

A NETWORK OF (AUTONOMIC) CLOCK OUTPUTS

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The circadian clock in the suprachiasmatic nuclei (SCN) is composed of thousands of oscillator neurons, each dependent on the cell-autonomous action of a defined set of circadian clock genes. A major question is still how these individual oscillators are organized into a biological clock that produces a coherent output capable of timing all the different daily changes in behavior and physiology. We investigated which anatomical connections and neurotransmitters are used by the biological clock to control the daily release pattern of a number of hormones. The picture that emerged shows projections contacting target neurons in the medial hypothalamus surrounding the SCN. The activity of these pre-autonomic and neuro-endocrine target neurons is controlled by differentially timed waves of vasopressin, GABA, and glutamate release from SCN terminals, among other factors. Together our data indicate that, with regard to the timing of their main release period within the LD cycle, at least four subpopulations of SCN neurons should be discernible. The different subgroups do not necessarily follow the phenotypic differences among SCN neurons. Thus, different subgroups can be found within neuron populations containing the same neurotransmitter. Remarkably, a similar distinction of four differentially timed subpopulations of SCN neurons was recently also discovered in experiments determining the temporal patterns of rhythmicity in individual SCN neurons by way of the electrophysiology or clock gene expression. Moreover, the specialization of the SCN may go as far as a single body structure, *i.e.*, the SCN seems to contain neurons that specifically target the liver, pineal gland, and adrenal gland.

Keywords Circadian clock, Suprachiasmatic nuclei, GABA, Glutamate, Vasopressin, Melatonin, Glucose

INTRODUCTION

In all circadian systems identified to date, three major components can be identified: (a) a light input pathway to a self-sustained master circadian pacemaker, (b) the circadian pacemaker itself, and (c) output pathways by

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which the circadian pacemaker regulates overt rhythms in biochemistry, physiology, and behavior. The issue of entrainment is very much at the heart of chronobiological research, and most of the early “circadian” research concentrated on the light input and entrainment pathways. In humans and other mammals, light information is exclusively processed by the retina and conveyed to the brain by the retinohypothalamic tract (RHT) in the optic nerve. Monosynaptic neural projections from the RHT terminate in the bilaterally paired suprachiasmatic nuclei (SCN) of the anterior hypothalamus, hosting the central pacemaker. The RHT comprises a distinct subset of retinal ganglion cells that recent studies have shown to contain a novel photopigment melanopsin (Opn4) and to be directly light sensitive (Fu et al., 2005). These ganglion cells appear to express and utilize pituitary adenylyl cyclase-activating peptide (PACAP) and glutamate as cotransmitters to communicate with the SCN (Fahrenkrug, 2006; Hannibal, 2006; Hannibal et al., 2000).

Conclusive evidence that the SCN indeed comprises the master circadian pacemaker came from lesion studies and transplantation experiments. Transplantation of SCN tissue from mutant donor animals into SCN-lesioned wild-type hosts conferred the mutant circadian phenotype to the host (Ralph et al., 1990; Sujino et al., 2003). Circadian rhythms were thought to be generated within SCN neurons through a set of interconnected positive and negative autoregulatory feedback loops of transcription and translation (Weaver, 1998). Finally, ten years ago, research into the core molecular mechanism of the central pacemaker was boosted by the discovery and mapping of the first mammalian clock gene, *i.e.*, CLOCK (Vitaterna et al., 1994). The transcription factors CLOCK and BMAL1 heterodimerize and promote the transcription of three *period* genes (*mPer1*, *mPer2*, *mPer3*) and two *cryptochrome* genes (*mCry1*, *mCry2*). The resulting mPER and mCRY proteins heterodimerize, translocate to the nucleus, and negatively regulate the transcription of the *mPer* and *mCry* genes by inhibiting the activity of CLOCK and BMAL1. The negative feedback cycle created by these molecular interactions takes approximately 24 h to complete and is responsible for the endogenous circadian rhythm. Deletion of any of these genes, or inhibition of the kinases that phosphorylate their encoded proteins, shortens or lengthens the circadian period and can result in arrhythmicity (Panda et al., 2002; Reppert and Weaver, 2002; Sato et al., 2004). Currently, at least ten core clock genes have been identified and characterized, and microarray studies indicate that many more may await identification (Takahashi, 2004). However, the link between these transcriptional and translational events, on one hand, and changes in neuronal firing, on the other, remains a mystery. Most, if not all, of the 16,000 to 20,000 neurons harbored in the paired rat SCN can independently generate a self-sustained circadian rhythm, for instance, when grown *in vitro*, but so can

