Electrophysiology of the suprachiasmatic nucleus
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Chapter 6

General Discussion
6. GENERAL DISCUSSION

In this chapter the following two questions will be discussed: 1) What is the contribution of electrophysiology to the research on SCN functioning? and 2) what can electrophysiology offer future research on SCN functioning? With the use of electrophysiological techniques, more insight in functioning of the SCN has been gained. The electrophysiological approach is mainly used in SCN research to elucidate the mechanisms underlying the circadian rhythm in spontaneous firing rate (section 2) or to study the integration of external and internal information signals that are involved in the entrainment function of the SCN (section 3). Although electrophysiological techniques are very suitable for studying how the SCN processes inputs and produces circadian output, it is necessary to familiarise ourselves with their technical limitations to avoid misinterpretation of results; a problem too often observed in electrophysiological research. For this purpose we will first address the "ins and outs" of the electrophysiological techniques used in SCN research to gain more insight in the advantages and disadvantages of the various electrophysiological approaches (section 1). Secondly, electrophysiological studies of the SCN will be critically reviewed (section 2 and 3) in order to address the contribution of electrophysiology to the research on SCN functioning (question 1, see above) and to position and discuss our findings in the general field of circadian research. In section 3.6, an important methodological problem concerning electrophysiological research on entrainment is put forward, which leads to our proposal that the current evidence is compatible with the hypothesis that nearly all known transmitter-induced phase shifts effects can be explained by effects on intracellular calcium concentration \([\text{Ca}^{2+}]\) of SCN clock neurons. This hypothesis is then corroborated by arguments and requires a re-evaluation of research results on entrainment.

Thirdly, we elaborated the question of which contribution electrophysiological techniques can offer future investigations on SCN functioning (section 4).

6.1 Electrophysiology of the SCN

Basically, the electrophysiological techniques used for investigating the SCN can be divided in extracellular and intracellular recording techniques. Extracellular recording techniques can be applied to SCN neurons in vivo and in vitro. Intracellular recording techniques have thus far only been used in vitro. The in vivo recording technique is an interesting tool for circadian research, because SCN activity can be correlated with behavioural activity and SCN responses to environmental manipulations can be monitored [38]. However, for detailed analysis of these input integration processes at the cellular level, in vitro electrophysiological studies are necessary. In vitro systems can be divided in acutely prepared brain slices and dissociated cells, and cultured brain slices (organotypic cultures) and cultured dissociated cells. Since circadian firing activity of SCN neurons is a cell-autonomous process and maintained
in vitro [24, 34–37, 39], all of these in vitro systems can be used for studying the clock-related activity of SCN neurons. Nevertheless, there are large differences between these systems. In acutely prepared SCN slices and dissociated cells, the SCN neurons are recorded within a few hours after preparation, and it has been shown that the pacemaker cells maintain their rhythm and phase after preparation during the subjective day and are not grossly distorted by the procedure [79]. By culturing slices or cells, the opportunity of neurons to recover from the preparation procedure is increased as well as the opportunity for non-physiological up- and down regulation of regulatory proteins and peptides and cell-specific cell death. Measuring SCN neurons in slices has the advantages of working with a semi-intact network (e.g. to investigate cellular communication) and of controlling the topographic location of the electrodes (to study different subpopulations of the SCN). Dissociated cells are largely stripped from their dendrites and axons and are suitable for studying receptor and ion channel function. One should take into account that receptor and channel function can be degraded by the enzymatic treatment prior to cell dissociation. However, acutely dissociated cells have the advantage of high electrotonic compactness (because neurons only retain somata and proximal dendrites), which prevent space-clamp errors: an important problem in whole-cell voltage-clamp studies. Dissociated cell cultures have the additional advantage that they can form a network, although this network is often not comparable with an intact SCN.

6.1.1 Extracellular recordings

Extracellular recording techniques are suitable for long-term recordings (> 24 hours), which is important for studying the circadian aspect of neuronal activity of SCN neurons. By studying single- and multi-unit activity of SCN neurons in vivo, effects of environmental or internal inputs on SCN behaviour can be investigated on different time scales; direct effects as well as effects on a circadian time scale can be observed. Investigation of SCN neurons in vitro has the advantage of nearly full control of the composition of the extracellular fluid at all times, which allows the application of receptor agonists and antagonists with a selected temporal relationship to cellular rhythms; a strategy often used in circadian research. However, in vitro SCN systems are also limited in the sense that slices are of course devoid of external inputs unless a researcher mimics them by applying electrical stimuli or transmitter agonists. The use of agonists can easily lead to a functional misinterpretation, because an agonist may not perfectly mimic an original input and this imperfection might lead to side-effects (e.g. massive shunting, Chapter 5) that affect the results.

