercellular signaling, are the most prominent signals from the immune system to the brain. During infections, cytokines can affect the brain to induce fever and neuroendocrine changes and so-called “sickness behavior”, which includes increased time spent in sleep (Konsman *et al.*, 2002). The most notable cytokines involved in such responses are tumor necrosis factor (TNF)- $\alpha$ ,

interleukin (IL)-1 $\beta$ , IFN- $\gamma$  and IL-6 (Konsman *et al.*, 2002). These and other cytokines can also be produced by resident brain glial cells, and TNF- $\alpha$  has recently been shown to play an important role in modulating neuronal function and homeostasis (Stellwagen & Malenka, 2006). Within the brain, microglia and astrocytes are the major sources of cytokines (Vitkovic *et al.*, 2000).

Among the cytokines, especially IL-1 $\beta$  and TNF- $\alpha$  are involved in regulating sleep (Krueger *et al.*, 2001; Bryant *et al.*, 2004). Intracerebroventricular (i.c.v.) injection of TNF- $\alpha$  enhances the duration of non-rapid eye movement (NREM) sleep (Shoham *et al.*, 1987), and inhibition of endogenous TNF- $\alpha$  with anti-TNF- $\alpha$  antibodies reduces NREM sleep (Takahashi *et al.*, 1995). Microinjections of TNF- $\alpha$  into the preoptic area in rats induces NREM sleep (Kubota *et al.*, 2002), but effects of TNF- $\alpha$  on other nuclei involved in sleep regulation remain to be analyzed. For instance, the SCN influences sleep regulation, but compared to the vast literature on how cytokines affect sleep, relatively little is known about the effects of inflammatory molecules on circadian rhythms and the SCN.

In the following sections the possible contribution of immune molecules to disturbances of circadian rhythms during aging and inflammatory conditions will be discussed.

#### 1.4.1 Cytokines can modulate circadian rhythms

The notion that cytokines can affect the circadian system stems from observations of circadian rhythm disturbances in conditions when cytokine levels are elevated. For instance, in rheumatoid arthritis circadian neuroendocrine rhythms of corticosterone are suppressed (Cardinali & Esquifino, 2003), in colorectal cancer dampened rest-activity rhythms are correlated with higher serum levels of cytokines (Rich *et al.*, 2005) and in human African trypanosomiasis or sleeping sickness there are altered circadian rhythms of cortisol, prolactin and IFN- $\gamma$  (Radomski *et al.*, 1994). Animal studies have shown that inflammatory challenges can cause physiological alterations in the SCN. Treatment with the bacterial wall toxin lipopolysaccharide (LPS), which may signal via Toll-like receptor 4 and release TNF- $\alpha$  and IL-1 $\beta$  from macrophages, causes changes in locomotor activity rhythms and induction of Fos, a marker for neuronal activation, in the dorsal SCN (Marpegan *et al.*, 2005). Also i.c.v. injection of IFN- $\gamma$  causes induction of Fos-protein in SCN neurons (Robertson *et al.*, 2000). Long-term treatment with LPS, representing a chronic inflammation, results in a diminished SCN response to photic

stimuli (Palomba & Bentivoglio, 2007), and subcutaneous administration of TNF- $\alpha$  for a prolonged period of time causes reduced locomotor activity and prolonged rest periods and interferes with expression of the clock-controlled gene *Dbp* in the SCN (Cavadini *et al.*, 2007). Peripherally administered interferons also reduce SCN clock gene expression and responses to photic stimuli, and weaken locomotor and body temperature rhythms (Ohdo *et al.*, 2001). Inflammatory molecules released in the periphery can thus affect circadian rhythms and alter SCN function. The arcuate nucleus in the hypothalamus has an increased accessibility to molecules in the blood circulation, and has been proposed to receive and transmit metabolic information from the body to the SCN (Buijs *et al.*, 2006; Deboer & Marks, 2006). Cytokines in the blood circulation could potentially also affect the SCN via this pathway. It should be noted that cytokine activities directly in peripheral tissues also can contribute to circadian behavioral effects (Cavadini *et al.*, 2007).

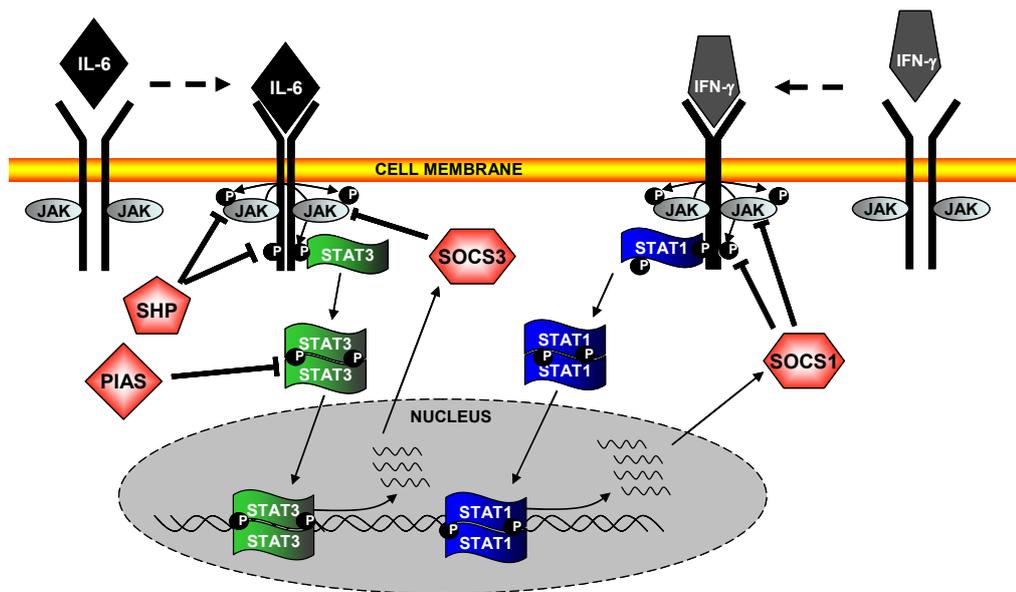
Cytokines can also affect the SCN in experimental *in vitro* systems. A cocktail of IFN- $\gamma$ , LPS and TNF- $\alpha$  disrupts SCN neuronal activity rhythms and can acutely affect SCN synaptic activity (Lundkvist *et al.*, 2002). The expression of TNF- $\alpha$  shows a circadian rhythm in the brain (Floyd & Krueger, 1997; Cearley *et al.*, 2003), and SCN-derived astrocytes in culture, which respond to TNF- $\alpha$  (and also IL-1 $\alpha$  and LPS) with increased expression of nuclear factor- $\kappa$ B, have been proposed to act as an interface for immune-circadian signaling (Leone *et al.*, 2006). TNF- $\alpha$  affects synaptic transmission and spontaneous firing activity in some areas of the brain (Shibata & Blatteis, 1991; Tancredi *et al.*, 1992; Katafuchi *et al.*, 1997), but it is not known if TNF- $\alpha$  can affect these parameters in the SCN.

Although a few studies show that circadian rhythms are affected during inflammatory conditions, relatively little is known about which cytokines may influence the SCN, and through which mechanisms they operate.

#### 1.4.2 Control of intracellular responses to cytokine signaling

Cytokines act as key communicators between immune cells and are vital for eliciting immune responses during infections. Many cytokines signal via the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to elicit cellular responses (Fig. 5). In order to avoid excessive signaling, at least three main classes of intracellular negative regulators that affect components of the JAK/STAT pathway have been identified. These include: tyrosine phosphatases, e.g. SH2-containing phosphatases (SHP) that dephosphorylate and thereby deactivate cytokine receptors,

protein inhibitors of activated STATs (PIAS), and suppressors of cytokine signaling (SOCS) (Fig. 5). The SHP and PIAS molecules are constitutively expressed, whereas SOCS molecules are induced in response to cytokines and act as negative feedback inhibitors (for review see Wormald & Hilton, 2004; Rakesh & Agrawal, 2005). SOCS molecules target the cytokine receptor signaling complex for ubiquitin-mediated degradation, but some of the SOCS proteins can also directly inhibit JAK tyrosine kinase activity by acting as pseudosubstrates (Yoshimura *et al.*, 2007). The SOCS family of proteins consists of eight members (CIS and SOCS1-SOCS7), with both overlapping and specific functions. Of these, SOCS1 and SOCS3 are rapidly induced and play important roles in regulating cellular responses to IFN- $\gamma$  and IL-6 respectively (Wormald *et al.*, 2006). SOCS1 is involved in regulating STAT1-signaling, and lack of the *Socs1* gene leads to embryonic lethality due to excessive inflammation (Naka *et al.*, 1998; Starr *et al.*, 1998). Also SOCS3 deficient mice show embryonic lethality (Marine *et al.*, 1999).



**Figure 5. Regulation of intracellular cytokine signaling.**

Cytokines exert their effects via the JAK/STAT pathway, here exemplified by IL-6 and IFN- $\gamma$ . Ligand binding leads to activation of JAK which phosphorylates the receptor to allow binding and subsequent activation of STAT molecules. Activated STAT molecules dimerize; the complex thereafter translocates to the nucleus and activates transcription of an array of genes. Different cytokines induce the expression of specific SOCS molecules; however there is considerable cross talk between these pathways and one cytokine may activate several STAT molecules and hence induce several SOCS proteins. SOCS proteins act as negative feedback inhibitors by targeting components of the JAK/STAT-pathway thereby inhibiting further intracellular signaling. SHP and PIAS are constitutive inhibitors that act on JAK and STAT molecules, respectively.

Relatively little is known about regulation of cytokine signaling during physiological conditions in the brain. PIAS1 may be an important regulator of IFN- $\gamma$  signaling via inhibition of activated STAT1 (Maier *et al.*, 2002), while SHP-1 has been localized to oligodendroglial cells (Massa *et al.*, 2000) and may regulate levels of IL-1 $\beta$  in the brain (Zhao & Lurie, 2004). SOCS genes are also expressed in the developing and adult brain. SOCS2 regulates neuronal differentiation and shows high expression in neurons, especially during development, whereas SOCS1 and SOCS3 expression is low both in the developing and adult brain, with a widespread distribution (Polizzotto *et al.*, 2000).

During inflammatory conditions, levels of cytokines can increase dramatically in the brain and the negative feed-back regulators SOCS could then play an important role in limiting the response to these potentially harmful signals. Especially SOCS1 and SOCS3 are induced in the brain during inflammation. In experimental allergic encephalomyelitis SOCS1 and SOCS3 transcripts have been restricted to infiltrating mononuclear cells (Maier *et al.*, 2002), whereas after a seizure, SOCS mRNA is up-regulated in the hippocampus (Rosell *et al.*, 2003). SOCS3 is expressed in the arcuate nucleus in the hypothalamus in response to leptin to regulate the sensitivity to this hormone (Mori *et al.*, 2004), and SOCS3 also regulates effects of TNF- $\alpha$  on cortical neurons *in vitro* (Yadav *et al.*, 2005). In peripheral neurons, a role has been described for SOCS1 in regulating IFN- $\gamma$ -induced MHC class I expression (Turnley *et al.*, 2002). In central nervous system (CNS) neurons, IFN- $\gamma$  can induce STAT1 phosphorylation (Jin *et al.*, 2004) and MHC class I expression after TTX treatment (Neumann *et al.*, 1995). However, it remains to be determined if SOCS1 plays a role in limiting responses of CNS neurons to cytokines

#### 1.4.3 Aging and neuroinflammation

The aging process is characterized by a dysfunction of the immune system often referred to as immunosenescence or “inflammaging” (Miller, 1996; Franceschi *et al.*, 2007). This state is associated with low-grade chronic inflammatory activity (Saurwein-Teissl *et al.*, 2000; Bruunsgaard *et al.*, 2001), with increased serum levels of the pro-inflammatory cytokines TNF- $\alpha$  (Paolisso *et al.*, 1998) and IL-6 (Ershler *et al.*, 1993; Hager *et al.*, 1994). The increased levels of these molecules indicates that a dysregulation of cytokine production occurs during aging that may be exacerbated by persistent subclinical infections or other chronic disorders in the aged individual (Bruunsgaard *et al.*, 2001; Nikolich-Zugich, 2005).

Gene-expression profiling of aged mouse brains indicates that the expression of genes involved in immune responses and cellular stress are upregulated as compared to young mouse brains (Lee *et al.*, 2000). Aging is also associated with altered basal levels of cytokines, as well as altered immune responses in the brain (Bodles & Barger, 2004). Levels of TNF- $\alpha$  and IL-6 are elevated in the cerebral cortex as well as in the thalamus, where also IL-1 $\beta$  and inducible NOS (iNOS) have been found to be higher in aged animals as compared to young ones (Sharman *et al.*, 2002; Sandhir *et al.*, 2004). These changes are probably a reflection of the increased basal expression of these cytokines in resident astrocytes and microglial cells (Yu *et al.*, 2002; Sierra *et al.*, 2007) that show an activated phenotype in the brain and spinal cord with advancing age (Rozovsky *et al.*, 1998; Kullberg *et al.*, 2001). There is also increased numbers of leukocytes in the aged brain (Stichel & Luebbert, 2007), which may contribute to the elevated cytokine production.

The responses of nervous tissues to inflammatory challenges are also changed during aging. Although some studies have found attenuated reactions in the aged brain or spinal cord in response to a peripheral LPS challenge or after traumatic lesions (Stuesse *et al.*, 2000; Utsuyama & Hirokawa, 2002), most reports indicate that aged animals exhibit exaggerated inflammatory responses with dramatically enhanced levels of cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (Sandhir *et al.*, 2004; Godbout *et al.*, 2005; Deng *et al.*, 2006). An enhanced inflammatory reactivity may be associated with the more marked and prolonged sickness behavior observed in aged as compared to young animals after peripheral or i.c.v. LPS injections (Imeri *et al.*, 2004; Godbout *et al.*, 2005; Huang *et al.*, 2007). Cytokines in the brain are not only involved in mediating immune responses to pathogens and sickness behavior of an animal, but also in modulating synaptic functions and sleep-wakefulness and in the pathogenesis of neurodegenerative diseases (for review see Munoz-Fernandez & Fresno, 1998; Vitkovic *et al.*, 2000; Tonelli *et al.*, 2005). Although an exaggerated immune response in the aged brain has been linked to prolonged sickness behavior (Huang *et al.*, 2007), relatively little is known about how neurons are affected by such exaggerated inflammation. It is therefore of interest to analyze the neuronal responses to cytokines in the aging brain. Importantly, it is not known whether inflammatory priming caused by senescence alters the SCN circadian clock and if this confers altered responses to an inflammatory challenge.



## 2 AIMS

The general aim of this thesis is to investigate how the circadian pacemaker, the SCN, changes during aging and to analyze the effects of pro-inflammatory cytokines on neurons in this and other brain structures, to possibly explain age-related changes in circadian rhythms.