many peripheral clocks. However, the SCN neurons are heavily interconnected and coupled. It is this ability to communicate and synchronize that provides the SCN with its ability to express long-term synchronized rhythms of electrical activity, humoral output, and gene expression (Long et al., 2005; Nagoshi et al., 2004; Welsh et al., 1995).

SCN OUTPUTS: NEURONAL OR HUMORAL?

Despite the recent, vast increase in knowledge on the underlying mechanisms that generate circadian rhythms, it is still mysterious how individual SCN neurons are assembled to create an integrated tissue pacemaker that governs the circadian behavior of the whole animal. Moreover, it is a mystery as to how this integrated pacemaker produces a non-uniform rhythm. For instance, although most endocrine functions show clear day-night rhythms, the time of their peaks varies widely throughout the 24 h period (Figure 1). Again, transplantation experiments turned out to be crucial. The demonstration that transplanted SCN tissue encaged in a semi-permeable membrane can still restore a circadian rhythm in locomotor activity proved that diffusible factors emanate from the transplanted SCN (Silver et al., 1996). The first SCN transmitter to be recognized was vasopressin (VP) (Burlet and Marchetti, 1975; Swaab et al., 1975; Vandesande et al., 1974). Due to its pronounced day-night rhythm in the cerebrospinal fluid or CSF (Reppert et al., 1981; Schwartz et al., 1983), VP was characterized as a humoral output of the SCN, and remains the only SCN output that has been demonstrated to be secreted in a circadian rhythm *in vivo*. However, it is still not clear whether VP in the cerebrospinal fluid or CSF really acts as a humoral factor or whether it is merely spill-over, *i.e.*, VP released as a neurotransmitter that is removed by diffusion. Despite its early discovery, the interest in VP as an important clock output rapidly disappeared when no gross abnormalities could be detected in the circadian rhythms of the Brattleboro rat (*i.e.*, a rat strain bearing a naturally occurring missense mutation in the gene encoding for VP) (Groblewski et al., 1981). More recently, TNF-alpha and prokineticin-2 were proposed as humoral outputs of the circadian pacemaker in mammals (Cheng et al., 2002; Kramer et al., 2001) in addition to VP (Tousson and Meissl, 2004). Together, these results suggest that there may be a mélange of secreted factors that is able to control a behavioral output under circadian control, such as locomotor activity.

Transplantation experiments, however, also unequivocally demonstrate the necessity of intact neural projections from the SCN to specific regional targets for complete restoration of function, since transplants that successfully restore circadian locomotor activity fail to restore neuroendocrine rhythms (Lehman et al., 1987; Meyer-Bernstein et al., 1999). Two very elegant recent experiments provide additional evidence