6.1.2 Intracellular recordings

Intracellular recordings techniques are suitable for detailed studies on membrane properties of SCN neurons, but it is technically still unfeasible to routinely perform long-term recordings (> 4 hours) with these techniques. Thus, investigation of circadian aspects of SCN neurons by intracellular recordings is still limited to a strategy in which neurons in different phases are recorded separately and then compared. Pioneering studies on membrane properties of SCN neurons were performed with sharp
electrodes [13, 26, 68, 110]. Sharp microelectrodes have the disadvantage of a high electrode resistance, which results in high signal filtering and thus reduced accuracy in recording membrane events with fast kinetics and/or small amplitudes [141]. The more recently used whole-cell patch-clamp technique avoids these problems by using electrodes with a low resistance [141]. This technique does allow accurate measurement of events with fast kinetics and small amplitudes. The large access of pipette fluid to the neuron in whole-cell patch-clamp recordings also entails intracellular dialysis [142]. Cytoplasmic modulators may be removed due to this dialysis, which could affect the state of ionic channels; a “side-effect” that makes this technique less suitable for studying mechanisms that crucially depend on cytoplasmic components. The perforated-patch clamp technique allows a relatively good access to the neuron (although not as high as with the whole-cell patch-clamp technique) without dilution of larger intracellular factors, by perforating the membrane patch with pore-forming proteins [111, 112, 143]. Due to these characteristics the perforated-patch clamp technique is preferable above the whole-cell patch-clamp and sharp electrode technique for cellular electrophysiology of SCN neurons. Its main drawback is that it does not permit accurate voltage-clamp studies on fast currents (e.g. Na⁺).

6.2 Circadian modulation of ion channels

It has been repeatedly demonstrated that the SCN contains circadian pacemaker cells [24, 34–37, 39]; these are neurons with an intrinsic mechanism for circadian rhythmogenesis. Although the fundamental molecular elements of the biological clock are being defined at a breathtaking pace, little is known about the mechanisms mediating the expression of circadian rhythm in spontaneous firing rate, the main "output parameter" of the SCN neurons. This circadian rhythm in neuronal excitability is likely to be mediated by ionic conductances (ion channels) that are under control of the circadian molecular machinery (Fig. 6.1.).

6.2.1 Ionic currents in the SCN; possible candidates for mediating the circadian rhythm in firing rate

In chapter 4, we showed (using a perforated-patch clamp technique) that SCN neurons express a circadian rhythm in spontaneous firing activity, membrane potential and input resistance. We concluded that at least one hyperpolarizing conductance (K⁺ or Cl⁻) would be open at night and closed during the day (see also [26]). Potassium currents are interesting candidates for mediating these circadian changes because in other brain areas they are known to contribute to firing patterns and there are many messenger systems modulating potassium channels [91–93].

Michel and colleagues (1993, [43]) showed, by using a perforated-patch clamp technique, that the circadian pacemaker cells of the mollusc Bulla gouldiana exhibited a circadian modulation of a K⁺ conductance. Although a delayed rectifier was characterized that contributes to this oscillation in K⁺ conductance, this current could not hold responsible for the circadian rhythm in excitability because it is not activated at the resting membrane potential of these neurons [44].
Fig. 6.1: Possible signal transduction pathways that regulate the circadian rhythm in excitability of SCN neurons. Circadian rhythm in excitability might be accomplished by circadian expression of ion channels, kinases or their regulatory elements, PAS-domain containing proteins. CCG's: clock controlled genes.

In chick pineal cells, which also exhibit an autonomous circadian rhythm, a cationic conductance (I_{LOT}, ‘long open time’) was identified by using cell-attached and inside-out patch-clamp techniques [144]. The channel is active at night and not during the day. Under constant darkness the circadian rhythm is maintained, indicating that the channel responsible for I_{LOT} is under circadian control. The gating of I_{LOT} does not depend on continued exposure to cytosolic messengers. Therefore, the molecular mechanism that controls the gating of this channel may be phosphorylation/dephosphorylation of the channel or a substance that remains associated with the plasma membrane only during the night time. However, the ion channel we are looking for in the SCN may well depend on continued exposure to cytosolic messengers, because whole-cell recordings with non-enriched pipette medium revealed that cell-dialysis abolished the circadian rhythm of neuronal activity [129].

