Electrophysiology of the suprachiasmatic nucleus
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Chapter 1

General Introduction
1. GENERAL INTRODUCTION

1.1 Circadian rhythms

The rotation of the earth on its polar axis generates a periodicity of sun illumination on the surface of the earth; a day-night cycle. Life on earth developed endogenous rhythmicities in behavioural and physiological processes close to this periodicity (circadian rhythm: circa, about; dies, a day). Fine-tuning of endogenous rhythms to the environmental day-night cycle is accomplished by small daily adjustments (entrainment). The advantage of entrained circadian rhythmicities is that behavioural and physiological activities of an organism will be performed in a temporal order and allows the organism to prepare for or anticipate the coming period [1].

In mammals, the main endogenous circadian rhythm is generated by the suprachiasmatic nucleus (SCN); a bilateral hypothalamic cluster of neurons on top of the optic chiasm [2–5]. This circadian rhythm is transmitted to target areas and imposes circadian rhythmicity on behaviours and physiological processes [6,7]. Entrainment of circadian rhythms in mammals can be accomplished by photic as well as non-photic stimuli, such as stress, activity and food [8]. Light is the strongest Zeitgeber (synchronizer) in mammals. Light information enters the SCN directly via the retinohypothalamic tract and uses glutamate as signal transmitter [9–13]. Indirectly, light information is transmitted to the intergeniculate leaflet (IGL) via the retinogeniculate tract and projections from the IGL enter the SCN via the geniculohypothalamic tract using γ-aminobutyric acid (GABA) and neuropeptide Y (NPY) -mediated neurotransmission [14]. Non-photic stimuli (like temperature, stress and physical activity) are also effective Zeitgebers [15]. The pathways by which non-photic information enters the SCN have not been completely elucidated yet, but likely run via the IGL or Raphe nuclei [16]. The circadian rhythm generated by the SCN is transmitted to target areas and imposes circadian rhythmicity on behaviours and physiological processes via these target areas. The main neuronal output pathways from the SCN project to the subparaventricular zone, paraventricular nucleus, dorso- and ventromedial nucleus of the hypothalamus, medial preoptic nucleus, anteroventral nucleus, lateral septum and bed nucleus of the stria terminalis, paratenial nucleus, lateral geniculate nucleus, periaqueductal gray and raphe nuclei [17,18].

In many studies on circadian physiology and behaviour, the SCN is considered to be a black box [19]. Input-output relationships do provide insight in the mechanism underlying circadian rhythms, but are not able to reveal the underlying molecular and cellular substrate. In this thesis, we will try to get a glimpse of the machinery underlying the regulation of day-night rhythms by using an electrophysiological approach. For this study, we used SCN neurons of the rat (a nocturnal animal). The firing activity of SCN neurons in diurnal and nocturnal species are similar: neuronal firing rates within the SCN are high during the day and low during the night in nocturnal as well as diurnal animals [20–24]. Thus, results obtained from studying
fundamental electrophysiological properties of SCN neurons of the rat may not only apply to nocturnal animals, but may also hold for diurnal animals.

1.2 The suprachiasmatic nucleus

1.2.1 Cellular structure of the Suprachiasmatic nucleus

The SCN in rats is a bilateral, paired nucleus that contains approximately 16,000 neurons [25]. The neurons of the SCN form a complex structure, containing many different cell types that are loaded with a variety of different neuroactive substances. Based on the characteristics of the dendritic tree [25-27], SCN neurons were classified into four types: monopolar, radial, simple bipolar and curly bipolar. Characterization based on electrophysiological properties led to a classification of 3 cell types (cluster I, II and III; [27]). No correlation was found between cell morphology and the electrophysiological characteristics of cell types, except that cluster III neurons possessed more axon collaterals [27].

Characterization of the peptidergic content of SCN neurons [28] revealed clear topographic differences in the distribution of cell phenotypes. In the dorsomedial part of the SCN most neurons contain vasopressin (VP), whereas neurons in the ventral part of the SCN contain vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI) or gastrin releasing peptide (GRP). The somatostatin (SOM)-containing neurons are located more centrally in the SCN. Immunocytochemical staining of electrophysiologically identified cells revealed that vasopressin-containing neurons can be defined as a subset of cluster I neurons [29].