The specific objectives are:

- To analyze whether the synaptic network of the aged SCN undergoes functional changes.
- To compare the response of young and aged SCN neurons to pro-inflammatory cytokines
- To investigate if the somnogenic cytokine TNF- $\alpha$  affects the electrical activity and clock gene expression in the SCN
- To analyze the expression of SOCS molecules in the SCN and how SOCS molecules may regulate the response of neurons to pro-inflammatory cytokines.



### 3 METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of the methods employed in this thesis are given in the original articles. Below, general aspects of the methods used are discussed.

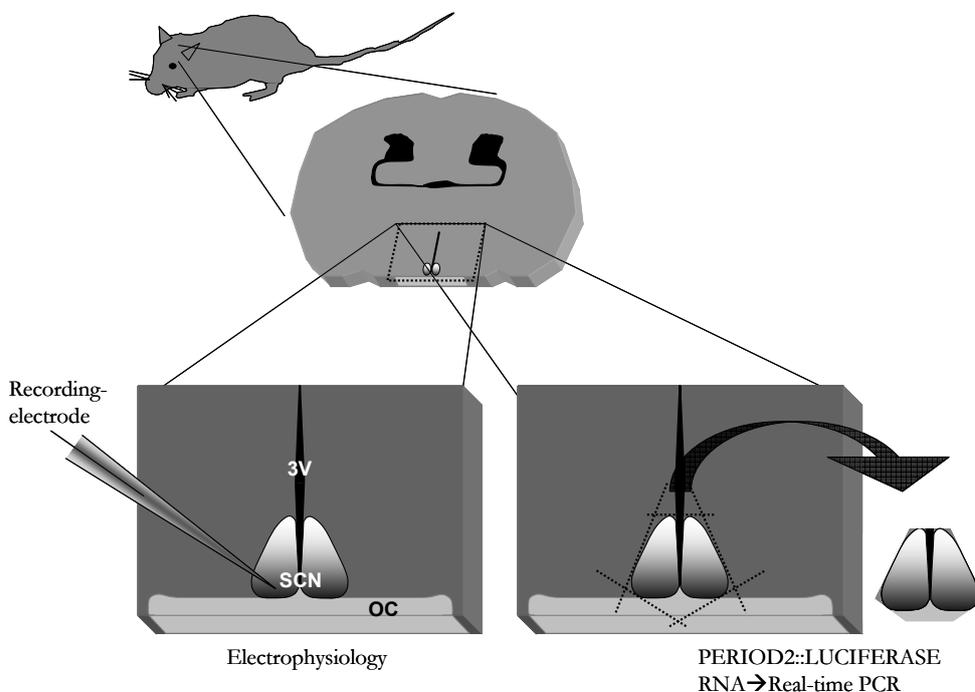
#### 3.1 ANIMALS

Individuals tend to age differently, which complicates studies of the aging process since large variations in collected data are seen also in a well-defined aged population. This becomes especially evident in studies of human subjects and has probably contributed to contradictory results in several studies. Using inbred laboratory rodents that are kept under pathogen free, strictly controlled conditions substantially reduces inter-individual variations and is therefore a valuable tool for neurobiological studies of aging. In addition to reducing inter-subject variability it allows investigations not possible in humans for practical and ethical reasons.

We have throughout this work used C57B6/J mice as experimental subjects. All animal procedures were approved by the local ethical committee (*Stockholms Norra Djurförsöksetiska Nämnd*; N71/03, N35/05, N158/06, N21/02, N34/05). Mice had *ad libitum* access to food and water and were kept on a 12/12 h LD schedule and were allowed to synchronize to the LD cycle for at least 10 days before an experiment. In the mouse SCN, synapse formation occurs until around postnatal day 10 (Moore & Bernstein, 1989). In the present work, mice aged 2-3 months were considered as young adults. At this age most aspects of the nervous system are fully developed and animals have reached sexual maturity. Terms such as “middle-aged” or “old” may not be directly applicable when referring to rodents. There are differences in life span, and hence the aging process, between rodent species and also between different strains. In addition, the life span may be influenced by feeding regimens and housing conditions (Coleman, 2004). Previous studies of age-related circadian disturbances indicate that alterations occur already at an age of 9-11 months in C3H mice (Aujard *et al.*, 2001). The average life span of most laboratory mice is 2-3 years (although mutant mice that live significantly longer have been reported) (Quarrie & Riabowol, 2004). In the present studies of age-related changes in the SCN, we used 14-21 months old mice in paper I and 15-17 months old mice in paper II.

### 3.2 THE SCN SLICE PREPARATION

The use of *in vitro* brain slice techniques has greatly advanced the knowledge and understanding of the nervous system. This methodology holds several advantages over studying neuronal function *in vivo*, the major ones being the better mechanical stability of the preparation, and the possibility to modify the extracellular environment and to easily apply drugs and other biologically active compounds. Although afferent inputs may suffer from the slice preparation process, the cyto-architecture within a slice is well preserved, enabling high qualitative studies of synaptic function. This is an advantage as compared to using dispersed cells, which implies rearrangement of synaptic connections and the use of embryonic or early postnatal tissue that may not have reached a fully mature state. In SCN slice preparations there is spontaneous inhibitory as well as excitatory synaptic activity (Kim & Dudek, 1991; Lundkvist *et al.*, 2002; Michel *et al.*, 2002), whereas in dispersed cells only inhibitory synaptic activity has been reported (Welsh *et al.*, 1995). For a structure such as the SCN that has a dense intra-nuclear synaptic network, the slice technique is an excellent model system since the rhythmic properties of the nucleus are well preserved (see Introduction). The importance of the intra-SCN network in the function of the SCN was also recently demonstrated (Liu *et al.*, 2007). In this thesis the SCN slice was therefore used for studying electrophysiological properties and clock gene expression in the SCN (Fig 6).



**Figure 6. The SCN slice.**

Coronal tissue slices (250-350  $\mu\text{m}$ ) were prepared from mouse brains and used for electrophysiology, real-time monitoring of clock protein expression and also for RNA extraction and subsequent gene expression analysis using real-time PCR.

### 3.3 ELECTROPHYSIOLOGY

With the development of the patch-clamp technique (Neher & Sakmann, 1976), which enables exact control of the intracellular environment of neurons, detailed investigations of membrane and cellular properties as well as synaptic mechanisms have become possible. One obvious difference with the brain slice technique compared to an *in vivo* recording setup is that afferent and efferent connections to the brain area of interest may no longer be present. When studying connectivity between brain areas this is obviously a major limitation, but it could also be considered an advantage in situations where afferent input is not a factor of interest. A part of the electrophysiological work was devoted to comparing the properties of the aged and young SCN. In this context acute slice preparations pose an advantage over cultured cells since culturing neurons from aged mice may require prolonged enzymatic treatment to separate the cells, which may compromise the cellular physiology of aged neurons (Verkhatsky & Toescu, 1998). However, the slice preparation procedure also confers mechanical insult to the tissue with consequent cellular stress, and may cause activation of microglial cells (Stence *et al.*, 2001), and such reactions may possibly differ in slices from young and aged animals.

The SCN generates a rhythm with high electrical activity during the subjective day and low activity during the subjective night, which was initially described by *in vivo* extracellular recordings (Inouye & Kawamura, 1979). Later studies demonstrated that this rhythmic activity of spontaneous action potentials persisted *in vitro* using extracellular single unit recordings in acute coronal slices of the SCN (Green & Gillette, 1982; Groos & Hendriks, 1982; Shibata *et al.*, 1982). Since then, SCN slice preparations have been used with great success in electrophysiological experiments to investigate the membrane and synaptic properties of SCN neurons, to determine circadian phase phenomena, and to analyze the effects of drugs and other molecules on the SCN.

Acute slice preparations were used in the present work to investigate if properties of the SCN neuronal network are affected by the aging process, and to analyze the effects of TNF- $\alpha$  on the SCN. Two recording techniques were used for these purposes, cell-attached recordings and whole-cell patch clamp recordings.

#### *Cell-attached recordings.*

For monitoring of spontaneous action potential firing rates, cell-attached recordings were used. Pipettes with a resistance of 5-9 M $\Omega$  were advanced blindly into the ventral

part of the SCN with a slight positive pressure applied. The current response to a voltage pulse was continuously monitored during this procedure. When the current response decreased, indicative of the approach of a cell, the pressure was released and slight suction was applied to achieve a seal of  $< 50 \text{ M}\Omega$ , a so-called loose-patch. This configuration allows high amplitude signals of spontaneous action potentials to be recorded for long time periods with minimal negative inference of the cell (Nunemaker *et al.*, 2003).

#### *Whole-cell patch clamp recordings.*

For recordings of spontaneous synaptic events, whole-cell patch clamping was used. As for cell-attached recordings the pipette was advanced blindly into the tissue until a cell was approached and negative pressure was applied to achieve a giga-seal (a seal with a resistance  $> 1 \text{ G}\Omega$ ). By increasing the suction briefly, the membrane was ruptured and access was gained to the intracellular milieu of the cell. This enables control of the cell membrane voltage and allows spontaneous activity of the cell and spontaneous synaptic input to be recorded. The voltage clamp mode, in which the membrane voltage is fixed and the current required to keep the membrane at this voltage is recorded, and the current clamp mode, where instead the voltage fluctuations are recorded, were used in this thesis.

### **3.4 PER2::LUCIFERASE RECORDINGS**

To monitor real-time clock gene activity we used a transgenic mouse knock-in line that produce a PER2::LUCIFERASE (PER2::LUC) fusion protein under the control of the *Per2* promoter (Yoo *et al.*, 2004). The level of expression of firefly luciferase provides a real-time report of PER2 expression of the cultured tissue. Importantly, the PER2::LUC protein is functional *in vivo* as these mice show normal circadian locomotor behavior. This system was employed to study SCN PER2 expression. The SCN slice was dissected in principle as for electrophysiological experiments but the non-SCN tissue was surgically removed. The bilateral SCN was separated and the two nuclei from each slice were prepared for organotypic culture.

### **3.5 IMMUNOHISTOCHEMISTRY**

Immunohistochemistry was used for the detection of proteins in both cultured cells and tissue sections. The technique is based on the highly selective affinity of antibodies to

their antigens, in this case specific proteins within cells or on their membrane surface. The technique was employed in paper II for detection of Fos protein in SCN tissue sections, and in paper IV for detection of cell type specific proteins in cultured cells (see the respective papers for details).

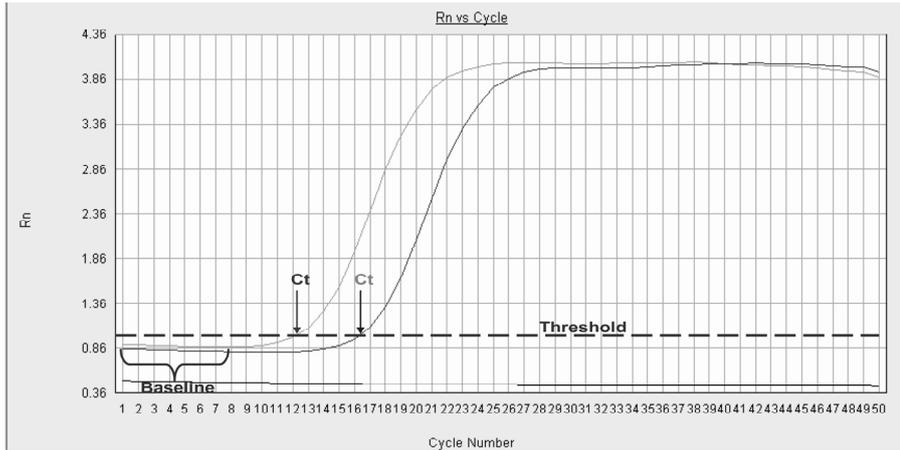
### **3.6 REAL-TIME PCR**

The introduction of the polymerase chain reaction (PCR) in the late 80s revolutionized molecular biology by providing a tool to perform gene cloning and study RNA (Mullis & Faloona, 1987). This technique allows a specific sequence of DNA to be amplified exponentially by using the heat stable enzyme Taq DNA polymerase originally isolated from the bacterium *Thermus Aquaticus*, and sequence specific primers binding only to certain segments of DNA (Saiki *et al.*, 1988). The application of this technique is very wide, but in this thesis it has been used to measure the expression of certain genes, which is reflected in the abundance of messenger RNA (mRNA) molecules encoded by these genes. The mRNA molecule is translated into a protein molecule based on its nucleotide sequence and the level of mRNA generally correlates to the level of protein.

In order to quantify RNA molecules in extracts from a cell-culture or tissue sample, the extracted total RNA is first transcribed into to a complementary DNA (cDNA) strand by adding randomly generated primers hybridizing to the RNA. These primers are subsequently extended along the template by a reverse transcriptase enzyme. The cDNA can then be used as template in the PCR reaction. The major advantage of this approach is that very low numbers of template molecules can be detected.

The PCR reaction procedure involves a number of cycles, each cycle consisting of heating steps that cause: 1) denaturation of double stranded DNA, 2) annealing of primers and 3) extension of annealed primers. By repeating this procedure the sequence delimited by the primers is amplified exponentially. In regular PCR the cycling is stopped after a predetermined number of cycles, and gel-electrophoresis is used to verify that the size of the product is correct. This procedure was employed to determine the genotype of embryos from interbred *Socs1<sup>+/-</sup>* mice that were used for hippocampal cultures (paper IV). Genomic DNA was then extracted from embryonic tissue and used as PCR template.

Real-time PCR follows the general procedure of the classical polymerase chain reaction, but the amount of DNA is quantified after each round of amplification



**Figure 7. Real-time PCR.**

The amplification curves for two samples are shown, with fluorescence intensity plotted versus cycle number. During the initial reaction-cycles the fluorescence is below the detection limit. The point at which the fluorescence starts increasing exponentially is related to the amount of starting template. The Ct value is defined as the fractional cycle when the fluorescence passes a threshold value that is set within the exponential phase of the reaction.

(i.e. in real-time) rather than at the end of a given number of rounds (Fig. 7). This feature offers the method a wide dynamic range of measurement. The quantification of the amplified product is commonly achieved by using a compound that fluoresces only when bound to double stranded DNA. A number of such molecules are used for real-time PCR and in the present work SYBR green was used. The read-out is based on the number of cycles required for the fluorescence signal of a sample to reach a certain threshold value, i.e. cycle threshold (Ct) value. This Ct-value is then usually compared to that of a stably expressed gene, not affected by the experimental conditions, a so-called endogenous control gene. By subtracting the Ct-values for the endogenous control and the gene of interest, a relative value ( $\Delta Ct$ ) of the expression of the gene of interest can be calculated.  $\Delta Ct$  values can then be compared between different samples (Livak & Schmittgen, 2001). Care must be taken to ensure that the endogenous control gene is not affected differentially between the experimental groups. The housekeeping genes GAPDH, 18S,  $\beta$ -Actin and cyclophilin are commonly used as endogenous controls. In the present work, the expression levels of several of these house-keeping genes were tested. The levels of transcripts encoding cyclophilin exhibited the least variation between samples and were not significantly different when comparing experimental and control groups. Cyclophilin was therefore subsequently used as an endogenous control in the presented experiments. The real time PCR method has been

employed both for SCN tissue samples (paper II) and cell culture samples (paper IV) to evaluate gene expression.