In the SCN, several K\(^+\) currents were identified and characterized to elucidate whether or not they were subject to circadian control. Evidence has been raised for the presence of a delayed rectifier [99, 145], a calcium-dependent K\(^+\) current [145], a transient outward K\(^+\) current (A-current; [99, 145], an H-current (mixed Na\(^+\)/K\(^+\) current) [68, 100] and an EAG2-like K\(^+\) current (chapter 3, [146]) in SCN neurons. Regarding the delayed rectifier, no correlation was found between its conductance and the circadian time of recording in a whole-cell patch-clamp study [99]. Although the conductance of the delayed rectifier was determined soon after impalement to avoid washout effects, a dialysis effect that abolishes the circadian rhythm of this
conductance cannot explicitly be excluded because of the fast dilution kinetics of the cytoplasmic circadian modulator(s) after membrane rupture [129] (cf. [147]). The kinetics and voltage dependence of the delayed rectifier suggest that this current does not affect the resting membrane potential and consequently the spontaneous firing rate, and it is more likely that this current is involved in the generation of the action potential waveform. Considering the voltage-dependence of the calcium-dependent K$^+$ current as measured in whole-cell patch-clamp mode, this current is unlikely to contribute to the resting membrane potential either [145]. However, both the delayed rectifier and the calcium-dependent K$^+$ current cannot be rejected as candidates solely based on their kinetics and voltage-dependence determined by whole-cell recordings, because the ion channel could assume a non-physiological unmodulated “null-state” due to the dialysis of cytosolic modulators. The kinetics and voltage dependence of the A-current, also determined under whole-cell patch-clamp conditions, suggest that this current may influence the spontaneous firing rate of SCN neurons, but circadian modulation of this current was never tested [99]. In the whole-cell patch-clamp recording study of chapter 2, we investigated the contribution of the H-current to the spontaneous firing activity of SCN neurons. The H-current did not affect the spontaneous firing and membrane potential of SCN neurons. Voltage-clamp studies revealed that the H-current, at the resting membrane potential, is probably too small in magnitude and too slow in activation to make a significant contribution to the spontaneous firing rate. The activation parameters of the H-current did not reveal a day-night difference. Our activation parameter values were similar to those reported by Akasu et al. (1993, 1994) [68, 87] obtained by using sharp electrodes. This similarity argues against the dialysis of an effective H-current modulator in our experimental setup, and consequently suggests the absence of circadian modulation of this current. The kinetics and voltage-dependence of the H-current suggest that in the SCN this current is more likely to be involved in termination of and recovery from large and long-lasting hyperpolarizations. In chapter 3, we identified and characterized a novel current in the SCN by using the whole-cell patch-clamp technique (with an enriched pipette solution). This EAG2-like K$^+$ current makes a large contribution to the resting membrane potential and the spontaneous firing rate of SCN neurons. This current has the properties of a fast activating outward rectifier and is active at levels below firing threshold, making this current an interesting candidate for transducing the molecular circadian rhythm into membrane excitability. An interesting property of EAG2 channels is that they have a PAS domain [97, 148], which allows PAS-mediated protein-protein dimerization. PAS-mediated protein-protein interactions play an important role in the feedback mechanism of the genetic clock (see Introduction Fig. 1.1.). PAS-mediated protein binding of EAG2(-like) channels is an attractive candidate mechanism for translating the molecular time-code into cyclic changes in membrane excitability (Fig. 6.1.). A tendency in day/night difference in the hyperpolarizing contribution to the resting membrane potential of the EAG2-like K$^+$ current was observed, but our results did not disclose a statistically significant difference. This should not be taken to imply that the current does not play any role in circadian rhythmicity because it is possible that a circadian modulation of this current was removed by washing out important cytoplasmic constituents due to
intracellular dialysis. Regardless of its role in circadian rhythmicity, the large contribution of this EAG2-like K\(^+\) current to the resting membrane potential of SCN neurons suggests an important role for this novel current in the genesis of the resting membrane potential.