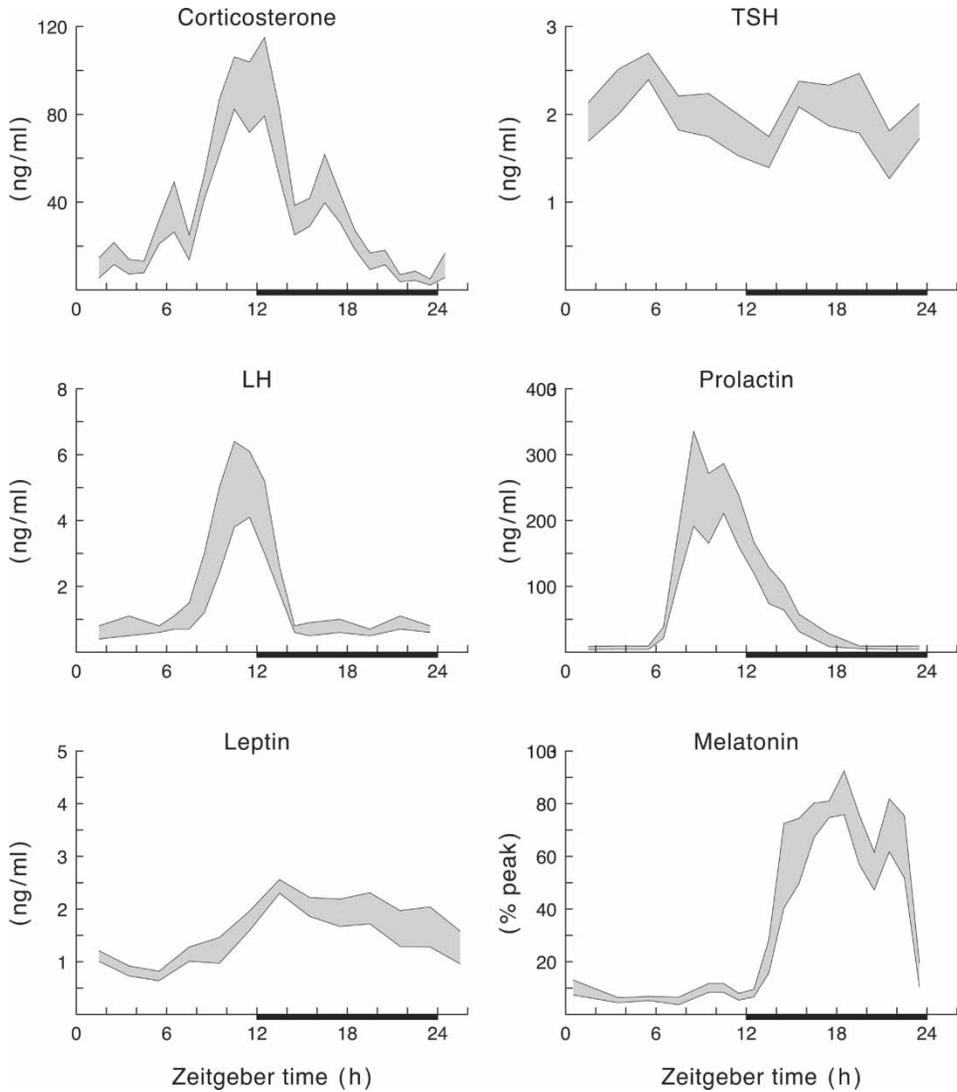


FIGURE 1 Day-night rhythms in plasma hormone concentrations of various endocrine systems in the rat. Note the diversity in the timing of the different acrophases (peak times of rhythms).

for the necessity of point-to-point neural connections in order to sustain neuroendocrine rhythms. Using behaviorally “split” female rats, de La Iglesia and coworkers (2000) not only showed an anti-phase of clock gene cycling in the left and right SCN, but also a pronounced left-right asymmetry in activated LHRH neurons, as indicated by *c-Fos* expression (de la Iglesia et al., 2003). Thus, the previously reported circa 12 h LH secretory surges (Swann and Turek, 1985) are the result of alternating left- and right-sided stimulation of LHRH neurons by point-to-point

axonal SCN projections. Also the experiments of Guo and colleagues (2005), using parabiosis between intact and SCN-lesioned animals, nicely demonstrate that non-neuronal mechanisms are not sufficient to reinstate circadian rhythms in all peripheral organs.