### **3.7 CELL CULTURE**

In order to investigate cellular responses to cytokines, and how these responses are regulated, we have employed primary cultures of neurons derived from the central nervous system as well as cell lines. An advantage with primary culture systems is that the conditions can be modified to achieve relatively pure cultures of one type of cell, so that effects can be differentiated between cell types, which may be problematic when using intact tissue. Furthermore, cells can be maintained for long periods of time, enabling long-term exposure to compounds. On the other hand, there is a close functional connection between neurons and glial cells in the nervous system and the properties of different cell types may change when isolated *in vitro*. Cell lines represent an experimental system that is completely free of contaminating cells, but instead, these immortalized cells may have altered functions and expression of cell-type specific molecules.

For neuronal cultures, hippocampus tissue was dissected from embryonic mice at gestation day 17. The tissue was dispersed and seeded into cell culture wells and grown in serum free conditions with the addition of B27 supplement to inhibit the growth of other cell types. Culture of dispersed hippocampal neurons is a well established *in vitro* experimental system, and these cells are known to form a dense synaptic network (e.g. Jin *et al.*, 2004). The neurons were allowed to differentiate for at least 10 days *in vitro* to reach a mature state before experiments were performed. To investigate the possible influence of contaminating glial cells in primary hippocampal cultures, primary neocortical glial cell cultures were also used for comparison.

## 4 RESULTS AND DISCUSSION

### 4.1 SYNAPTIC PROPERTIES OF THE SCN IN YOUNG AND AGED MICE (PAPER I)

Numerous changes occur in circadian rhythms during aging and there are indications that alterations in the function of the SCN may account for some of these changes. We therefore wanted to investigate if properties of the SCN neuronal network are altered during aging. Electrophysiological recordings were made in SCN-slices from young adult (2-3 months old) and aged (14-21 months old) mice during the subjective day (*Zeitgeber* time (ZT) 4–10), and night (ZT 14–20). Cell-attached recordings were performed to analyze spontaneous action potentials. When analyzing all spontaneously active cells, both young and aged animals showed a significant day/night difference in spontaneous firing rate. Although the firing rate day/night ratio was lower in aged mice, this difference was not statistically significant. This finding contrasts previous results from 23-28 months old Long-Evans rats (Satinoff *et al.*, 1993) and 24 months old hamsters (Watanabe *et al.*, 1995) in which a more pronounced decrease in firing rate amplitude was observed. In addition to differences in species and age of the experimental animals, our analysis was restricted to the ventrolateral part of the SCN, which contains a significantly lower proportion of neurons displaying rhythmic activity compared to the dorsomedial region (Nakamura *et al.*, 2001), while in the previous studies recordings were made throughout the SCN (Satinoff *et al.*, 1993; Watanabe *et al.*, 1995). Furthermore, silent cells, i.e. cells that did not fire spontaneous action potentials, were not included in the present analysis of spontaneous firing rate, which also had an impact on the amplitude of the firing rate rhythm. Young animals showed a significant rhythm in proportion of silent cells which were more numerous during the subjective night as compared to the day, in line with previous reports (Schaap *et al.*, 1999). In aged animals on the other hand, this rhythm was lost and an increased proportion of silent cells was observed during the day. The increased number of silent cells in aged mice may reflect alterations in the pacemaking properties of SCN neurons, i.e. the ionic currents that drive depolarization of the neuron to spike threshold and cause spontaneous firing. In the SCN, slowly inactivating persistent Na<sup>+</sup> channels (Jackson *et al.*, 2004; Kononenko *et al.*, 2004) and L-type voltage dependent Ca<sup>2+</sup> channels (Pennartz *et al.*, 2002) are involved in this process, and it would therefore be of interest to analyze if the expression or properties of these channels change in the

aged SCN. An increased proportion of cells that do not fire spontaneous action potentials in aged animals could potentially lead to a reduced output from the ventrolateral SCN, which would affect target areas including the cells in the dorsomedial SCN and also extra-SCN areas.

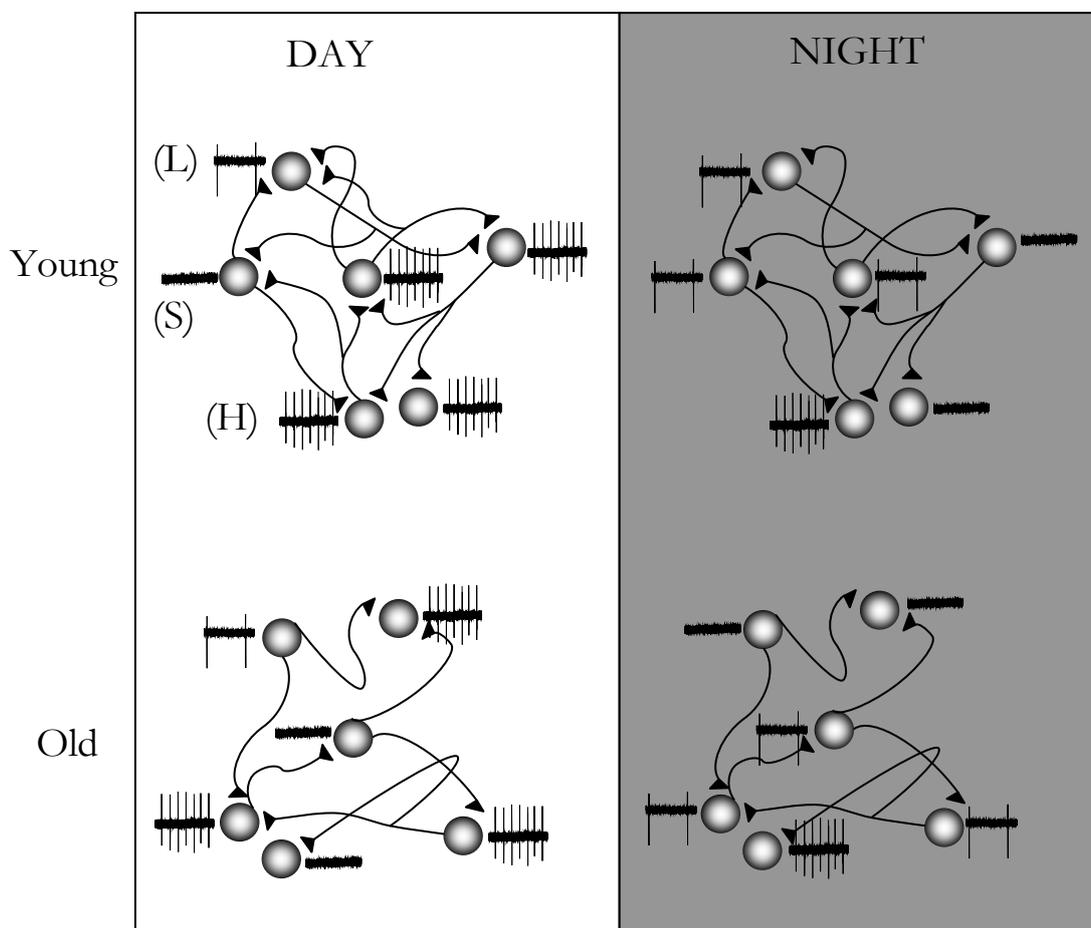
Whole-cell recordings were performed to investigate whether synaptic transmission in the SCN is altered in aged mice. Inhibitory postsynaptic currents (IPSCs) were predominant in the recorded cells whereas excitatory postsynaptic currents (EPSCs) were seen at low frequency or not at all and were therefore not subjected to analysis. Picrotoxin blocked all IPSCs demonstrating that they were GABAergic currents. No difference in the IPSC frequency was seen between day and night in either the young or aged mice. This result is consistent with previous characterizations of inhibitory SCN currents that report no day/night rhythm in IPSC frequency in the ventral SCN (Lundkvist *et al.*, 2002; Itri & Colwell, 2003; Itri *et al.*, 2004). In aged mice a clear reduction was seen in IPSC frequency both during the subjective day and night as compared to young mice. One modulator of inhibitory activity in the SCN is VIP, that can increase IPSC frequency (Itri & Colwell, 2003; Itri *et al.*, 2004). VIP is confined to the ventral SCN (Abrahamson & Moore, 2001) and its expression is reduced in aged rodents (Kawakami *et al.*, 1997; Duncan *et al.*, 2001; Kallo *et al.*, 2004). A reduction of VIP expression may therefore account for the reduced IPSC frequency currently observed. Since a large part of inhibitory synaptic input to ventral SCN neurons is derived from other SCN neurons (Strecker *et al.*, 1997), the larger number of silent cells in aged animals may also contribute to the observed reduction of IPSC frequency.

Pharmacological blockade of GABAergic activity leads to an increase in spontaneous firing in the ventral SCN (Albus *et al.*, 2005), and aged SCN neurons, which receive less GABAergic inhibition, would therefore be expected to increase their firing rate. An increased firing rate was however not observed, indicating that additional factors that regulate spontaneous firing may be affected in aged SCN neurons. Considering that GABAergic signaling is vital for regional coupling within the SCN during phase shifts (Albus *et al.*, 2005) and that GABA may affect synchronization of SCN neurons (Liu & Reppert, 2000), it is likely that the loss of synaptic network activity has negative consequences for the functioning of the SCN.

We have also analyzed the synaptic network in the aged SCN using quantitative confocal imaging analysis of tissue sections incubated with antibodies against synaptic terminal proteins. In support of a reduced GABAergic activity described above, we

have found by immunohistochemistry, a reduced density of synaptic terminals, including GABAergic synaptic terminals, in the SCN of aged mice (Palomba M, Nygård M, Kristensson K, Bentivoglio M, unpublished observations). This suggests that the observed reduction of synaptic transmission may in part be related to morphological changes in the aged SCN.

*In summary, SCN neurons in aged mice showed normal spontaneous firing, but a larger proportion of silent cells and markedly reduced GABAergic synaptic transmission. The underlying cause of these changes may involve a reduced density of GABAergic presynaptic terminals and possibly changes in pacemaking properties of aged SCN neurons. The described changes may contribute to reduced SCN output signals and alterations of circadian rhythms during aging.*



**Figure 8. Schematic summary of changes in the aging SCN synaptic network.**

Cells firing action potentials with high frequency (H), low frequency (L) and silent cells, firing no action potentials (S) are shown. In young mice the proportion of silent cells was lower during the day than during the night. Such a rhythm was not found in aged mice that had an elevated number of silent cells during the day. Furthermore, the density of the synaptic network was decreased in the aged SCN.

## 4.2 PRO-INFLAMMATORY CYTOKINES – EFFECTS ON THE YOUNG AND AGED SCN (PAPER II)

Since both TNF- $\alpha$  and IFN- $\gamma$  can affect the function of SCN neurons (paper III and Ohdo *et al.*, 2001; Lundkvist *et al.*, 2002), we investigated if young and aged animals responded differently to cytokine exposure and if SOCS molecules are involved in regulating responses of the SCN to such exposure.

The response of SCN neurons to pro-inflammatory cytokines was analyzed by immunohistochemical labelling for Fos protein, which is a marker for neuronal activation. After i.c.v. injections of a mixture of TNF- $\alpha$  and IFN- $\gamma$ , both young and aged mice showed a stronger induction of Fos after injections performed during early subjective night compared to injections during early subjective day. This coincided with the time point when the lowest basal expression of SOCS mRNA molecules was detected. SOCS1 and -3 mRNA levels precedes SOCS protein levels with ~30 min after *in vitro* cytokine stimulation (Wormald *et al.*, 2006), indicating that SOCS protein levels in the SCN also will be low at this time point. Thus, low expression of SOCS molecules during early subjective night may present a temporal window when inflammatory molecules may act on the SCN. In fact, interferons have stronger effects on clock gene expression in the SCN when injected during early night than during early day (Ohdo *et al.*, 2001), and also LPS phase shifts the locomotor activity rhythm only when injected during early night (Marpegan *et al.*, 2005).

The Fos response to i.c.v. injections was similar in the two age groups during early day but blunted in aged animals as compared to young animals after injections performed during early night. At this time point the Fos expression in young animals was seen throughout the SCN with very strong immunolabelling in the ventral part of the nucleus. In aged mice on the other hand, labelling in the ventral part of the SCN was weak.

There was no significant difference between young and aged mice in the basal expression of SOCS1 and SOCS3 mRNA at any time point in the SCN. It has been reported that SOCS3 levels increase in the aged hypothalamus of rats, which may contribute to leptin-resistance during aging (Wang *et al.*, 2001; Peralta *et al.*, 2002). The elevated SOCS3 level is thought to be caused by age-associated hyperleptinemia. Although not determined, the previously reported increases in SOCS3 levels most likely reflect elevated expression in the arcuate nucleus which is the site of action of leptin in the hypothalamus (Wang *et al.*, 2001; Peralta *et al.*, 2002). Our results indicate

no change in SOCS1 or -3 levels in the SCN during aging. The expression of IFN- $\gamma$  receptor (IFN- $\gamma$ R) and TNF- $\alpha$  receptor 2 (TNF- $\alpha$ R) mRNA was also analyzed. TNF- $\alpha$ R mRNA did not show changes with aging whereas the IFN- $\gamma$ R expression was lower in aged as compared to young animals during early subjective night. Thus, the low IFN- $\gamma$ R expression may underlie low Fos induction in aged animals at this time point. Interestingly, the expression of IFN- $\gamma$ R protein is confined to neurons in the ventral SCN (Lundkvist *et al.*, 1998) where the most marked difference in Fos expression was seen between young and aged animals. In the current study cytokine receptor mRNA expression was analyzed in RNA-samples extracted from the entire SCN, which does not give information on the subregional expression in the SCN. It would however be interesting to determine the *in situ* expression and localisation of IFN- $\gamma$ R protein in the aged SCN.

Inflammatory stimuli often cause stronger and more prolonged responses in glial cells of the aged brain (Deng *et al.*, 2006). We have preliminary data indicating that this is the case also in the aged SCN, where microglial cells as well as astrocytes show enhanced responses after i.c.v. IFN- $\gamma$ /TNF- $\alpha$  injection (Bentivoglio *et al.*, 2006). Glial cells play an important role in regulating neuronal activity, and the enhanced glial response and blunted neuronal expression of Fos in the SCN of aged mice after cytokine challenge may be related phenomena.