Although chapter 4 indicates that at least one hyperpolarizing conductance would contribute to the circadian rhythm in firing activity, this does not exclude a contribution by other types of ion channels to the circadian rhythm in firing activity. A slowly inactivating component of Na\(^+\) current [106], the low-threshold Ca\(^{2+}\) current [13,68] and the high-threshold Ca\(^{2+}\) current [105] have been suggested to regulate spontaneous firing of SCN neurons. The slowly inactivating component of Na\(^+\) current studied with the whole-cell patch-clamp technique [106] can be activated well below firing threshold. The voltage dependence and the kinetics of the slowly inactivating component suggest that it could contribute to the depolarizing ramp that precedes an action potential. Thus, this current has been inferred to be important for the initiation of action potentials and thus for the spontaneous firing rate of SCN neurons. Day-night modulation of this component was investigated in dissociated cells, but no correlation was found between its conductance and the circadian time of recording (Pennartz, unpublished observations). Intriguing are also the low- and high-threshold Ca\(^{2+}\) currents in SCN neurons. Low-threshold Ca\(^{2+}\) current underlies the generation of the rebound depolarization [68], but this current is unlikely to regulate spontaneous firing behaviour of SCN neurons under physiological conditions, because it is inactive at the normal resting membrane potential (perforated-patch and sharp electrode recordings: [13,105]). Colwell (2000) [149] showed with a Calcium imaging technique that SCN neurons exhibit a circadian rhythm in intracellular Ca\(^{2+}\) levels. This rhythm was abolished by tetrodotoxin (TTX; a blocker of the fast Na\(^+\) current) and by methoxyverapamil (a blocker of high-threshold Ca\(^{2+}\) current), suggesting that firing activity of SCN neurons generates the circadian rhythm in intracellular Ca\(^{2+}\) levels via Ca\(^{2+}\) influx through high-threshold Ca\(^{2+}\) channels. Perforated-patch recordings under TTX conditions revealed slow Ca\(^{2+}\)-channel mediated membrane potential oscillations (2-6 Hz) in SCN neurons during their subjective day phase, and these slow oscillations were not found and could not be evoked in SCN neurons during their subjective night phase. These slow Ca\(^{2+}\)-mediated oscillations were also generated by high-threshold Ca\(^{2+}\) currents [150]. At first glance this observation might seem to be contradictory to the results of Colwell. However, the Ca\(^{2+}\)-mediated oscillations observed by Pennartz et al. [150] do not necessarily contribute to the whole-cell intracellular Ca\(^{2+}\) concentration, because oscillation-induced changes in [Ca\(^{2+}\)]\(_i\) can be spatially limited to zones just underneath the plasma membrane. The circadian modulation of these channels is currently under investigation.

Overall, no ion channel species has been identified yet that contributes to the circadian rhythm in spontaneous firing rate of SCN neurons. However, our advanced knowledge on SCN electrophysiology has led to a better understanding of firing activity behaviour of SCN neurons and therefore to SCN functioning in general.
6.2.2 Research strategies and their problems

As stated above, it has been shown that the circadian rhythm in neuronal activity is abolished during whole-cell patch-clamp recordings using non-enriched pipette medium [129], whereas the circadian rhythm in neuronal activity is maintained during perforated-patch clamp recordings (chapter 4; [94]). It is not yet know whether the loss of rhythmicity in whole-cell mode is due to the dialysis of specific clock components or to dialysis of metabolic components leading to a more generalized change in membrane physiology. Preventing the latter, by using for example an enriched pipette solution, may indicate whether or not the ion channels responsible for the circadian rhythm in neuronal activity require continued contact with cytosolic clock components. The outcome will determine the usefulness of the whole-cell patch-clamp technique in the search for circadian modulation of ion channels.

Thus far, researchers investigating the circadian modulation of ionic currents used a sample strategy; neurons were sampled either in their subjective day or night phase and statistically compared. The foremost problem of this research strategy is that an unknown amount of non-pacemaker neurons are enclosed in the sample cohort, resulting in a "contaminated" data set. Because the percentage of pacemaker cells in the SCN is not exactly known, it is impossible to determine the degree of contamination. In our own study it has been feasible to identify and exclude all cluster II cells (non-pacemaker neurons; [27, 129]) from the data set, but this does not remove all non-pacemaker cells. In order to obtain an uncontaminated data set, it would be preferable to determine whether a recorded cell is a circadian pacemaker neuron prior to or after the experiment. Selecting circadian pacemaker neurons from the SCN can already be established on the basis of long-term recording of electrical activity, for example by culturing SCN neurons on multi-electrode plates (MEP's) [37, 64, 96]. Preselecting the neurons in this way also allows the researcher to choose a cell-specific circadian phase for recording. This new approach would certainly increase the chance of successfully determining circadian modulation of ion currents of SCN pacemaker neurons. Post-experimental selection of pacemaker neurons by immunocytochemical markers of clock cells or mRNA amplification of clock products may also result in a more pure dataset, but this method still needs to be developed.