ANATOMY OF NEURONAL SCN OUTPUTS

Information on the distribution of SCN projections was initially obtained from neuro-anatomical studies using tracing, immunocytochemistry, SCN-lesions, or a combination of these methods. Together, these studies show that the outflow of SCN information is mainly restricted to the medial hypothalamus. Within the hypothalamus, at least three main targets for the SCN efferents can be discerned: endocrine neurons, pre-autonomic neurons, and intermediate neurons (Figure 2). The first group (*i.e.*, endocrine neurons), such as those containing corticotropin-releasing hormone (CRH) and gonadotropin-releasing hormone (GnRH), seems a logical target for the biological clock. Anatomical experiments, however, have until now only provided limited evidence for direct connections (Buijs et al., 1993; Vrang et al., 1995). The second group of SCN targets consists of the pre-autonomic neurons in the hypothalamus, *i.e.*, the neurons that lie at the origin of long descending projections to

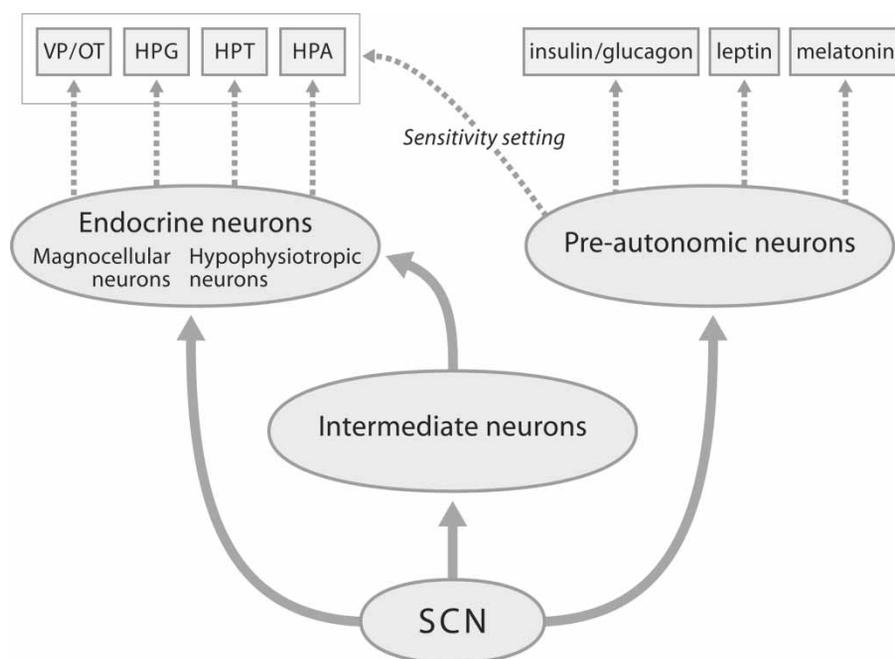


FIGURE 2 Review scheme of the various SCN projections controlling the rhythmicity of endocrine functions (from Perreau-Lenz et al., 2004).

pre-ganglionic parasympathetic and sympathetic neurons in the brainstem and spinal cord, respectively. Viral tracing studies, in combination with labeling of SCN efferents, provide clear evidence for a direct SCN input to these neurons (Tecler-Mesbah et al., 1997). The third category of neurons reached by the SCN efferents consists of the so-called intermediate neurons (*e.g.*, in the dorsomedial hypothalamus, the medial preoptic area, and subparaventricular zone) which probably integrate circadian information with other hypothalamic inputs before passing it on to the endocrine (and autonomic) neurons (Saper et al., 2005).

Immunocytochemical staining of known transmitters reveals a clear heterogeneity in SCN neurons with respect to the major neuropeptides expressed. So far, vasopressin, vasoactive intestinal peptide (VIP), and gastrin-releasing peptide (GRP) seem to be the most abundantly present neuropeptides, but neurons expressing somatostatin, cholecystokinin, calcitonin, calbindin, and neurotensin were demonstrated as well. In addition to these neuropeptides, many SCN neurons also contain GABA and/or glutamate. In most species studied to date, the dorsomedial region of the SCN is dominated by vasopressin-containing neurons, while overlapping sets of neurons in the ventrolateral part of the SCN express both VIP and GRP. As of now, no evidence connects a certain transmitter to one specific SCN output (de La Iglesia and Schwartz, 2002; Kriegsfeld et al., 2004; Leak and Moore, 2001; Munch et al., 2002). Overall, the gross anatomic substructures and major neuronal connections of the SCN are conserved from rodents to humans, implying conservation of the neuroendocrine and autonomic control of rhythms by the SCN across species (Dai et al., 1997, 1998a, 1998b).