*In summary, the expression of SOCS1 and -3 mRNA showed a day/night variation in the SCN, with low levels at early night which corresponded to enhanced responses to the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ . In aged mice the induction of Fos protein during early night was blunted with an altered subregional distribution pattern, which may be related to an observed decreased level of IFN- $\gamma$ R mRNA. The altered responses of SCN neurons to pro-inflammatory cytokines may contribute to impaired regulation of circadian rhythms in aged animals.*

#### **4.3 EFFECTS OF TNF- $\alpha$ ON THE SCN (PAPER III)**

As mentioned in the introduction, there is certain evidence that cytokines can affect circadian rhythms. We therefore wanted to determine if TNF- $\alpha$ , a molecule that has been ascribed an important role in regulation of sleep and synaptic strength, can also affect the activity of SCN neurons. Spontaneous firing was monitored by loose patch extracellular recordings of neurons in the ventral SCN. At ZT 4-7 variable responses

were seen after exposure to TNF- $\alpha$  for 10 min and many neurons did not respond at all. At ZT 10-14 on the other hand, most neurons responded to TNF- $\alpha$  exposure with an increased spontaneous firing. In total, the effects persisted in 78 % of the responding neurons throughout the recording periods that lasted up until 30 min after TNF- $\alpha$  incubation. Previous studies where neurons in the ventromedial hypothalamus and *organum vasculosum laminae terminalis* were exposed to TNF- $\alpha$  also report both excitatory and inhibitory responses (Shibata & Blatteis, 1991; Katafuchi *et al.*, 1997). The mixed effects of TNF- $\alpha$  on firing rates in the SCN could reflect the functional heterogeneity that has been described within the SCN with respect to electrophysiological properties, chemoarchitecture and connectivity (Pennartz *et al.*, 1998; Abrahamson & Moore, 2001; Morin *et al.*, 2006). Notably, the effects of TNF- $\alpha$  were more homogenous at ZT 10-14 than at ZT 4-7.

It has been suggested that the gaseous transmitter molecule NO could mediate some effects of TNF- $\alpha$  in the central nervous system (Kubota *et al.*, 2002; Davies *et al.*, 2006). To determine if NO was involved in eliciting the responses to TNF- $\alpha$  in the SCN, extracellular recordings were performed at ZT 10-14 with the non-selective NOS inhibitor L-NAME added to the aCSF. Under this condition, TNF- $\alpha$  had no significant effect on the firing rate of SCN neurons, indicating that NO mediates the observed effects of TNF- $\alpha$  in the SCN. NO levels fluctuate with a circadian rhythm in the SCN (Mitome *et al.*, 2001), and NO can mediate light induced phase shifts (Ding *et al.*, 1994), and may also function as an intra-SCN messenger (Menger *et al.*, 2007; Plano *et al.*, 2007). Several isoforms of NOS have been described in the SCN of various animal species. In the mouse SCN, neuronal NOS (nNOS) has been described throughout the nucleus (Wang & Morris, 1996), but the expression of other NOS isoforms has not been investigated. However inducible NOS (iNOS) transcripts have been demonstrated in the SCN of guinea pig (Starkey *et al.*, 2001) and rat (Menger *et al.*, 2007), and endothelial NOS (eNOS) is expressed in astrocytes in the hamster and rat SCN (Caillol *et al.*, 2000). In this study we used L-NAME that blocks all NOS isoforms and it is therefore not possible to conclude which NOS isoform might mediate the observed effects of TNF- $\alpha$ . NO can exert a multitude of effects on neuronal function (Prast & Philippu, 2001), including changes in membrane properties that may cause changes in spontaneous firing lasting > 40 min (Smith & Otis, 2003). It is therefore possible that the observed increase in firing rate is due to an effect on membrane properties mediated by a TNF- $\alpha$ -induced NO release.

At ZT 10-14 we also analyzed effects of TNF- $\alpha$  on spontaneous synaptic transmission using whole-cell recordings. Both EPSCs and IPSCs were clearly decreased in frequency and also IPSC amplitude was decreased by TNF- $\alpha$ . This effect is probably not related to changes in synaptic strength since no significant differences, either in frequency or amplitude of mIPSCs or mEPSCs, could be detected between TNF- $\alpha$ -treated and vehicle-treated SCN slices. This contrasts effects of TNF- $\alpha$  in the hippocampus, where this molecule can modulate the surface expression of AMPA- and GABA<sub>A</sub>-receptors in cultured neurons as well as mIPSCs and mEPSCs in hippocampal slices (Stellwagen *et al.*, 2005; Stellwagen & Malenka, 2006). The lack of TNF- $\alpha$  induced synaptic scaling in the SCN may be related to a low degree of synaptic plasticity in the SCN as compared to the hippocampus. Exposure to TNF- $\alpha$  was made at the transition between day and night. Short-term synaptic plasticity (Gompf & Allen, 2004) that may be induced in GABAergic synapses at daytime in the SCN, are not, or only rarely, elicited at night, and it can therefore not be ruled out that TNF- $\alpha$ -dependent synaptic scaling may be induced in the SCN during other intervals of the circadian cycle.

Retrograde suppression of GABAergic input has been described in the ventral SCN as a consequence of postsynaptic depolarization (Gompf *et al.*, 2006). At ZT 10-14 most neurons responded to TNF- $\alpha$  exposure with an increased firing rate that may be associated with a depolarization of the neuron. The observed decrease in GABAergic input may therefore be the result of a depolarizing effect of TNF- $\alpha$  with an associated increase in postsynaptic Ca<sup>2+</sup> levels that could cause retrograde suppression. Such synaptic suppression may be NO-mediated, as has been described in the hippocampus and at the neuromuscular junction (Wang *et al.*, 1995; Makara *et al.*, 2007). Considering the mixed inhibitory and excitatory effects of TNF- $\alpha$  in our and other studies, it is also possible that a primary inhibitory effect of TNF- $\alpha$  on neurons projecting to the ventral SCN also could have contributed to a reduction of inhibitory synaptic input and increased firing rate in these cells. In SCN slice preparations, prepared as in our protocol, functional connections of GABAergic neurons projecting to the ventral SCN are preserved, including dorsal SCN neurons and neurons in the supraoptic nucleus (Cui *et al.*, 1997; Saeb-Parsy *et al.*, 2000).

We also tested whether TNF- $\alpha$  could influence PER2 expression using SCN slices from PER2::LUC mice. No effects of TNF- $\alpha$  on PER2 expression regarding circadian phase or amplitude was observed, indicating that the subpopulation of neurons

responding to TNF- $\alpha$  with altered neural firing may not be involved in phase shifts of the SCN molecular clockwork. The observed modulation of spontaneous firing may therefore occur downstream of the molecular clock and affect neuronal output from the SCN to target areas.

*In summary, these results show that TNF- $\alpha$  can modulate spontaneous firing and synaptic transmission in the SCN. This effect was dependent on NO and may involve modulation of membrane properties and transmitter release. The described signaling pathway may contribute to circadian rhythm disturbances during conditions associated with elevated levels of TNF- $\alpha$  such as inflammatory diseases.*

#### **4.4 SOCS-MEDIATED REGULATION OF CYTOKINE RESPONSES IN NEURONS (PAPER IV)**

The effects of cytokines are under tight control in cells of the immune system, and the family of SOCS proteins has proven to be crucial in limiting responses to cytokines by acting as negative feedback inhibitors. To investigate if SOCS molecules are expressed in cells of the central nervous system, and how they may influence the sensitivity of neurons to cytokines, we used primary cultures of hippocampal neurons and neocortical glial cells, as well as the hypothalamic neuronal cell line GT1-1, as model systems. IFN- $\gamma$  caused a dose dependent induction of SOCS1 and SOCS3 mRNA in hippocampal cultures. During continuous IFN- $\gamma$  exposure, SOCS3 was transiently induced whereas SOCS1 showed a sustained expression. Similar induction profiles were also seen in primary glial cultures and GT1-1 cells. Basal SOCS1 and SOCS3 mRNA levels were similar in glial and neuronal cultures, indicating that the small percentage of contaminating glial cells in the neuronal cultures is not the source of cytokine-induced SOCS mRNA expression. Thus, both glial cells and neurons of the central nervous system can express SOCS molecules in response to cytokines.

The different temporal profiles of SOCS1 and SOCS3 mRNA induction were not caused by differences in mRNA stability that was similar for the two molecules. Induction of SOCS1 was completely dependent on STAT1, whereas SOCS3 expression was only partly STAT1 dependent. The observed induction and degradation profiles of these molecules were similar to the temporal profiles previously observed in hepatocytes (Wormald *et al.*, 2006), indicating that the kinetics and regulation of these

signaling pathways may be similar across a range of cell types, including CNS neurons and glial cells.

To analyze the function of SOCS1 in neurons, the responses to IFN- $\gamma$  in WT and *Socs1*<sup>-/-</sup> hippocampal cultures were compared. IFN- $\gamma$ -inducible transcripts showed enhanced induction in *Socs1*<sup>-/-</sup> hippocampal cultures as compared to WT cultures. This included molecules involved in antigen presentation such as proteasomal components low molecular weight protein (LMP) 2 and LMP7, and classical and non-classical MHC class I molecules H2D<sup>b</sup> and Qa1<sup>b</sup>. Also iNOS and IFN- $\gamma$  inducible protein (IP-10), that are usually produced by macrophages/microglia in response to IFN- $\gamma$ , but under some circumstances have been reported in neurons (Wang *et al.*, 1998; Heneka & Feinstein, 2001), were elevated in *Socs1*<sup>-/-</sup> cultures. The enhanced responses to IFN- $\gamma$  indicates that SOCS1 limits cytokine signaling in neurons in a manner similar to other cell types (Wormald *et al.*, 2006).

The enhanced transcriptional responses after IFN- $\gamma$  treatment suggested that antigen presentation may be enhanced in *Socs1*<sup>-/-</sup> neurons as compared to WT cells. IFN- $\gamma$  treated neurons that are electrophysiologically silenced with TTX, express MHC class I molecules (Neumann *et al.*, 1995) to become targets for killing by sensitized T cells (Medana *et al.*, 2000). To evaluate if SOCS1 is involved in regulating antigen presentation by neurons we used an experimental system with ovalbumin (OVA)-specific T cell receptor transgenic T cells (OT-1). After pulsing cells with the OVA-derived peptide SIINFEKL, cells were co-incubated with OT-1 cells. To evaluate whether T cells became activated, cell culture supernatants were analyzed for the presence of T cell secreted cytokines. The supernatants from both WT and *Socs1*<sup>-/-</sup> cultures contained IFN- $\gamma$  and IL-2 when cultures had been pulsed with peptide and co-incubated with OT-1 cells. Pretreatment of neurons with IFN- $\gamma$  increased the subsequent secretion of IFN- $\gamma$  by T cells, and a high number of T cells in relation to neurons raised the level of IFN- $\gamma$  production even more. Using the high T cell:neuron ratio, an almost complete destruction of the neuronal network in the *Socs1*<sup>-/-</sup> cultures was observed, whereas only some fragmentation of neurites was seen in WT cultures.

Production of cytokines was similar in WT and *Socs1*<sup>-/-</sup> cultures indicating that T cells become activated in both cases. However, destruction of neurons was only seen in *Socs1*<sup>-/-</sup> cultures. This may reflect a higher density of MHC class I molecules on the neuronal membrane induced by IFN- $\gamma$  in *Socs1*<sup>-/-</sup> as compared to WT cultures which causes a stronger activation of T cells to mediate cytotoxic lysis (Khanna *et al.*, 2004).

SOCS1 may therefore play an important role in limiting the response of CNS neurons to IFN- $\gamma$  which was here demonstrated as an increased susceptibility of *Socs1*<sup>-/-</sup> neuronal cultures to T-cell mediated attack after IFN- $\gamma$  exposure. Other cytokines may also be subject to SOCS1 regulation. For instance, *Socs1*<sup>-/-</sup> fibroblast are hypersensitive to TNF- $\alpha$ , and consequently SOCS1 may limit also neuronal responses to TNF- $\alpha$  and other immune molecules.

*In summary, SOCS1 and SOCS3 mRNA could be induced in relatively pure cultures of hippocampal neurons as well as in a neuronal cell line, GT1-1, and lack of SOCS1 enhanced IFN- $\gamma$ -induced transcriptional responses of neurons. IFN- $\gamma$ -stimulation rendered *Socs1*<sup>-/-</sup> neurons more sensitive to subsequent T cell recognition and attack, possibly as a consequence of elevated surface levels of MHC class I as compared to WT neurons. The findings indicate that SOCS1 may play a role in down-regulating cytokine responses in CNS neurons and thereby limit functional disturbances and reduce the risk of cytolytic T cell attack.*

## 5 CONCLUDING REMARKS

In this thesis I observed that the SCN in aged mice exhibits an increased proportion of silent neurons and a markedly reduced synaptic transmission and density of synaptic terminals. The reduced intra-SCN neurotransmission may impair the ability of the SCN to adapt to changes in the external environment, which could contribute to slow resynchronization after phase shifts and an advanced phase of circadian rhythms frequently seen during aging. In addition, these alterations are likely to result in reduced circadian output signals that may contribute to age-related dampening of circadian rhythms.

Aging is frequently associated with a low grade chronic inflammation with increased levels of pro-inflammatory cytokines, both in the periphery and the brain. This thesis demonstrates that the pro-inflammatory cytokine TNF- $\alpha$  affects neuronal functions in the SCN. Interestingly, TNF- $\alpha$  caused a reduction of synaptic transmission in the SCN, similar to that observed in the aged SCN, suggesting that the reduced synaptic transmission in the aged SCN may be the result of a long-term exposure to pro-inflammatory cytokines. However, the temporal aspect of these experiments must be taken into consideration. The aged mice might have been subjected to long-term exposure of elevated cytokine levels, which contrasts the relatively brief exposure to TNF- $\alpha$  that was made to SCN tissue slices. Long-term effects of cytokines may be regulated by homeostatic responses, both at the level of synaptic function and intracellular signaling pathways. We did not observe homeostatic synaptic scaling in the SCN in response to TNF- $\alpha$ , which has been previously described in the hippocampus (Stellwagen & Malenka, 2006). However, it is possible that other molecules, such as BDNF that mediates synaptic homeostasis in cortical GABAergic neurons (Rutherford *et al.*, 1998; Turrigiano & Nelson, 2004), may play such a role in the SCN.

SOCS molecules maintain intracellular homeostasis by regulating responses to cytokines. Analysis of SOCS molecules in hippocampal neurons demonstrated that SOCS1 plays a functional role in neurons of the central nervous system. SOCS1 and SOCS3 mRNA were expressed in the SCN with a day/night rhythm and this variation may regulate the sensitivity of the SCN to pro-inflammatory cytokines. Although aged mice showed no alteration in basal SOCS expression in the SCN, neurons in the SCN of aged and young mice responded differently to TNF- $\alpha$  and IFN- $\gamma$  exposure. The

altered responses in the aged SCN may be due to altered expression of cytokine receptors. In addition, it remains to be studied whether induction of SOCS molecules in response to cytokines is altered in aged SCN neurons or not.