6.3 Signal integration in the Suprachiasmatic nucleus

Basically, 3 main neuronal pathways are known to enter the SCN and supply information to it about the environment and the internal state of brain and body: projections from the retina, the intergeniculate leaflet (IGL) and the Raphe nuclei. The circadian clock can be affected by signals entering the SCN via these projections. The circadian clock is also regulated by the many different neuroactive substances that are released by SCN neurons themselves [28, 61, 117, 151]. Moreover, the circadian clock can be manipulated by substances circulating through the cardiovascular system (hormones). In order to elucidate the integration of external and internal information signals involved in the entrainment function of SCN, it is necessary to determine the capacity of "all" well-characterized modulators of the biological clock (see Table
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6.1.) Therefore, we review phase-shift potency, electrophysiological characteristics, signal transduction pathways presumed to be involved in phase-shifting and position in SCN circuitry of all well known modulators of the biological clock (section 3.1-3.5). In section 3.6, we will discuss the interpretations of results concerning the putative entrainment mechanism of these modulators.

6.3.1 Retinal inputs to the SCN

Glutamatergic neurotransmission plays a predominant role in the signal transfer from retina to SCN. Glutamate is released from stimulated optic nerves [11] and glutamate administration to *in vitro* SCN slices induces phase shifts similar to those elicited by light *in vivo* [54]. Phase-shifts induced by light, optic nerve stimulation or glutamate application, can be blocked by antagonists of NMDA as well as AMPA/Kainate receptors [12, 54, 152]. Intracellular recordings with sharp electrodes revealed that optic nerve stimulation evokes excitatory postsynaptic potentials (EPSPs) in SCN neurons. These evoked EPSPs were depressed in their late phase by an NMDA antagonist (D-AP5) at membrane potentials between -20 and -55 mV, whereas evoked EPSPs at membrane potentials between -60 and -100 mV were blocked by an AMPA/Kainate antagonist (DNQX: [153]). Moreover, the NMDA component is modulated in a circadian manner (recorded under whole-cell conditions, [47]). This component was found more frequently and was significantly larger in magnitude during the night than day period, whereas the AMPA/kainate component did not significantly change. Apparently the NMDA component does not require continued contact with cytosolic clock components or is less susceptible to a possible generalized change in cell physiology. The night-restricted domain of light-induced phase shifting and the "dead zone" during the subjective day may at least in part be explained by this circadian modulation of NMDA function. By way of this modulation the transfer of photic input to the phase shifting mechanism may be restricted to the subjective night period of the circadian cycle [47]. Activation of the NMDA receptor channel allows the entry of Ca\(^{2+}\) [154]. Increment of the intracellular Ca\(^{2+}\) concentration is thought to be the initial step of a signal transduction cascade by which light can cause a phase-shift of the circadian clock [55, 155].

Ca\(^{2+}\) release from intracellular stores via activation of ryanodine receptors also contributes to the resetting of the circadian clock by light [155]. There are indications that the increment of intracellular Ca\(^{2+}\) concentration might directly affect the molecular clock mechanism [156]. However, there is also evidence for a more expanded signal transduction cascade in which sequentially calcium influx, NO production, activation of mitogen-activated protein kinase (MAPK) and phosphorylation of CREB are necessary to obtain a light-induced phase shift [54-56, 135].

Glutamate release can also activate metabotropic glutamate receptors in the SCN [157-159]. Whether the activation of metabotropic glutamate receptors induces phase-shifts still needs to be determined. Although the activation of metabotropic glutamate receptors inhibits ionotrophic glutamate receptor-mediated neurotransmission in the SCN [160], its role in photic transduction in the SCN still needs to be specified. Next to glutamate the neuroactive peptides substance P and PACAP are also present in the retinohypothalamic tract [161-163]. Both peptides induce phase-shifts similar
to those induced by light [164,165]. Furthermore, they are both considered neuromodulators that can potentiate retinohypothalamic glutamatergic transmission [165–167]. The precise function of these neuroactive substances in retinohypothalamic transmission still needs to be elucidated. The mere amount of regulatory substances indicates the modulatory potency and the complexity of this system.