The presence of distinct cell types in the SCN suggests that different functions may be mediated by different subpopulations of SCN neurons. Although it is unknown how different cell types contribute to SCN function, some interesting postulates have been proposed. First, the vasopressin-containing neurons are thought to be output neurons, which are closely coupled to the circadian pacemaker mechanism [30,31], even though vasopressin-deficient (Brattleboro) rats retain rhythmicity. Second, the VIP-containing neurons are believed to be relay neurons and to process retinal inputs [32]. Third, the GRP and PHI containing neurons are thought to have a prominent role in light-induced phase shifts [33].

1.2.2 Physiology and Electrophysiology of the suprachiasmatic nucleus

Pacemaker neurons

Neurons of the SCN exhibit circadian rhythms in spontaneous firing rate in vivo as well as in vitro [24,34-38]. Blocking the neuronal firing of SCN neurons does not prevent the circadian clock from running [37,39,40], indicating that these neurons have an intrinsic, spike-independent mechanism for circadian rhythmogenesis. Further evidence for an intrinsic mechanism (cell autonomy) was provided by in vitro firing activity measurements of individual SCN neurons, showing single-cell circadian firing rhythms that were not synchronized within the same culture [37].
The pacemaker neurons contain a genetic clock function based on transcriptional and translational feedback loops of clock gene products (Box 1.). Long-term multi-electrode recordings of homozygous Clock mutant SCN neurons revealed arrhythmic firing activity patterns, similar to the behavioural activity of the animal, suggesting that the CLOCK protein is required for the circadian firing behaviour of individual SCN neurons [41]. The intracellular mechanisms that connect the genetic clock with membrane excitability are not yet known [42]. Thus far, little progress has been made in identifying day-night differences in membrane properties as a basis for explaining the circadian rhythm in spontaneous firing of SCN neurons. However, in the mollusc *Bulla gouldiana*, the circadian rhythm of basal retinal neurons appears to be driven by a rhythm in membrane conductance [43]. The membrane conductance is inversely correlated with the circadian rhythm in the frequency of action potentials, indicating the involvement of circadian switching of ion channel configurations or ion channel availability. Mechanisms comparable to those in pacemaker neurons of the mollusc may underlie expression of a circadian rhythm in SCN firing activity. In the mollusc *Bulla gouldiana*, the delayed rectifier channel does exhibit a circadian rhythm in conductance in phase with the general circadian rhythm in membrane conductance [44]. However, this current could not be held responsible for the circadian rhythm in excitability because it is not activated at the resting membrane potential of these neurons [44].

*Integration of external and internal information signals in the SCN*

Environmental and internal time cues can entrain circadian rhythms. This information needs to enter the SCN and adjust the circadian rhythm of SCN pacemaker neurons. By plotting the magnitude of the adjustment of the circadian rhythm (phase shift) against the circadian timing of the stimuli (time cue), a phase-response curve (PRC) is generated: PRC’s are often used in circadian research to investigate entrainment characteristics of stimuli [19]. Light affects the circadian rhythm only during the night. Light in the beginning of the night delays the phase of the circadian rhythm, whereas light at the end of the night advances the phase of the circadian rhythm [46]. *In vivo* recordings showed that light increases the firing activity of SCN neurons in a circadian manner [23]. Strong light-induced responses of SCN neurons were found during the night period, whereas light-induced responses of SCN neurons during the day period were weak or absent. These results indicate a mechanism that controls the gain of retinal-to-SCN signalling. Transmission of light information to pacemaker cells appears to be facilitated during the night but reduced during the day (see also [47]; day: small NMDA, night: high NMDA). This idea is supported by light-induced SCN profiles of clock gene expression (e.g. mPer1) and activity markers (e.g. c-fos) propagating throughout the SCN in a spatio-temporal manner measured during the subjective night [48]. However, the mechanism underlying transmission of photic information throughout the SCN is still unknown.