SCN OUTPUT PATHWAYS

The first SCN output pathway to be clearly linked to a neuroendocrine rhythm is the one describing the connection between the rhythmic activity generated in the SCN and the rhythmic release of melatonin from the pineal gland. The major anatomical details of the neural pathway between the biological clock and the pineal gland were already revealed soon after the identification of the SCN as the central pacemaker in mammals (Klein and Moore, 1979; Moore and Klein, 1974). Yet, it was only with the help of transneuronal viral tracer injections in the pineal gland that the multi-synaptic nature of the connection between the paraventricular nucleus of the hypothalamus (PVN), pre-ganglionic sympathetic neurons in the spinal cord, and the noradrenalin-containing neurons in the superior cervical ganglion finally could be confirmed (Larsen et al., 1998; Tecler-Mesbah et al., 1999). Unfortunately, viral tracing studies were unable to discern the exact trajectory of the PVN fibers on their way to the spinal cord, *i.e.*, the dorsomedial route

along the central gray or the ventrolateral route within the medial forebrain bundle (Luiten et al., 1985), although the early lesion studies favor the pathway along the medial forebrain bundle (Moore, 1996).

The first physiological experiments to identify the SCN neurotransmitters involved in the control of the daily melatonin rhythm, however, were not very successful. Notwithstanding its reverse relation to the melatonin rhythm and its clear effects on the daily corticosterone rhythm (see below), we could find no effects whatsoever of hypothalamic vasopressin on the melatonin rhythm (Kalsbeek et al., 1993, 2000). Also, VIP and oxytocin, when applied at the level of the PVN, proved to be ineffective in changing melatonin release from the pineal gland (Kalsbeek et al., 1993); however, manipulation of the GABAergic transmission in the PVN proved to be very effective. The concomitant changes in pineal melatonin release and extracellular pineal noradrenaline levels provide clear evidence for the involvement of sympathetic innervation of the pineal in these PVN-induced changes (Kalsbeek et al., 1996a), although they do not exclude, of course, a possible presynaptic effect of central pinealopetal projections (Fink-Jensen and Møller, 1990). In a number of subsequent experiments, we showed that GABAergic neurons in the SCN are involved in the direct inhibitory effects of light on melatonin release as well as in the circadian control of the melatonin rhythm (Kalsbeek et al., 1996a, 1999, 2000). An endogenous rhythm of GABA release, with an increased release during the light period, fits well with the daily rhythm of multi-unit electrical and metabolic activity in the SCN (Flood and Gibbs, 1982; Green and Gillette, 1982; Groos and Hendriks, 1982; Schwartz and Gainer, 1977; Schwartz et al., 1980; Shibata et al., 1982) and the daily rhythm of vasopressin release from SCN terminals (Schwartz and Reppert, 1985; Gillette and Reppert, 1987; Schwartz et al., 1983).

Additional experiments indicated that, together with the inhibitory control by GABA, the SCN lesion also removes an important stimulatory input to the melatonin-rhythm-generating-system (Perreau-Lenz et al., 2003). However, the nocturnal peak of melatonin release means that, in order to provide this stimulatory input, SCN neurons should be active during the dark period, *i.e.*, in contrast with the suggested nocturnal silence of SCN neurons indicated by electrophysiological recordings and 2-deoxy-glucose studies. Nevertheless, the immediate decrease of pineal melatonin release upon bilateral administration of TTX in the SCN clearly demonstrates the necessity of a nocturnal activity of SCN neurons (Perreau-Lenz et al., 2004). Moreover, blockade of the glutamatergic input to the PVN by local administration of a receptor-antagonist for the N-methyl-D-aspartate (NMDA) receptors also causes an immediate decrease in nocturnal melatonin release (Perreau-Lenz et al., 2004). Therefore, although the nocturnal (electrical) activity of SCN neurons may seem rather weak, it is sufficient, and even necessary, to stimulate

nocturnal melatonin release. The nocturnal electrical activity of SCN neurons likely remained unnoticed for such a long time because of its restriction to only a limited number of neurons.