### 6.3.2 Geniculohypothalamic inputs to the SCN

The projection from the IGL, thought to be important for non-photic as well as photic entrainment [208,209], contains the neurotransmitters NPY, enkephalin (species specific: hamster) and GABA [14,210,211]. Extracellular recordings in SCN slices revealed that NPY produces two distinct actions on neurons of the SCN; phase shifting and direct neuronal inhibition. The NPY induced phase-shifting seems to be mediated by Y2 receptors, whereas the direct NPY neuronal inhibition seems to be mediated by Y5 receptors [49,181,212]. The NPY induced phase-response relationship (PRC) is similar to a non-photic PRC [180], but see [213]), suggesting the involvement of the NPY Y2 receptor in non-photic entrainment. The functional significance of NPY mediated inhibition is still unclear, but might perhaps reduce the cellular responsiveness to other stimuli [49]. Enkephalines act on opioid receptors (μ, δ and κ receptors). Systemic injection of a δ-opioid agonist [183,184] and a μ-opioid agonist [182] in hamsters induced phase-advances when applied during the day period. However, with extracellular recordings of single SCN neurons (also in hamsters) Cutler and colleagues (1999) [185] showed that opioid receptor agonists did not affect SCN neuronal firing activity in vitro. The GABAergic input from the IGL also affects the SCN, see section 3.4.

### 6.3.3 Raphe inputs to the SCN

The Serotonergic projection from the median raphe innervates the SCN [214]. This projection is also thought to be important for photic and non-photic entrainment [16]. Activation of serotonin receptors in the subjective day phase advances the circadian clock, whereas activation of the serotonin receptors in the subjective night phase delays the circadian clock [51,189,191,215,216], similar to non-photic stimuli. Serotonin exerts multiple cellular actions in the SCN [187]. Local administration of 5-HT₁₅/₇ agonists into the SCN region inhibits behavioural light-induced phase-shifts [217] and a 5-HT₁₅ antagonist, applied systemically, is able to potentiate light-induced phase shifts [218], indicating that serotonin modulates the response of the SCN circadian clock to photic stimuli. Retinohypothalamic responses evoked by electrical stimulation were inhibited by serotonin, whereas glutamate-induced current responses were not affected by serotonin [187], indicating a presynaptic site of action. Activation of presynaptically located serotonin receptors in SCN slices inhibits retinohypothalamic as well as GABAergic transmission as assessed in whole-cell recordings [187,219]. The presynaptic serotonergic inhibition of retinohypothalamic inputs is most likely caused by activation of 5-HT₁₃ receptors [186,219], whereas the receptor responsible for the presynaptic serotonergic inhibition of GABAergic transmission is not yet known. Thus, serotonin can modulate the response of the circadian clock to photic stim-
uli by presynaptic inhibition of glutamate release from retinohypothalamic terminals via 5-HT$_{1B}$ receptors or by modulating GABAergic transmission presynaptically (i.e. blocking the propagation of photic information through the SCN; [220]). Serotonin also induced in- and outward currents and these current responses were unaffected by TTX application, indicating a postsynaptic site of action as well. Postsynaptically, serotonin activates a K$^+$ conductance [190], probably via the 5-HT$_7$ receptor [187]. Prosser and colleagues (1994) [190] revealed with extracellular recordings in SCN slices that serotonin-mediated phase-advances can be blocked by K$^+$ channel blockers (Ba$^{2+}$ and apamin), suggesting an involvement of the postsynaptic 5-HT$_7$ receptor in this process. The serotonergic system might provide the SCN with a modulation mechanism in which photic and non-photic information can be integrated to generate a weighted compound input (i.e. serotonin may balance photic against non-photic inputs before they are relayed to pacemaker neurons).

6.3.4 Intrinsic transmitters of the SCN

Neurons of the SCN are loaded with many different neuroactive substances [28] that are locally released and able to affect the circadian clock. GABA$_A$-receptor mediated neurotransmission has been shown to be prominent in the SCN [26,119] and is at least partly of intranuclear origin [62], indicating that SCN neurons are interconnected by GABAergic synapses (see also [61]). Multiple functions are assigned to GABA$_A$-mediated transmission in the SCN. Local in vivo administration of the GABA$_A$ agonist muscimol in the subjective day period phase-advanced the circadian clock [172,173] and this effect was blocked by local administration of the GABA$_A$ antagonist bicuculline [221]. Locally applied bicuculline in vivo also potentiates light-induced phase delays [222], but has no effect on light-induced phase advances [223]. Furthermore, extracellular recordings in SCN slices revealed that the GABA$_A$ receptor agonist muscimol phase-advanced the circadian rhythm in neural activity when applied in the subjective day period [173]. These results indicate a role for GABA$_A$ receptors in entrainment mechanisms of the SCN.