In the SCN, NO can mediate light induced phase shifts and appears to play an important role in rhythm generation and coupling of the dorsal and ventral SCN (Ding *et al.*, 1994; Menger *et al.*, 2007; Plano *et al.*, 2007). In the present study, the effects of TNF- $\alpha$  on neuronal firing in the SCN were found to be NO-mediated. It is therefore possible that altered NO levels in the SCN mediate disturbances of circadian rhythms during inflammation. Notably, SOCS1 was found to regulate IFN- $\gamma$ -mediated transcription of iNOS in hippocampal neurons. The day/night oscillation of SOCS1 observed in the SCN suggests that this molecule may play a similar role in SCN neurons.

Despite marked changes in function of the SCN during aging, there are indications that age-related circadian rhythm disturbances can be improved by various types of treatment. Light therapy and melatonin administration are two major treatment strategies to reactivate the aged circadian system (reviewed by Wu & Swaab, 2007). Environmental light is the major entrainment signal for the SCN, and aged individuals are often exposed to lower levels of environmental light as compared to young individuals. Interestingly, light exposure can counteract age-related decrease of VP in the aged rat SCN (Lucassen *et al.*, 1995), and human studies indicate that bright light treatment may normalize affected rest-activity and melatonin rhythms in aged individuals (reviewed by Wu & Swaab, 2007). Some studies report positive results of melatonin treatment on insomnia with few adverse effects (Roth *et al.*, 2006). It is interesting to note that melatonin has potent anti-inflammatory actions, and significantly reduces age-related inflammation in the brain (Bondy *et al.*, 2004). These effects may contribute to the beneficial effects of melatonin on circadian rhythm disturbances in aged individuals. However, the existing treatment strategies have also yielded negative results in several studies and there is clearly a need for alternative therapeutic strategies.

The results of this thesis indicate that inflammatory molecules can affect the SCN and may contribute to circadian rhythm disturbances. It would therefore be of interest to investigate how anti-inflammatory treatment affects the circadian system in aged subjects with a chronic low-grade inflammation. However, before addressing such potential treatment strategies, the effects and role of inflammatory molecules in the SCN should be investigated in more detail.

An interesting question that arises from this work, is to what extent a chronic inflammation may contribute to the observed decrease in synapse number in the aged SCN, and whether such structural changes can be reversed or not. The regenerative capacity of the aged SCN is a largely unexplored area, but it has been shown that nerve growth factor may restore the expression of VP and VIP in aged rat SCN neurons (Pereira *et al.*, 2005). It would therefore be interesting to investigate if these effects could be associated with functional and morphological restoration of the SCN synaptic network and improvement of impaired circadian rhythms.

## 6 ACKNOWLEDGEMENTS

Many people have supported me and contributed to this work. I would especially like to express my gratitude to:

### *Supervisors, co-supervisors and co-authors*

Professor Krister Kristensson, my supervisor, for taking me on as a student and for trusting in me. For always taking time to discuss projects and for teaching me so much about neuroscience.

Dr Martin Rottenberg for always spreading good mood and for all the constructive comments on experiments.

Dr Russell Hill for valuable comments on manuscripts, for always having time to discuss electrophysiology, and for your support.

Dr Gabriella Lundkvist for help with manuscripts, good discussions about circadian biology and for efficient speed-slicing sessions.

Professor Marina Bentivoglio for fruitful scientific collaborations and endless e-mail conversations about sentences and statistics.

Dr Håkan Karlsson for teaching me about PCR and how to amplify something from tiny amounts of starting material.

Dr Martin Wikström for helping me out in the beginning, with slice preparations and patch-clamp problems.

Lars Vedin, for valuable advice and nice lunch meetings.

### *People at KI*

Present and former members of the Kristensson group: Gia Luhr, Elin Nordström, Johan Brask, Linnea Asp, Christoffer Nellåker, Nirvana Pilay, Daniel Amin, Willias Masocha, Eva Backström, Malin Sandberg, Fredrik Aronsson and Margareta Widing. Thanks for creating a nice work atmosphere, for bringing me food when I did not have time to go for lunch, and for being so supportive!

The animal facility and administrative staff at the Department of Neuroscience, for taking good care of the department and the mice. Thanks' also all PhD-students at the department for making it such a nice work-place!

## *Family and friends*

All friends at KI and outside the world of science. Thank you for distracting me and for all the good times! In particular I would like to thank: Jesper Ericsson for priceless friendship, for good company in the lab and for all the fun outside the lab! Keep up the good spirit! Olle Andersson for convincing me to come to KI in the first place, for being so laid back and for all the fun throughout the years, Fredrik Andersson for the nice road trips, Daniel Nykvist for good times in slopes and bars, Jakob Ribbing for just being you and for all the trips we did and parties we've had, Sven Kilander for good mountainbiking, hospitality and friendship, Karin Mjömark for ski- and boat-trips lots of laughter and nice dinners!, Henrik Sundberg for good advice and lot's of fun, Jonas Haldén for your genuine friendship and all the good talks we've had. Johan Perols for your generosity and friendship, you're up! Pär Skoglund for humour and kindness, Erik Edgren for good times! Thanks also to all the old Pharmacists and all the G.I.S. and U.I.S.-B.L.O.G. Hope to see more of you all now!

My relatives, all the Nygårds and Skoogs that keep asking me how it's going. Thanks for your support and interest.

My extended family, for taking interest in my work. Special thanks to Jan and Maud Höijer for lending their apartment during the fall, it would never have worked out otherwise!

My family for endless support and care and for always backing me up.

Karin, my everything ♥

This work was supported by: Loo och Hans Ostermans stiftelse, CD Carlssons stiftelse, Karl Jeppsons mine, Sigurd och Elsa Goljes stiftelse, EC grants LSHM-CT-2005-518189, FP6-2004-INCO-DEV-3 032324 and QLRT-2001-02258 and the Swedish Research Council.

## 7 REFERENCES

- Abrahamson, E.E. & Moore, R.Y. (2001) Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Res*, **916**, 172-191.
- Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H. & Kyriacou, C.P. (2002) Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr Biol*, **12**, 540-550.
- Albus, H., Bonnefont, X., Chaves, I., Yasui, A., Doczy, J., van der Horst, G.T. & Meijer, J.H. (2002) Cryptochrome-deficient mice lack circadian electrical activity in the suprachiasmatic nuclei. *Curr Biol*, **12**, 1130-1133.
- Albus, H., Vansteensel, M.J., Michel, S., Block, G.D. & Meijer, J.H. (2005) A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Curr Biol*, **15**, 886-893.
- Antle, M.C. & Silver, R. (2005) Orchestrating time: arrangements of the brain circadian clock. *Trends Neurosci*, **28**, 145-151.
- Arima, H., House, S.B., Gainer, H. & Aguilera, G. (2002) Neuronal activity is required for the circadian rhythm of vasopressin gene transcription in the suprachiasmatic nucleus in vitro. *Endocrinology*, **143**, 4165-4171.
- Asai, M., Yoshinobu, Y., Kaneko, S., Mori, A., Nikaido, T., Moriya, T., Akiyama, M. & Shibata, S. (2001) Circadian profile of Per gene mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and pineal body of aged rats. *J Neurosci Res*, **66**, 1133-1139.
- Aton, S.J., Colwell, C.S., Harmar, A.J., Waschek, J. & Herzog, E.D. (2005) Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nat Neurosci*, **8**, 476-483.
- Aton, S.J. & Herzog, E.D. (2005) Come together, right...now: synchronization of rhythms in a mammalian circadian clock. *Neuron*, **48**, 531-534.
- Aton, S.J., Huettner, J.E., Straume, M. & Herzog, E.D. (2006) GABA and Gi/o differentially control circadian rhythms and synchrony in clock neurons. *Proc Natl Acad Sci U S A*, **103**, 19188-19193.
- Aujard, F., Herzog, E.D. & Block, G.D. (2001) Circadian rhythms in firing rate of individual suprachiasmatic nucleus neurons from adult and middle-aged mice. *Neuroscience*, **106**, 255-261.
- Bentivoglio, M., Deng, X.H., Nygard, M., Sadki, A. & Kristensson, K. (2006) The aging suprachiasmatic nucleus and cytokines: functional, molecular, and cellular changes in rodents. *Chronobiol Int*, **23**, 437-449.
- Bodles, A.M. & Barger, S.W. (2004) Cytokines and the aging brain - what we don't know might help us. *Trends Neurosci*, **27**, 621-626.
- Bondy, S.C., Lahiri, D.K., Perreau, V.M., Sharman, K.Z., Campbell, A., Zhou, J. & Sharman, E.H. (2004) Retardation of brain aging by chronic treatment with melatonin. *Ann N Y Acad Sci*, **1035**, 197-215.
- Brown, T.M., Hughes, A.T. & Piggins, H.D. (2005) Gastrin-releasing peptide promotes suprachiasmatic nuclei cellular rhythmicity in the absence of vasoactive intestinal polypeptide-VPAC2 receptor signaling. *J Neurosci*, **25**, 11155-11164.
- Brunnsgaard, H., Pedersen, M. & Pedersen, B.K. (2001) Aging and proinflammatory cytokines. *Curr Opin Hematol*, **8**, 131-136.
- Bryant, P.A., Trinder, J. & Curtis, N. (2004) Sick and tired: Does sleep have a vital role in the immune system? *Nat Rev Immunol*, **4**, 457-467.
- Buijs, R.M., Scheer, F.A., Kreier, F., Yi, C., Bos, N., Goncharuk, V.D. & Kalsbeek, A. (2006) Organization of circadian functions: interaction with the body. *Prog Brain Res*, **153**, 341-360.
- Burke, S.N. & Barnes, C.A. (2006) Neural plasticity in the ageing brain. *Nat Rev Neurosci*, **7**, 30-40.

- Caillois, M., Devinoy, E., Lacroix, M.C. & Schirar, A. (2000) Endothelial and neuronal nitric oxide synthases are present in the suprachiasmatic nuclei of Syrian hamsters and rats. *Eur J Neurosci*, **12**, 649-661.
- Card, J.P., Brecha, N., Karten, H.J. & Moore, R.Y. (1981) Immunocytochemical localization of vasoactive intestinal polypeptide-containing cells and processes in the suprachiasmatic nucleus of the rat: light and electron microscopic analysis. *J Neurosci*, **1**, 1289-1303.
- Cardinali, D.P. & Esquifino, A.I. (2003) Circadian disorganization in experimental arthritis. *Neurosignals*, **12**, 267-282.
- Cavadini, G., Petrzilka, S., Kohler, P., Jud, C., Tobler, I., Birchler, T. & Fontana, A. (2007) TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proc Natl Acad Sci U S A*, **104**, 12843-12848.
- Cearley, C., Churchill, L. & Krueger, J.M. (2003) Time of day differences in IL1beta and TNFalpha mRNA levels in specific regions of the rat brain. *Neurosci Lett*, **352**, 61-63.
- Chee, C.A., Roozendaal, B., Swaab, D.F., Goudsmit, E. & Mirmiran, M. (1988) Vasoactive intestinal polypeptide neuron changes in the senile rat suprachiasmatic nucleus. *Neurobiol Aging*, **9**, 307-312.
- Cheng, M.Y., Bullock, C.M., Li, C., Lee, A.G., Bermak, J.C., Belluzzi, J., Weaver, D.R., Leslie, F.M. & Zhou, Q.Y. (2002) Prokineticin 2 transmits the behavioral circadian rhythm of the suprachiasmatic nucleus. *Nature*, **417**, 405-410.
- Chou, T.C., Bjorkum, A.A., Gaus, S.E., Lu, J., Scammell, T.E. & Saper, C.B. (2002) Afferents to the ventrolateral preoptic nucleus. *J Neurosci*, **22**, 977-990.
- Chou, T.C., Scammell, T.E., Gooley, J.J., Gaus, S.E., Saper, C.B. & Lu, J. (2003) Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. *J Neurosci*, **23**, 10691-10702.
- Coleman, P.D. (2004) How old is old? *Neurobiol Aging*, **25**, 1.
- Colwell, C.S. (2000) Circadian modulation of calcium levels in cells in the suprachiasmatic nucleus. *Eur J Neurosci*, **12**, 571-576.
- Cui, L.N., Saeb-Parsy, K. & Dyball, R.E. (1997) Neurones in the supraoptic nucleus of the rat are regulated by a projection from the suprachiasmatic nucleus. *J Physiol*, **502 ( Pt 1)**, 149-159.
- Cutler, D.J., Haraura, M., Reed, H.E., Shen, S., Sheward, W.J., Morrison, C.F., Marston, H.M., Harmar, A.J. & Piggins, H.D. (2003) The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro. *Eur J Neurosci*, **17**, 197-204.
- Czeisler, C.A., Dumont, M., Duffy, J.F., Steinberg, J.D., Richardson, G.S., Brown, E.N., Sanchez, R., Rios, C.D. & Ronda, J.M. (1992) Association of sleep-wake habits in older people with changes in output of circadian pacemaker. *Lancet*, **340**, 933-936.
- d'Ortous de Mairan, J.J. (1729) Observation botanique. *Histoire de l'Academie Royale des Sciences*, 35-36.
- Davidson, A.J., Sellix, M.T., Daniel, J., Yamazaki, S., Menaker, M. & Block, G.D. (2006a) Chronic jet-lag increases mortality in aged mice. *Curr Biol*, **16**, R914-916.
- Davidson, A.J., Yamazaki, S., Arble, D.M., Menaker, M. & Block, G.D. (2006b) Resetting of central and peripheral circadian oscillators in aged rats. *Neurobiol Aging*.
- Davies, A.L., Hayes, K.C. & Shi, R. (2006) Recombinant human TNFalpha induces concentration-dependent and reversible alterations in the electrophysiological properties of axons in mammalian spinal cord. *J Neurotrauma*, **23**, 1261-1273.
- Davies, I.R. & Mason, R. (1994) Tau-mutant hamster SCN clock neurones express a 20 h firing rate rhythm in vitro. *Neuroreport*, **5**, 2165-2168.
- Deboer, M.D. & Marks, D.L. (2006) Cachexia: lessons from melanocortin antagonism. *Trends Endocrinol Metab*, **17**, 199-204.
- Deng, X.H., Bertini, G., Xu, Y.Z., Yan, Z. & Bentivoglio, M. (2006) Cytokine-induced activation of glial cells in the mouse brain is enhanced at an advanced age. *Neuroscience*, **141**, 645-661.