The second SCN output pathway to be clearly linked to a neuroendocrine rhythm is the VP-containing projection to the PVN/DMH area and its control of the daily rhythm in corticosterone release from the adrenal cortex. The prominent rhythm of VP release from SCN terminals and the close proximity of these terminals to the corticotrophin-releasing hormone (CRH)-containing endocrine neurons in the PVN led us to start investigating the functional significance of this anatomical pathway. Our initial physiology experiments showed that the daily rhythm of corticosterone release is controlled by at least two SCN transmitters, *i.e.*, vasopressin release within the DMH, which inhibits the activity of the HPA-axis during the early part of the light period, and a thus far unknown SCN transmitter that stimulates the activity of the HPA-axis from the middle of the light period onward until the onset of the dark period (Kalsbeek *et al.*, 1992, 1996b, 1996c). Recently, Neuromedin-U appeared as a possible candidate for this stimulatory SCN transmitter (Graham *et al.*, 2005). In addition to this neuroendocrine route, the SCN also proved to be connected to the adrenal via a separate polysynaptic projection involving the pre-autonomic neurons in the PVN and spinal cord. This separate pathway enables the SCN to influence the sensitivity of the adrenal cortex to ACTH (Buijs *et al.*, 1999).

Additional SCN output pathways to be connected to (neuroendocrine) rhythms involve the VIP and VP-containing projections to the gonadotropin-releasing and estrogen nuclear receptor-containing neurons of the preoptic area (de La Iglesia *et al.*, 1995; Funabashi *et al.*, 2000; Palm *et al.*, 1999, 2001; Van Der Beek *et al.*, 1997; Watson *et al.*, 1995;), which may control the reproductive cycle in females. Sparse projections from the SCN to the ventrolateral preoptic area may participate in the circadian modulation of the sleep-wake system, and they are likely to use GABA and glutamate as a transmitter (Novak and Nunez, 2000; Sun *et al.*, 2000, 2001). Finally, SCN projections to the orexin-containing neurons of the lateral hypothalamus may serve as a critical relay to mediate the arousal function of the circadian timing system (Abrahamson *et al.*, 2001; Deboer *et al.*, 2004; Zhang *et al.*, 2004).

THE AUTONOMIC CONNECTION

For a number of years, we have been investigating the relation between energy homeostasis and circadian rhythms, in addition to the neuroendocrine rhythms mentioned above. In order to understand how the hypothalamic biological clock conveys its circadian message to the homeostatic system(s) that regulate energy balance, we focused our

attention on the daily control of glucose metabolism. Peak plasma glucose concentrations are attained every day shortly before awakening. Peripheral plasma glucose concentrations are the result of glucose influx from the gut and glucose production by the liver, and glucose efflux by its uptake in brain, muscle, and adipose tissue. Indeed, glucose uptake varies during the LD cycle, with the highest glucose disappearance rates measured at the time of awakening (Kalsbeek et al., 2003; La Fleur et al., 2001). The simultaneous increase of glucose uptake and plasma glucose concentrations, even under the conditions of our regular 6 meals/day feeding schedule, indicate also that (hepatic) glucose production should be increased at this time of the day. Additional experiments reveal that the daily rhythms in glucose uptake and glucose production are not a consequence of daily rhythms in the release of the pancreatic hormones insulin and glucagon, respectively (La Fleur et al., 1999; Ruiters et al., 2003); rather, they implicate an important role for the autonomic (sympathetic) innervation of the liver in the genesis of the anticipatory morning rise in plasma glucose concentrations, probably by controlling hepatic glucose production. As previously reported for the sympathetic input to the pineal gland, the activity of the pre-autonomic sympathetic liver neurons (involved in glucose production) also are controlled by the combined continuous glutamatergic and rhythmic GABAergic input from the SCN (Kalsbeek et al., 2004).