In 1997, Wagner and colleagues [123] presented data suggesting that GABA acts as an inhibitory neurotransmitter during the subjective night and switches to an excitatory neurotransmitter during the subjective day. This novel mechanism would amplify the rhythm in spontaneous firing rate of SCN neurons, which could be of importance for enhancing the outputs from the SCN [123,224]. However, three independent laboratories were unable to confirm the excitatory nature of GABA in the SCN during the subjective day by using several different electrophysiological techniques [127], as a result of which a controversy arose about GABA function in the SCN. In chapter 5, we showed that an overwhelming majority of the SCN neurons receive inhibitory GABA$_A$-mediated inputs during the day and that a subgroup of SCN neurons (approximately 50%) receive excitatory GABA$_A$-mediated inputs during the night. Both studies ( [123,174]: chapter 5) reveal a circadian time domain in which GABA is excitatory, although the time domains are roughly opposite. We also investigated several mechanisms that could underlie this discrepancy, but to solve this issue completely additional research is necessary. Our results, obtained by using the perforated-patch clamp technique, support the idea that the GABAergic network
of the SCN behaves as an active filter that passes excitatory inputs during the night but cuts them off during the day. The inhibitory nature of GABAergic transmission during the day may counteract the integration of timing cues by arresting the propagation of these signals throughout the SCN. In contrast, during the night the excitatory nature of the GABAergic transmission in a subset of cells may promote signal dissemination throughout a restricted domain of the SCN network and beyond. Another function assigned to GABAergic neurotransmission in the SCN is that of a coupling agent that is capable of synchronizing clock neurons [225]. In dispersed SCN cell cultures with different cell densities an increased synchrony of circadian rhythms in firing rate was found in high as compared to low density cultures [59], indicating the existence of a synchronizing mechanism in the SCN depending on cell density and associated parameters such as ephaptic, synaptic or gap junction contacts. Recently, Liu and Reppert (2000) [64] showed that the circadian rhythms in firing activity of individual clock cells, monitored by extracellular recordings (culturing clock cells on multielectrode plates), can be synchronized by daily applications of high levels of exogenous GABA (100 μM). This result supports the hypothesis that GABA acts as a synchronizer in the SCN. However, in these experiments neuronal firing of the clock cells was always completely inhibited during the application of GABA. It cannot be excluded that the long-lasting application of GABA (time = 3h; 100 μM) induced side-effects like prolonged shunting, desensitization of the GABA_A receptor or shifting of the GABA_A reversal potential. For instance, the inhibition of neuronal firing by GABA may have been caused by massive and prolonged shunting (chapter 5) and the synchronization of the clock cells might, subsequently, be the result of such a non-physiological side-effect (see point 3.6). Thus, the synchronization hypothesis of GABA still needs to be proven.

The neurons of the SCN also express GABA_B receptors. Extracellular recordings in vitro revealed that the GABA_B agonist baclofen induces phase-shifts of the circadian rhythm in firing activity; phase-advances were induced during the subjective day period and phase delays during the subjective night period [175]. These GABA_B receptor mediated phase-shifts can be blocked by TTX indicating that these receptors are not located on SCN pacemaker neurons [63]. In patch-clamp studies, presynaptic GABA_B-mediated inhibition of glutamatergic transmission and GABA_B modulation of a postsynaptic K^+ current were found in the SCN [176]. GABA_B receptor stimulation may induce phase shifts by modulation of neurotransmitter release, or through modulating the activity of non-pacemaker cells that synapse onto pacemaker cells [63]. However, it should be noted that slow GABA_B-mediated postsynaptic potentials have not yet been observed in the SCN [13,119,176]. The primary action of GABA_B receptors, as far as revealed by patch-clamp recordings, appears to regulate the relay of excitatory signals to SCN pacemaker neurons.