- Ding, J.M., Chen, D., Weber, E.T., Faiman, L.E., Rea, M.A. & Gillette, M.U. (1994) Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. *Science*, **266**, 1713-1717.
- Duffy, J.F., Dijk, D.J., Klerman, E.B. & Czeisler, C.A. (1998) Later endogenous circadian temperature nadir relative to an earlier wake time in older people. *Am J Physiol*, **275**, R1478-1487.
- Duncan, M.J., Herron, J.M. & Hill, S.A. (2001) Aging selectively suppresses vasoactive intestinal peptide messenger RNA expression in the suprachiasmatic nucleus of the Syrian hamster. *Brain Res Mol Brain Res*, **87**, 196-203.
- Ershler, W.B., Sun, W.H., Binkley, N., Gravenstein, S., Volk, M.J., Kamoske, G., Klopp, R.G., Roecker, E.B., Daynes, R.A. & Weindruch, R. (1993) Interleukin-6 and aging: blood levels and mononuclear cell production increase with advancing age and in vitro production is modifiable by dietary restriction. *Lymphokine Cytokine Res*, **12**, 225-230.
- Floyd, R.A. & Krueger, J.M. (1997) Diurnal variation of TNF alpha in the rat brain. *Neuroreport*, **8**, 915-918.
- Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., Panourgia, M.P., Invidia, L., Celani, L., Scurti, M., Cevenini, E., Castellani, G.C. & Salvoli, S. (2007) Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mech Ageing Dev*, **128**, 92-105.
- Godbout, J.P., Chen, J., Abraham, J., Richwine, A.F., Berg, B.M., Kelley, K.W. & Johnson, R.W. (2005) Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *Faseb J*, **19**, 1329-1331.
- Gompf, H.S. & Allen, C.N. (2004) GABAergic synapses of the suprachiasmatic nucleus exhibit a diurnal rhythm of short-term synaptic plasticity. *Eur J Neurosci*, **19**, 2791-2798.
- Gompf, H.S., Irwin, R.P. & Allen, C.N. (2006) Retrograde suppression of GABAergic currents in a subset of SCN neurons. *Eur J Neurosci*, **23**, 3209-3216.
- Green, D.J. & Gillette, R. (1982) Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice. *Brain Res*, **245**, 198-200.
- Groos, G. & Hendriks, J. (1982) Circadian rhythms in electrical discharge of rat suprachiasmatic neurones recorded in vitro. *Neurosci Lett*, **34**, 283-288.
- Guilding, C. & Piggins, H.D. (2007) Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain? *Eur J Neurosci*, **25**, 3195-3216.
- Hager, K., Machein, U., Krieger, S., Platt, D., Seefried, G. & Bauer, J. (1994) Interleukin-6 and selected plasma proteins in healthy persons of different ages. *Neurobiol Aging*, **15**, 771-772.
- Harmar, A.J., Marston, H.M., Shen, S., Spratt, C., West, K.M., Sheward, W.J., Morrison, C.F., Dorin, J.R., Piggins, H.D., Reubi, J.C., Kelly, J.S., Maywood, E.S. & Hastings, M.H. (2002) The VPAC(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell*, **109**, 497-508.
- Hastings, M.H., Reddy, A.B. & Maywood, E.S. (2003) A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci*, **4**, 649-661.
- Heneka, M.T. & Feinstein, D.L. (2001) Expression and function of inducible nitric oxide synthase in neurons. *J Neuroimmunol*, **114**, 8-18.
- Herzog, E.D. (2007) Neurons and networks in daily rhythms. *Nat Rev Neurosci*, **8**, 790-802.
- Herzog, E.D., Takahashi, J.S. & Block, G.D. (1998) Clock controls circadian period in isolated suprachiasmatic nucleus neurons. *Nat Neurosci*, **1**, 708-713.
- Hof, P.R. & Morrison, J.H. (2004) The aging brain: morphomolecular senescence of cortical circuits. *Trends Neurosci*, **27**, 607-613.
- Hofman, M.A. & Swaab, D.F. (2006) Living by the clock: the circadian pacemaker in older people. *Ageing Res Rev*, **5**, 33-51.
- Hofman, M.A., Zhou, J.N. & Swaab, D.F. (1996) Suprachiasmatic nucleus of the human brain: an immunocytochemical and morphometric analysis. *Anat Rec*, **244**, 552-562.

- Huang, Y., Henry, C.J., Dantzer, R., Johnson, R.W. & J.P., G. (2007) Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. *Neurobiol Aging*, **in press**.
- Hurd, M.W., Zimmer, K.A., Lehman, M.N. & Ralph, M.R. (1995) Circadian locomotor rhythms in aged hamsters following suprachiasmatic transplant. *Am J Physiol*, **269**, R958-968.
- Imeri, L., Ceccarelli, P., Mariotti, M., Manfredi, A., Opp, M.R. & Mancia, M. (2004) Sleep, but not febrile responses of Fisher 344 rats to immune challenge are affected by aging. *Brain Behav Immun*, **18**, 399-404.
- Inouye, S.T. & Kawamura, H. (1979) Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc Natl Acad Sci U S A*, **76**, 5962-5966.
- Itri, J. & Colwell, C.S. (2003) Regulation of inhibitory synaptic transmission by vasoactive intestinal peptide (VIP) in the mouse suprachiasmatic nucleus. *J Neurophysiol*, **90**, 1589-1597.
- Itri, J., Michel, S., Waschek, J.A. & Colwell, C.S. (2004) Circadian rhythm in inhibitory synaptic transmission in the mouse suprachiasmatic nucleus. *J Neurophysiol*, **92**, 311-319.
- Itri, J.N., Michel, S., Vansteensel, M.J., Meijer, J.H. & Colwell, C.S. (2005) Fast delayed rectifier potassium current is required for circadian neural activity. *Nat Neurosci*, **8**, 650-656.
- Jackson, A.C., Yao, G.L. & Bean, B.P. (2004) Mechanism of spontaneous firing in dorsomedial suprachiasmatic nucleus neurons. *J Neurosci*, **24**, 7985-7998.
- Jin, Y., Lundkvist, G., Dons, L., Kristensson, K. & Rottenberg, M.E. (2004) Interferon-gamma mediates neuronal killing of intracellular bacteria. *Scand J Immunol*, **60**, 437-448.
- Jobst, E.E., Robinson, D.W. & Allen, C.N. (2004) Potential pathways for intercellular communication within the calbindin subnucleus of the hamster suprachiasmatic nucleus. *Neuroscience*, **123**, 87-99.
- Joly, E., Mucke, L. & Oldstone, M.B. (1991) Viral persistence in neurons explained by lack of major histocompatibility class I expression. *Science*, **253**, 1283-1285.
- Kallo, I., Kalamatianos, T., Piggins, H.D. & Coen, C.W. (2004) Ageing and the diurnal expression of mRNAs for vasoactive intestinal peptide and for the VPAC2 and PAC1 receptors in the suprachiasmatic nucleus of male rats. *J Neuroendocrinol*, **16**, 758-766.
- Kalsbeek, A. & Buijs, R.M. (2002) Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. *Cell Tissue Res*, **309**, 109-118.
- Katafuchi, T., Motomura, K., Baba, S., Ota, K. & Hori, T. (1997) Differential effects of tumor necrosis factor-alpha and -beta on rat ventromedial hypothalamic neurons in vitro. *Am J Physiol*, **272**, R1966-1971.
- Kawakami, F., Okamura, H., Tamada, Y., Maebayashi, Y., Fukui, K. & Ibata, Y. (1997) Loss of day-night differences in VIP mRNA levels in the suprachiasmatic nucleus of aged rats. *Neurosci Lett*, **222**, 99-102.
- Khanna, K.M., Lepisto, A.J. & Hendricks, R.L. (2004) Immunity to latent viral infection: many skirmishes but few fatalities. *Trends Immunol*, **25**, 230-234.
- Kim, Y.I. & Dudek, F.E. (1991) Intracellular electrophysiological study of suprachiasmatic nucleus neurons in rodents: excitatory synaptic mechanisms. *J Physiol*, **444**, 269-287.
- Ko, C.H. & Takahashi, J.S. (2006) Molecular components of the mammalian circadian clock. *Hum Mol Genet*, **15 Spec No 2**, R271-277.
- Kolker, D.E., Fukuyama, H., Huang, D.S., Takahashi, J.S., Horton, T.H. & Turek, F.W. (2003) Aging alters circadian and light-induced expression of clock genes in golden hamsters. *J Biol Rhythms*, **18**, 159-169.
- Kononenko, N.I., Shao, L.R. & Dudek, F.E. (2004) Riluzole-sensitive slowly inactivating sodium current in rat suprachiasmatic nucleus neurons. *J Neurophysiol*, **91**, 710-718.
- Konopka, R.J. & Benzer, S. (1971) Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*, **68**, 2112-2116.

- Konsman, J.P., Parnet, P. & Dantzer, R. (2002) Cytokine-induced sickness behaviour: mechanisms and implications. *Trends Neurosci*, **25**, 154-159.
- Krajnak, K., Kashon, M.L., Rosewell, K.L. & Wise, P.M. (1998) Aging alters the rhythmic expression of vasoactive intestinal polypeptide mRNA but not arginine vasopressin mRNA in the suprachiasmatic nuclei of female rats. *J Neurosci*, **18**, 4767-4774.
- Kramer, A., Yang, F.C., Snodgrass, P., Li, X., Scammell, T.E., Davis, F.C. & Weitz, C.J. (2001) Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science*, **294**, 2511-2515.
- Kraves, S. & Weitz, C.J. (2006) A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity. *Nat Neurosci*, **9**, 212-219.
- Krout, K.E., Kawano, J., Mettenleiter, T.C. & Loewy, A.D. (2002) CNS inputs to the suprachiasmatic nucleus of the rat. *Neuroscience*, **110**, 73-92.
- Krueger, J.M., Obal, F.J., Fang, J., Kubota, T. & Taishi, P. (2001) The role of cytokines in physiological sleep regulation. *Ann N Y Acad Sci*, **933**, 211-221.
- Kubota, T., Li, N., Guan, Z., Brown, R.A. & Krueger, J.M. (2002) Intrapreoptic microinjection of TNF-alpha enhances non-REM sleep in rats. *Brain Res*, **932**, 37-44.
- Kullberg, S., Aldskogius, H. & Ulfhake, B. (2001) Microglial activation, emergence of ED1-expressing cells and clusterin upregulation in the aging rat CNS, with special reference to the spinal cord. *Brain Res*, **899**, 169-186.
- Larsen, P.J., Enquist, L.W. & Card, J.P. (1998) Characterization of the multisynaptic neuronal control of the rat pineal gland using viral transneuronal tracing. *Eur J Neurosci*, **10**, 128-145.
- Leak, R.K., Card, J.P. & Moore, R.Y. (1999) Suprachiasmatic pacemaker organization analyzed by viral transsynaptic transport. *Brain Res*, **819**, 23-32.
- Lee, C.K., Weindruch, R. & Prolla, T.A. (2000) Gene-expression profile of the ageing brain in mice. *Nat Genet*, **25**, 294-297.
- Lehman, M.N., Silver, R., Gladstone, W.R., Kahn, R.M., Gibson, M. & Bittman, E.L. (1987) Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. *J Neurosci*, **7**, 1626-1638.
- Leone, M.J., Marpegan, L., Bekinschtein, T.A., Costas, M.A. & Golombek, D.A. (2006) Suprachiasmatic astrocytes as an interface for immune-circadian signalling. *J Neurosci Res*, **84**, 1521-1527.
- Lewy, A.J., Lefler, B.J., Emens, J.S. & Bauer, V.K. (2006) The circadian basis of winter depression. *Proc Natl Acad Sci U S A*, **103**, 7414-7419.
- Li, H. & Satinoff, E. (1998) Fetal tissue containing the suprachiasmatic nucleus restores multiple circadian rhythms in old rats. *Am J Physiol*, **275**, R1735-1744.
- Li, X., Sankrithi, N. & Davis, F.C. (2002) Transforming growth factor-alpha is expressed in astrocytes of the suprachiasmatic nucleus in hamster: role of glial cells in circadian clocks. *Neuroreport*, **13**, 2143-2147.
- Liu, A.C., Welsh, D.K., Ko, C.H., Tran, H.G., Zhang, E.E., Priest, A.A., Buhr, E.D., Singer, O., Meeker, K., Verma, I.M., Doyle, F.J., 3rd, Takahashi, J.S. & Kay, S.A. (2007) Intercellular coupling confers robustness against mutations in the SCN circadian clock network. *Cell*, **129**, 605-616.
- Liu, C. & Reppert, S.M. (2000) GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron*, **25**, 123-128.
- Liu, C., Weaver, D.R., Strogatz, S.H. & Reppert, S.M. (1997) Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei. *Cell*, **91**, 855-860.
- Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-408.
- Long, M.A., Jutras, M.J., Connors, B.W. & Burwell, R.D. (2005) Electrical synapses coordinate activity in the suprachiasmatic nucleus. *Nat Neurosci*, **8**, 61-66.
- Lu, J., Zhang, Y.H., Chou, T.C., Gaus, S.E., Elmquist, J.K., Shiromani, P. & Saper, C.B. (2001) Contrasting effects of ibotenate lesions of the paraventricular nucleus and subparaventricular zone on sleep-wake cycle and temperature regulation. *J Neurosci*, **21**, 4864-4874.