HETEROGENEITY OF (AUTONOMIC) CLOCK OUTPUTS

For many years, the prevailing model of the SCN has been a “monophasic” one, in which the SCN neurons formed a single population, with a coherent phase and an activity peak at \sim CT6. More recently, however, both molecular and electrophysiological evidence has challenged this “monophasic” view. Defined cell groups in the rat SCN exhibit different day-night rhythms of single unit activity *in vivo* (SaebParsy and Dyball, 2003), and some *in vitro* studies even indicate the existence of SCN neurons with an opposite phase of activity (Herzog et al., 1997; Nakamura et al., 2001). In addition, experiments using phase-shifting protocols and PER1/2 immunocytochemistry indicate a differential timing of the circadian clock work in different subgroups of the SCN (Nagano et al., 2003; Reddy et al., 2002; Yan and Silver, 2002). Our data on the SCN control of neuroendocrine rhythms provide clear evidence not only that the SCN consists of phenotypically (*i.e.*, according to neurotransmitter content) different subpopulations of neurons, but also that subpopulations should be discerned based on the acrophase (peak time) of their electrical activity.

Thus far, our physiological findings indicate the existence of at least four subpopulations of SCN neurons with a differential timing of their acrophase in electrical activity. The first population (acrophase at \sim ZT2)

contains vasopressin and GABA and inhibits the release of corticosterone and hepatic glucose production, respectively. The second and probably main population (acrophase at \sim ZT6) contains at least for some part GABA and is responsible for the inhibition of melatonin release during the light period. The third population (acrophase at \sim ZT10) contains an as yet unknown transmitter and is responsible for the activation of the HPA-axis during its daily peak. Finally, the fourth population consists of neurons that are mainly active during the subjective dark period (acrophase at \sim ZT18), with at least a part of them containing glutamate, are responsible for the nocturnal peak in melatonin release. Remarkably, a similar distinction of discrete phase groups of SCN neurons also was recently defined on the basis of molecular and electrophysiological recordings. Schaap and colleagues (2003) showed that subpopulations of SCN neurons have surprisingly short periods (\sim 5 h) of enhanced electrical activity, with peak activities occurring at different phases of the circadian cycle. The summed activity of these subpopulations accounts for the neuronal ensemble pattern of SCN activity. At about the same time, Quintero and coworkers (2003) presented their results on the time-lapse imaging of SCN slices from mice with a short half-life green fluorescent protein (GFP) reporter of *Per1* gene. They discerned four groups of cellular oscillators, with the majority of neurons (50% to 60%) forming a main phase group at \sim CT6, with two additional groups (10% to 20%) peaking 3 to 4 h earlier or later. In addition, a less numerous group of neurons cycled with peak times primarily in the dark phase.

Finally, the differential timing of the acrophases for hepatic glucose production and pineal melatonin release implicates a different timing of the trough of GABA release from SCN terminals that target the pre-autonomic neurons controlling the sympathetic input to the liver versus the ones controlling the sympathetic input to the pineal gland. Therefore, these data indicate that, even within neurons with the same phenotype (*i.e.*, GABAergic), subpopulations with a differential timing may exist. In addition, our previous tracing studies indicate that the SCN not only contains separate neurons to control the sympathetic and parasympathetic branches of the autonomic nervous system, but also contains separate neurons for the control of abdominal *versus* subcutaneous fat tissue (Kreier et al., 2002). But if single neurons in the cortex are devoted to recognizing the face of specific individuals (Quiroga et al., 2005), then separate SCN neurons or neuron populations for every bodily structure may be not that extraordinary.

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