In non-photic entrainment, two inputs to the SCN are considered to be of importance: the NPY-containing projections from the IGL and the serotonin-containing projections from the Raphe nuclei [16]. Both neurotransmitters (NPY and serotonin) inhibit firing activity of SCN neurons and induce phase shifts resembling non-photic
Box 1. The genetic clock*

mPER2 activates the Bmal gene. BMAL binds to constitutively active CLOCK and after a delay the dimer binds to a CACGTG E-box element to activate transcription of mPer2 and mCry. mPER2 and mCRY proteins are formed. These clock gene products can heterodimerize with each other by PAS domain protein-protein interactions (like BMAL-CLOCK dimers). This heterodimer interaction leads to the nuclear translocation of mPER2 and mCRY. After entering the nucleus mPER2 and mCRY split their duties. mPER2 will activate Bmal again, while mCRY shuts down the transcription of mPer2, mCry and CCG's. The roles of mPER1 and mPER3 in the genetic clock are not yet known. CCG’s are clock controlled genes.

Fig. 1.1: Schematic representation of the genetic clock in mammals. The circadian rhythm of clock proteins is generated due to interaction of positive and negative transcription-translation feedback loops (with delay components). * Based on the model of Shearman and co-workers [45]. It should be noted that the work on the genetic clock proceeds at a breathtaking pace, which results in a high turnover of genetic clock models.
PRC's [49–51].

In the complex structure of the SCN, the timing of photic and non-photic cues is compared to the phase of the endogenous circadian rhythm. The endogenous circadian clock will be adjusted when periodic photic and/or non-photic time cues are not in phase with the endogenous circadian rhythm. To adjust the endogenous circadian clock a perturbation of the molecular clock is necessary [52,53]. One may propose that glutamate (photic) influences the molecular clock by activating Ca$^{2+}$-dependent signal transduction pathways [54–56], whereas serotonin and NPY (non-photic) influence the molecular clock by activating G-protein coupled signal transduction pathways.

Although a lot of progress is currently being made towards understanding the molecular mechanism underlying circadian oscillators [45,58], the way in which oscillators can coordinate circadian outputs still needs to be elucidated. In dispersed SCN cell cultures with different cell densities an increased synchrony of circadian rhythms in firing rates was found in the high as opposed to low density cultures [59], indicating the importance of SCN cell communication for the synchronization of multiple circadian oscillators. A mechanism for intranuclear communication between pacemaker neurons can be established via chemical or electrical (gap junctions) transmission, but the coupling interaction between pacemaker neurons is still unknown. The neurotransmitter GABA is an interesting candidate as a synchronizing (coupling) transmitter for pacemaker neurons. Since (I) GABA is present in almost all neurons throughout the whole SCN [60,61], (II) GABA is involved in intranuclear transmission between SCN neurons [61,62] and (III) GABA affects pacemaker neurons via GABA$_A$-mediated receptors [63], it may have properties necessary to perform a function as a "synchronizer" [64].

1.2.3 Scope of this Thesis

The general aim of our studies was to investigate the circadian electrophysiological behaviour of SCN neurons and their communication mechanisms. At a cellular level, we studied mechanisms underlying the cell-autonomous circadian rhythm in firing activity. By using the electrophysiological whole-cell patch-clamp technique, ion channels were investigated that modulate the excitability of SCN neurons and may thus influence the circadian rhythm in spontaneous firing activity (Chapter II and III). With the use of the more advanced gramicidin-perforated patch-clamp technique, day-night differences in membrane properties were investigated (Chapter IV).

At an intercellular level, we characterized the role of the neurotransmitter GABA in the SCN. By using the gramicidin-perforated-patch clamp technique, GABAergic transmission in the SCN was investigated both in the day and night phase and we observed a circadian modulation of GABA$_A$-receptor function in the SCN (Chapter V). This circadian modulation of GABA function could be of importance for synchronizing pacemakers and for relaying information throughout the SCN in a restricted time domain (Chapter V). This thesis is concluded by a General Discussion (Chapter VI).