- Lu, T., Pan, Y., Kao, S.Y., Li, C., Kohane, I., Chan, J. & Yankner, B.A. (2004) Gene regulation and DNA damage in the ageing human brain. *Nature*, **429**, 883-891.
- Lucassen, P.J., Hofman, M.A. & Swaab, D.F. (1995) Increased light intensity prevents the age related loss of vasopressin-expressing neurons in the rat suprachiasmatic nucleus. *Brain Res*, **693**, 261-266.
- Lundkvist, G.B., Hill, R.H. & Kristensson, K. (2002) Disruption of circadian rhythms in synaptic activity of the suprachiasmatic nuclei by African trypanosomes and cytokines. *Neurobiol Dis*, **11**, 20-27.
- Lundkvist, G.B., Kwak, Y., Davis, E.K., Tei, H. & Block, G.D. (2005) A calcium flux is required for circadian rhythm generation in mammalian pacemaker neurons. *J Neurosci*, **25**, 7682-7686.
- Lundkvist, G.B., Robertson, B., Mhlanga, J.D., Rottenberg, M.E. & Kristensson, K. (1998) Expression of an oscillating interferon-gamma receptor in the suprachiasmatic nuclei. *Neuroreport*, **9**, 1059-1063.
- Madeira, M.D., Sousa, N., Santer, R.M., Paula-Barbosa, M.M. & Gundersen, H.J. (1995) Age and sex do not affect the volume, cell numbers, or cell size of the suprachiasmatic nucleus of the rat: an unbiased stereological study. *J Comp Neurol*, **361**, 585-601.
- Maier, J., Kincaid, C., Pagenstecher, A. & Campbell, I.L. (2002) Regulation of signal transducer and activator of transcription and suppressor of cytokine-signaling gene expression in the brain of mice with astrocyte-targeted production of interleukin-12 or experimental autoimmune encephalomyelitis. *Am J Pathol*, **160**, 271-288.
- Makara, J.K., Katona, I., Nyiri, G., Nemeth, B., Ledent, C., Watanabe, M., de Vente, J., Freund, T.F. & Hajos, N. (2007) Involvement of nitric oxide in depolarization-induced suppression of inhibition in hippocampal pyramidal cells during activation of cholinergic receptors. *J Neurosci*, **27**, 10211-10222.
- Marine, J.C., McKay, C., Wang, D., Topham, D.J., Parganas, E., Nakajima, H., Penderville, H., Yasukawa, H., Sasaki, A., Yoshimura, A. & Ihle, J.N. (1999) SOCS3 is essential in the regulation of fetal liver erythropoiesis. *Cell*, **98**, 617-627.
- Marpegan, L., Bekinschtein, T.A., Costas, M.A. & Golombek, D.A. (2005) Circadian responses to endotoxin treatment in mice. *J Neuroimmunol*, **160**, 102-109.
- Massa, P.T., Saha, S., Wu, C. & Jarosinski, K.W. (2000) Expression and function of the protein tyrosine phosphatase SHP-1 in oligodendrocytes. *Glia*, **29**, 376-385.
- Maywood, E.S., O'Neill, J., Wong, G.K., Reddy, A.B. & Hastings, M.H. (2006a) Circadian timing in health and disease. *Prog Brain Res*, **153**, 253-269.
- Maywood, E.S., Reddy, A.B., Wong, G.K., O'Neill, J.S., O'Brien, J.A., McMahon, D.G., Harmar, A.J., Okamura, H. & Hastings, M.H. (2006b) Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. *Curr Biol*, **16**, 599-605.
- Medana, I.M., Gallimore, A., Oxenius, A., Martinic, M.M., Wekerle, H. & Neumann, H. (2000) MHC class I-restricted killing of neurons by virus-specific CD8<sup>+</sup> T lymphocytes is effected through the Fas/FasL, but not the perforin pathway. *Eur J Immunol*, **30**, 3623-3633.
- Melyan, Z., Tarttelin, E.E., Bellingham, J., Lucas, R.J. & Hankins, M.W. (2005) Addition of human melanopsin renders mammalian cells photoresponsive. *Nature*, **433**, 741-745.
- Menaker, M. (1968) Extraretinal light perception in the sparrow. I. Entrainment of the biological clock. *Proc Natl Acad Sci U S A*, **59**, 414-421.
- Menger, G.J., Allen, G.C., Neuendorff, N., Nahm, S.S., Thomas, T.L., Cassone, V.M. & Earnest, D.J. (2007) Circadian profiling of the transcriptome in NIH/3T3 fibroblasts: comparison with rhythmic gene expression in SCN2.2 cells and the rat SCN. *Physiol Genomics*, **29**, 280-289.
- Meredith, A.L., Wiler, S.W., Miller, B.H., Takahashi, J.S., Fodor, A.A., Ruby, N.F. & Aldrich, R.W. (2006) BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nat Neurosci*, **9**, 1041-1049.
- Meyer-Bernstein, E.L., Jetton, A.E., Matsumoto, S.I., Markuns, J.F., Lehman, M.N. & Bittman, E.L. (1999) Effects of suprachiasmatic transplants on circadian

- rhythms of neuroendocrine function in golden hamsters. *Endocrinology*, **140**, 207-218.
- Michel, S., Geusz, M.E., Zaritsky, J.J. & Block, G.D. (1993) Circadian rhythm in membrane conductance expressed in isolated neurons. *Science*, **259**, 239-241.
- Michel, S., Itri, J. & Colwell, C.S. (2002) Excitatory mechanisms in the suprachiasmatic nucleus: the role of AMPA/KA glutamate receptors. *J Neurophysiol*, **88**, 817-828.
- Miller, J.D., Morin, L.P., Schwartz, W.J. & Moore, R.Y. (1996) New insights into the mammalian circadian clock. *Sleep*, **19**, 641-667.
- Miller, R.A. (1996) The aging immune system: primer and prospectus. *Science*, **273**, 70-74.
- Mitome, M., Shirakawa, T., Oshima, S., Nakamura, W. & Oguchi, H. (2001) Circadian rhythm of nitric oxide production in the dorsal region of the suprachiasmatic nucleus in rats. *Neurosci Lett*, **303**, 161-164.
- Moga, M.M. & Moore, R.Y. (1997) Organization of neural inputs to the suprachiasmatic nucleus in the rat. *J Comp Neurol*, **389**, 508-534.
- Moore, R.Y. & Bernstein, M.E. (1989) Synaptogenesis in the rat suprachiasmatic nucleus demonstrated by electron microscopy and synapsin I immunoreactivity. *J Neurosci*, **9**, 2151-2162.
- Moore, R.Y. & Eichler, V.B. (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res*, **42**, 201-206.
- Moore, R.Y. & Klein, D.C. (1974) Visual pathways and the central neural control of a circadian rhythm in pineal serotonin N-acetyltransferase activity. *Brain Res*, **71**, 17-33.
- Moore, R.Y. & Lenn, N.J. (1972) A retinohypothalamic projection in the rat. *J Comp Neurol*, **146**, 1-14.
- Moore, R.Y. & Speh, J.C. (1993) GABA is the principal neurotransmitter of the circadian system. *Neurosci Lett*, **150**, 112-116.
- Moore, R.Y., Speh, J.C. & Leak, R.K. (2002) Suprachiasmatic nucleus organization. *Cell Tissue Res*, **309**, 89-98.
- Mori, H., Hanada, R., Hanada, T., Aki, D., Mashima, R., Nishinakamura, H., Torisu, T., Chien, K.R., Yasukawa, H. & Yoshimura, A. (2004) Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. *Nat Med*, **10**, 739-743.
- Morin, L.P. (2007) SCN organization reconsidered. *J Biol Rhythms*, **22**, 3-13.
- Morin, L.P. & Blanchard, J.H. (2001) Neuromodulator content of hamster intergeniculate leaflet neurons and their projection to the suprachiasmatic nucleus or visual midbrain. *J Comp Neurol*, **437**, 79-90.
- Morin, L.P., Shivers, K.Y., Blanchard, J.H. & Muscat, L. (2006) Complex organization of mouse and rat suprachiasmatic nucleus. *Neuroscience*, **137**, 1285-1297.
- Morton, A.J., Wood, N.I., Hastings, M.H., Hurelbrink, C., Barker, R.A. & Maywood, E.S. (2005) Disintegration of the sleep-wake cycle and circadian timing in Huntington's disease. *J Neurosci*, **25**, 157-163.
- Mullis, K.B. & Faloona, F.A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol*, **155**, 335-350.
- Munch, M., Knoblauch, V., Blatter, K., Schroder, C., Schnitzler, C., Krauchi, K., Wirz-Justice, A. & Cajochen, C. (2005) Age-related attenuation of the evening circadian arousal signal in humans. *Neurobiol Aging*, **26**, 1307-1319.
- Munoz-Fernandez, M.A. & Fresno, M. (1998) The role of tumour necrosis factor, interleukin 6, interferon-gamma and inducible nitric oxide synthase in the development and pathology of the nervous system. *Prog Neurobiol*, **56**, 307-340.
- Naka, T., Matsumoto, T., Narazaki, M., Fujimoto, M., Morita, Y., Ohsawa, Y., Saito, H., Nagasawa, T., Uchiyama, Y. & Kishimoto, T. (1998) Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice. *Proc Natl Acad Sci U S A*, **95**, 15577-15582.
- Nakamura, W., Honma, S., Shirakawa, T. & Honma, K. (2001) Regional pacemakers composed of multiple oscillator neurons in the rat suprachiasmatic nucleus. *Eur J Neurosci*, **14**, 666-674.

- Neher, E. & Sakmann, B. (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature*, **260**, 799-802.
- Neumann, H., Cavalie, A., Jenne, D.E. & Wekerle, H. (1995) Induction of MHC class I genes in neurons. *Science*, **269**, 549-552.
- Niederhorn, J.Y. (2006) See no evil, hear no evil, do no evil: the lessons of immune privilege. *Nat Immunol*, **7**, 354-359.
- Nikolich-Zugich, J. (2005) T cell aging: naive but not young. *J Exp Med*, **201**, 837-840.
- Nitabach, M.N., Blau, J. & Holmes, T.C. (2002) Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. *Cell*, **109**, 485-495.
- Nunemaker, C.S., DeFazio, R.A. & Moenter, S.M. (2003) A targeted extracellular approach for recording long-term firing patterns of excitable cells: a practical guide. *Biol Proceed Online*, **5**, 53-62.
- Ohdo, S., Koyanagi, S., Suyama, H., Higuchi, S. & Aramaki, H. (2001) Changing the dosing schedule minimizes the disruptive effects of interferon on clock function. *Nat Med*, **7**, 356-360.
- Ohta, H., Yamazaki, S. & McMahon, D.G. (2005) Constant light desynchronizes mammalian clock neurons. *Nat Neurosci*, **8**, 267-269.
- Okamura, H., Berod, A., Julien, J.F., Geffard, M., Kitahama, K., Mallet, J. & Bobillier, P. (1989) Demonstration of GABAergic cell bodies in the suprachiasmatic nucleus: in situ hybridization of glutamic acid decarboxylase (GAD) mRNA and immunocytochemistry of GAD and GABA. *Neurosci Lett*, **102**, 131-136.
- Pachter, J.S., de Vries, H.E. & Fabry, Z. (2003) The blood-brain barrier and its role in immune privilege in the central nervous system. *J Neuropathol Exp Neurol*, **62**, 593-604.
- Pakhotin, P., Harmar, A.J., Verkhatsky, A. & Piggins, H. (2006) VIP receptors control excitability of suprachiasmatic nuclei neurones. *Pflugers Arch*, **452**, 7-15.
- Palomba, M. & Bentivoglio, M. (2007) Chronic inflammation affects the photic response of the suprachiasmatic nucleus. *Journal of Neuroimmunology*, doi:10.1016/j.jneuroim.2007.1009.1002.
- Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S. & Hogenesch, J.B. (2002a) Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*, **109**, 307-320.
- Panda, S., Hogenesch, J.B. & Kay, S.A. (2002b) Circadian rhythms from flies to human. *Nature*, **417**, 329-335.
- Panda, S., Nayak, S.K., Campo, B., Walker, J.R., Hogenesch, J.B. & Jegla, T. (2005) Illumination of the melanopsin signaling pathway. *Science*, **307**, 600-604.
- Paolisso, G., Rizzo, M.R., Mazziotti, G., Tagliamonte, M.R., Gambardella, A., Rotondi, M., Carella, C., Giugliano, D., Varricchio, M. & D'Onofrio, F. (1998) Advancing age and insulin resistance: role of plasma tumor necrosis factor-alpha. *Am J Physiol*, **275**, E294-299.
- Partonen, T., Treutlein, J., Alpman, A., Frank, J., Johansson, C., Depner, M., Aron, L., Rietschel, M., Wellek, S., Soronen, P., Paunio, T., Koch, A., Chen, P., Lathrop, M., Adolfsson, R., Persson, M.L., Kasper, S., Schalling, M., Peltonen, L. & Schumann, G. (2007) Three circadian clock genes *Per2*, *Arntl*, and *Npas2* contribute to winter depression. *Ann Med*, **39**, 229-238.
- Peng, Z.C. & Bentivoglio, M. (2004) The thalamic paraventricular nucleus relays information from the suprachiasmatic nucleus to the amygdala: a combined anterograde and retrograde tracing study in the rat at the light and electron microscopic levels. *J Neurocytol*, **33**, 101-116.
- Pennartz, C.M., de Jeu, M.T., Bos, N.P., Schaap, J. & Geurtsen, A.M. (2002) Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. *Nature*, **416**, 286-290.
- Pennartz, C.M., De Jeu, M.T., Geurtsen, A.M., Sluiter, A.A. & Hermes, M.L. (1998) Electrophysiological and morphological heterogeneity of neurons in slices of rat suprachiasmatic nucleus. *J Physiol*, **506 ( Pt 3)**, 775-793.
- Peralta, S., Carrascosa, J.M., Gallardo, N., Ros, M. & Arribas, C. (2002) Ageing increases SOCS-3 expression in rat hypothalamus: effects of food restriction. *Biochem Biophys Res Commun*, **296**, 425-428.

- Pereira, P.A., Cardoso, A. & Paula-Barbosa, M.M. (2005) Nerve growth factor restores the expression of vasopressin and vasoactive intestinal polypeptide in the suprachiasmatic nucleus of aged rats. *Brain Res*, **1048**, 123-130.
- Peters, A., Moss, M.B. & Sethares, C. (2001) The effects of aging on layer 1 of primary visual cortex in the rhesus monkey. *Cereb Cortex*, **11**, 93-103.
- Plano, S.A., Agostino, P.V. & Golombek, D.A. (2007) Extracellular nitric oxide signaling in the hamster biological clock. *FEBS Lett*.
- Polizzotto, M.N., Bartlett, P.F. & Turnley, A.M. (2000) Expression of "suppressor of cytokine signalling" (SOCS) genes in the developing and adult mouse nervous system. *J Comp Neurol*, **423**, 348-358.
- Prast, H. & Philippu, A. (2001) Nitric oxide as modulator of neuronal function. *Prog Neurobiol*, **64**, 51-68.
- Prolo, L.M., Takahashi, J.S. & Herzog, E.D. (2005) Circadian rhythm generation and entrainment in astrocytes. *J Neurosci*, **25**, 404-408.
- Qiu, X., Kumbalasisri, T., Carlson, S.M., Wong, K.Y., Krishna, V., Provencio, I. & Berson, D.M. (2005) Induction of photosensitivity by heterologous expression of melanopsin. *Nature*, **433**, 745-749.
- Quarrie, J.K. & Riabowol, K.T. (2004) Murine models of life span extension. *Sci Aging Knowledge Environ*, **2004**, re5.
- Quintero, J.E., Kuhlman, S.J. & McMahan, D.G. (2003) The biological clock nucleus: a multiphasic oscillator network regulated by light. *J Neurosci*, **23**, 8070-8076.
- Radomski, M.W., Buguet, A., Bogui, P., Doua, F., Lonsdorfer, A., Tapie, P. & Dumas, M. (1994) Disruptions in the secretion of cortisol, prolactin, and certain cytokines in human African trypanosomiasis patients. *Bull Soc Pathol Exot*, **87**, 376-379.
- Rakesh, K. & Agrawal, D.K. (2005) Controlling cytokine signaling by constitutive inhibitors. *Biochem Pharmacol*, **70**, 649-657.
- Ralph, M.R., Foster, R.G., Davis, F.C. & Menaker, M. (1990) Transplanted suprachiasmatic nucleus determines circadian period. *Science*, **247**, 975-978.
- Ralph, M.R. & Menaker, M. (1988) A mutation of the circadian system in golden hamsters. *Science*, **241**, 1225-1227.
- Rash, J.E., Olson, C.O., Pouliot, W.A., Davidson, K.G., Yasumura, T., Furman, C.S., Royer, S., Kamasawa, N., Nagy, J.I. & Dudek, F.E. (2007) Connexin36 vs. connexin32, "miniature" neuronal gap junctions, and limited electrotonic coupling in rodent suprachiasmatic nucleus. *Neuroscience*, **149**, 350-371.
- Reed, H.E., Cutler, D.J., Brown, T.M., Brown, J., Coen, C.W. & Piggins, H.D. (2002) Effects of vasoactive intestinal polypeptide on neurones of the rat suprachiasmatic nuclei in vitro. *J Neuroendocrinol*, **14**, 639-646.
- Rich, T., Innominato, P.F., Boerner, J., Mormont, M.C., Iacobelli, S., Baron, B., Jasmin, C. & Levi, F. (2005) Elevated serum cytokines correlated with altered behavior, serum cortisol rhythm, and dampened 24-hour rest-activity patterns in patients with metastatic colorectal cancer. *Clin Cancer Res*, **11**, 1757-1764.
- Robertson, B., Kong, G., Peng, Z., Bentivoglio, M. & Kristensson, K. (2000) Interferon-gamma-responsive neuronal sites in the normal rat brain: receptor protein distribution and cell activation revealed by Fos induction. *Brain Res Bull*, **52**, 61-74.
- Rosell, D.R., Akama, K.T., Nacher, J. & McEwen, B.S. (2003) Differential expression of suppressors of cytokine signaling-1, -2, and -3 in the rat hippocampus after seizure: implications for neuromodulation by gp130 cytokines. *Neuroscience*, **122**, 349-358.
- Roth, T., Seiden, D., Sainati, S., Wang-Weigand, S., Zhang, J. & Zee, P. (2006) Effects of ramelteon on patient-reported sleep latency in older adults with chronic insomnia. *Sleep Med*, **7**, 312-318.
- Rozovsky, I., Finch, C.E. & Morgan, T.E. (1998) Age-related activation of microglia and astrocytes: in vitro studies show persistent phenotypes of aging, increased proliferation, and resistance to down-regulation. *Neurobiol Aging*, **19**, 97-103.
- Rutherford, L.C., Nelson, S.B. & Turrigiano, G.G. (1998) BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. *Neuron*, **21**, 521-530.

- Saeb-Parsy, K., Lombardelli, S., Khan, F.Z., McDowall, K., Au-Yong, I.T. & Dyball, R.E. (2000) Neural connections of hypothalamic neuroendocrine nuclei in the rat. *J Neuroendocrinol*, **12**, 635-648.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487-491.
- Sandhir, R., Puri, V., Klein, R.M. & Berman, N.E. (2004) Differential expression of cytokines and chemokines during secondary neuron death following brain injury in old and young mice. *Neurosci Lett*, **369**, 28-32.
- Saper, C.B., Lu, J., Chou, T.C. & Gooley, J. (2005) The hypothalamic integrator for circadian rhythms. *Trends Neurosci*, **28**, 152-157.
- Satinoff, E., Li, H., Tchong, T.K., Liu, C., McArthur, A.J., Medanic, M. & Gillette, M.U. (1993) Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones? *Am J Physiol*, **265**, R1216-1222.
- Saurwein-Teissl, M., Blasko, I., Zisterer, K., Neuman, B., Lang, B. & Grubeck-Loebenstien, B. (2000) An imbalance between pro- and anti-inflammatory cytokines, a characteristic feature of old age. *Cytokine*, **12**, 1160-1161.
- Schaap, J., Albus, H., VanderLeest, H.T., Eilers, P.H., Detari, L. & Meijer, J.H. (2003) Heterogeneity of rhythmic suprachiasmatic nucleus neurons: Implications for circadian waveform and photoperiodic encoding. *Proc Natl Acad Sci U S A*, **100**, 15994-15999.
- Schaap, J., Bos, N.P., de Jeu, M.T., Geurtsen, A.M., Meijer, J.H. & Pennartz, C.M. (1999) Neurons of the rat suprachiasmatic nucleus show a circadian rhythm in membrane properties that is lost during prolonged whole-cell recording. *Brain Res*, **815**, 154-166.
- Schwartz, W.J., Gross, R.A. & Morton, M.T. (1987) The suprachiasmatic nuclei contain a tetrodotoxin-resistant circadian pacemaker. *Proc Natl Acad Sci U S A*, **84**, 1694-1698.
- Sharman, K.G., Sharman, E.H., Yang, E. & Bondy, S.C. (2002) Dietary melatonin selectively reverses age-related changes in cortical cytokine mRNA levels, and their responses to an inflammatory stimulus. *Neurobiol Aging*, **23**, 633-638.
- Shibata, M. & Blatteis, C.M. (1991) Human recombinant tumor necrosis factor and interferon affect the activity of neurons in the organum vasculosum laminae terminalis. *Brain Res*, **562**, 323-326.
- Shibata, S., Oomura, Y., Kita, H. & Hattori, K. (1982) Circadian rhythmic changes of neuronal activity in the suprachiasmatic nucleus of the rat hypothalamic slice. *Brain Res*, **247**, 154-158.
- Shoham, S., Davenne, D., Cady, A.B., Dinarello, C.A. & Krueger, J.M. (1987) Recombinant tumor necrosis factor and interleukin 1 enhance slow-wave sleep. *Am J Physiol*, **253**, R142-149.
- Sierra, A., Gottfried-Blackmore, A.C., McEwen, B.S. & Bulloch, K. (2007) Microglia derived from aging mice exhibit an altered inflammatory profile. *Glia*, **55**, 412-424.
- Silver, R., LeSauter, J., Tresco, P.A. & Lehman, M.N. (1996) A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. *Nature*, **382**, 810-813.
- Smith, S.L. & Otis, T.S. (2003) Persistent changes in spontaneous firing of Purkinje neurons triggered by the nitric oxide signaling cascade. *J Neurosci*, **23**, 367-372.
- Starkey, S.J., Grant, A.L. & Hagan, R.M. (2001) A rapid and transient synthesis of nitric oxide (NO) by a constitutively expressed type II NO synthase in the guinea-pig suprachiasmatic nucleus. *Br J Pharmacol*, **134**, 1084-1092.
- Starr, R., Metcalf, D., Elefanty, A.G., Brysha, M., Willson, T.A., Nicola, N.A., Hilton, D.J. & Alexander, W.S. (1998) Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. *Proc Natl Acad Sci U S A*, **95**, 14395-14399.
- Stellwagen, D., Beattie, E.C., Seo, J.Y. & Malenka, R.C. (2005) Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor- $\alpha$ . *J Neurosci*, **25**, 3219-3228.

- Stellwagen, D. & Malenka, R.C. (2006) Synaptic scaling mediated by glial TNF-alpha. *Nature*, **440**, 1054-1059.
- Stence, N., Waite, M. & Dailey, M.E. (2001) Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia*, **33**, 256-266.
- Stephan, F.K. & Zucker, I. (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci U S A*, **69**, 1583-1586.
- Stichel, C.C. & Luebbert, H. (2007) Inflammatory processes in the aging mouse brain: participation of dendritic cells and T-cells. *Neurobiol Aging*, **28**, 1507-1521.
- Storch, K.F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F.C., Wong, W.H. & Weitz, C.J. (2002) Extensive and divergent circadian gene expression in liver and heart. *Nature*, **417**, 78-83.
- Strecker, G.J., Wuarin, J.P. & Dudek, F.E. (1997) GABAA-mediated local synaptic pathways connect neurons in the rat suprachiasmatic nucleus. *J Neurophysiol*, **78**, 2217-2220.
- Stuesse, S.L., Cruce, W.L., Lovell, J.A., McBurney, D.L. & Crisp, T. (2000) Microglial proliferation in the spinal cord of aged rats with a sciatic nerve injury. *Neurosci Lett*, **287**, 121-124.
- Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. & Lee, C.C. (1997) RIGUI, a putative mammalian ortholog of the *Drosophila* period gene. *Cell*, **90**, 1003-1011.
- Swaab, D.F., Fliers, E. & Partiman, T.S. (1985) The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. *Brain Res*, **342**, 37-44.
- Takahashi, S., Kapas, L., Fang, J. & Krueger, J.M. (1995) An anti-tumor necrosis factor antibody suppresses sleep in rats and rabbits. *Brain Res*, **690**, 241-244.
- Tanaka, M., Hayashi, S., Tamada, Y., Ikeda, T., Hisa, Y., Takamatsu, T. & Ibata, Y. (1997) Direct retinal projections to GRP neurons in the suprachiasmatic nucleus of the rat. *Neuroreport*, **8**, 2187-2191.
- Tancredi, V., D'Arcangelo, G., Grassi, F., Tarroni, P., Palmieri, G., Santoni, A. & Eusebi, F. (1992) Tumor necrosis factor alters synaptic transmission in rat hippocampal slices. *Neurosci Lett*, **146**, 176-178.
- Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. & Sakaki, Y. (1997) Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. *Nature*, **389**, 512-516.
- Tonelli, L.H., Postolache, T.T. & Sternberg, E.M. (2005) Inflammatory genes and neural activity: involvement of immune genes in synaptic function and behavior. *Front Biosci*, **10**, 675-680.
- Tsukahara, S., Tanaka, S., Ishida, K., Hoshi, N. & Kitagawa, H. (2005) Age-related change and its sex differences in histoarchitecture of the hypothalamic suprachiasmatic nucleus of F344/N rats. *Exp Gerontol*, **40**, 147-155.
- Turnley, A.M., Starr, R. & Bartlett, P.F. (2002) Failure of sensory neurons to express class I MHC is due to differential SOCS1 expression. *J Neuroimmunol*, **123**, 35-40.
- Turrigiano, G.G. & Nelson, S.B. (2004) Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci*, **5**, 97-107.
- Ueda, H.R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K., Suzuki, Y., Sugano, S., Iino, M., Shigeyoshi, Y. & Hashimoto, S. (2002) A transcription factor response element for gene expression during circadian night. *Nature*, **418**, 534-539.
- Utsuyama, M. & Hirokawa, K. (2002) Differential expression of various cytokine receptors in the brain after stimulation with LPS in young and old mice. *Exp Gerontol*, **37**, 411-420.
- Valentinuzzi, V.S., Scarbrough, K., Takahashi, J.S. & Turek, F.W. (1997) Effects of aging on the circadian rhythm of wheel-running activity in C57BL/6 mice. *Am J Physiol*, **273**, R1957-1964.
- van Esseveldt, K.E., Lehman, M.N. & Boer, G.J. (2000) The suprachiasmatic nucleus and the circadian time-keeping system revisited. *Brain Res Brain Res Rev*, **33**, 34-77.

- Vandesande, F., Dierickx, K. & DeMey, J. (1975) Identification of the vasopressin-neurophysin producing neurons of the rat suprachiasmatic nuclei. *Cell Tissue Res*, **156**, 377-380.
- Wang, H. & Morris, J.F. (1996) Presence of neuronal nitric oxide synthase in the suprachiasmatic nuclei of mouse and rat. *Neuroscience*, **74**, 1059-1068.
- Wang, T., Xie, Z. & Lu, B. (1995) Nitric oxide mediates activity-dependent synaptic suppression at developing neuromuscular synapses. *Nature*, **374**, 262-266.
- Wang, X., Ellison, J.A., Siren, A.L., Lysko, P.G., Yue, T.L., Barone, F.C., Shatzman, A. & Feuerstein, G.Z. (1998) Prolonged expression of interferon-inducible protein-10 in ischemic cortex after permanent occlusion of the middle cerebral artery in rat. *J Neurochem*, **71**, 1194-1204.
- Wang, Z.W., Pan, W.T., Lee, Y., Kakuma, T., Zhou, Y.T. & Unger, R.H. (2001) The role of leptin resistance in the lipid abnormalities of aging. *Faseb J*, **15**, 108-114.
- Watanabe, A., Shibata, S. & Watanabe, S. (1995) Circadian rhythm of spontaneous neuronal activity in the suprachiasmatic nucleus of old hamster in vitro. *Brain Res*, **695**, 237-239.
- Weale, R.A. (1988) Age and the transmittance of the human crystalline lens. *J Physiol*, **395**, 577-587.
- Weinert, D. (2000) Age-dependent changes of the circadian system. *Chronobiol Int*, **17**, 261-283.
- Weinert, H., Weinert, D., Schurov, I., Maywood, E.S. & Hastings, M.H. (2001) Impaired expression of the mPer2 circadian clock gene in the suprachiasmatic nuclei of aging mice. *Chronobiol Int*, **18**, 559-565.
- Welsh, D.K., Logothetis, D.E., Meister, M. & Reppert, S.M. (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron*, **14**, 697-706.
- Verkhatsky, A. & Toescu, E.C. (1998) Calcium and neuronal ageing. *Trends Neurosci*, **21**, 2-7.
- Vitkovic, L., Bockaert, J. & Jacque, C. (2000) "Inflammatory" cytokines: neuromodulators in normal brain? *J Neurochem*, **74**, 457-471.
- Wormald, S. & Hilton, D.J. (2004) Inhibitors of cytokine signal transduction. *J Biol Chem*, **279**, 821-824.
- Wormald, S., Zhang, J.G., Krebs, D.L., Mielke, L.A., Silver, J., Alexander, W.S., Speed, T.P., Nicola, N.A. & Hilton, D.J. (2006) The comparative roles of suppressor of cytokine signaling-1 and -3 in the inhibition and desensitization of cytokine signaling. *J Biol Chem*, **281**, 11135-11143.
- Wrona, D. (2006) Neural-immune interactions: an integrative view of the bidirectional relationship between the brain and immune systems. *J Neuroimmunol*, **172**, 38-58.
- Wu, Y.H. & Swaab, D.F. (2007) Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease. *Sleep Med*, **8**, 623-636.
- Yadav, A., Kalita, A., Dhillon, S. & Banerjee, K. (2005) JAK/STAT3 pathway is involved in survival of neurons in response to insulin-like growth factor and negatively regulated by suppressor of cytokine signaling-3. *J Biol Chem*, **280**, 31830-31840.
- Yamaguchi, S., Isejima, H., Matsuo, T., Okura, R., Yagita, K., Kobayashi, M. & Okamura, H. (2003) Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science*, **302**, 1408-1412.
- Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M. & Block, G.D. (2002) Effects of aging on central and peripheral mammalian clocks. *Proc Natl Acad Sci U S A*, **99**, 10801-10806.
- Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepk, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M. & Takahashi, J.S. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A*, **101**, 5339-5346.
- Yoshimura, A., Naka, T. & Kubo, M. (2007) SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol*, **7**, 454-465.

- Yu, W.H., Go, L., Guinn, B.A., Fraser, P.E., Westaway, D. & McLaurin, J. (2002) Phenotypic and functional changes in glial cells as a function of age. *Neurobiol Aging*, **23**, 105-115.
- Zhang, Y., Kornhauser, J.M., Zee, P.C., Mayo, K.E., Takahashi, J.S. & Turek, F.W. (1996) Effects of aging on light-induced phase-shifting of circadian behavioral rhythms, fos expression and CREB phosphorylation in the hamster suprachiasmatic nucleus. *Neuroscience*, **70**, 951-961.
- Zhao, J. & Lurie, D.I. (2004) Loss of SHP-1 phosphatase alters cytokine expression in the mouse hindbrain following cochlear ablation. *Cytokine*, **28**, 1-9.
- Zhou, J.N., Hofman, M.A. & Swaab, D.F. (1995) VIP neurons in the human SCN in relation to sex, age, and Alzheimer's disease. *Neurobiol Aging*, **16**, 571-576.