Besides GABA, several SCN neuropeptides are locally active in the SCN. The Vasopressin V1 receptor is present on both VP and VIP containing SCN neurons [205]. In the SCN, the extracellular VP level cycles in a circadian manner with high levels of VP during the subjective day phase [226-229]. Local application of vasopressin into the suprachiasmatic region did not induce phase-shifts of the circadian clock [206]. Application of VP to SCN cells in vitro resulted in an increase of firing rate via the
activation of V1 receptors in approximately 50% of the neurons (extracellular recordings: [203,230]), whereas the application of a VP V1 antagonist on SCN neurons in vitro decreases the firing activity in half of the SCN neurons (i.e. indicating the presence of an endogenous excitatory tone, [204]). These results indicate that VP may boost the amplitude of the rhythm, generating a more pronounced output.

The neuropeptides VIP, PHI, GRP and SOM are all able to induce phase-shifts more or less similar to those elicited by light [177,196,200] and they all modestly increase the spontaneous firing rate (or 2DG uptake) of SCN neurons [196,198,201], indicating that all of these neuropeptides play a role in photic entrainment.

Immunoelectron microscopy revealed that VIP-containing neurons, which are thought to receive retinal input [231], form synapses on VP-containing neurons [232]. VIP administration to an in vitro SCN slice culture increases VP release [233], suggesting that VIP may be used to relay retinal information to other SCN neurons (e.g. VP-containing neurons). Activation of VIP receptors increases cAMP levels in SCN [234,235], which may subsequently result in the modulation of ion-channels (e.g. [236]). Since PHI and VIP are products of the same precursor and are therefore colocalized throughout the entire SCN at a cellular level [237], and PHI and VIP both bind to the same receptor [238], is it likely that PHI will have similar biological actions as VIP. However, it should be noted that VIP receptors have a higher affinity for VIP than for PHI [238].

Intracellular recordings of SCN neurons in slices showed that GRP depolarizes the membrane of SCN neurons and consequently increases the spontaneous firing rate [199]. Nonetheless, GRP administration to an in vitro SCN slice culture reduces the release of both VP [233] and serotonin [239] from SCN neurons. These GRP actions are probably indirect and mediated by interneurons, although this still needs to be determined. GRP induced phase-shifts of the circadian rhythm in firing activity, determined by extracellular recordings in vitro, were completely blocked by a selective bombesin BB2 receptor antagonist [200]. BB2 receptors are linked to G-proteins and are able to activate protein kinase A, protein kinase C, phospholipase C and MAPK [200,202]. These kinases and lipase are signal transduction molecules that are often referred to in relation to SCN entrainment mechanisms [55,56,190,240].

Immunoelectron microscopy revealed that Somatostatin (SOM) containing neurons of the SCN project to the VIP immunoreactive neurons of the SCN ( [241,242], see also [151]). The SOM inputs to VIP-containing neurons might be able to affect the transmission of photic information to the pacemaker neurons.

Overall, the locally active neuropeptides of the SCN engage in interactions that are likely to control information flow throughout the SCN, however, the precise function of each neuropeptide in the SCN still needs to be elucidated. Other interesting substances that have been reported in the rat SCN but are beyond the scope of this review are: angiotensin II [243], calcitonin gene-related peptide [244], corticotrophin releasing factor [245], dynorphin [246], galanin [247], thyrotropin-releasing homone [248] and VGF (vesicular protein regulated by growth factors) [249].
Tab. 6.1: Neuroactive substances acting on SCN neurons

<table>
<thead>
<tr>
<th>Transmitter</th>
<th>Receptor</th>
<th>Mechanism (activation)</th>
<th>Phase-shifting capacity (PRC)</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>NMDA</td>
<td>Na⁺/K⁺/Ca²⁺-ion channel</td>
<td>Photic-like-PRC</td>
<td>$</td>
<td>V_M$</td>
</tr>
<tr>
<td></td>
<td>AMPA/Kainate</td>
<td>Na⁺/K⁺-ion channel</td>
<td>Photic-like-PRC?</td>
<td>$</td>
<td>V_M$</td>
</tr>
<tr>
<td></td>
<td>MGlu type I, II, III</td>
<td>G-protein PKC/AC</td>
<td>?</td>
<td>$</td>
<td>sf_r$</td>
</tr>
<tr>
<td>GABA</td>
<td>GABAₐ</td>
<td>Cl⁻-ion channel</td>
<td>Non-photic-like PRC</td>
<td>$</td>
<td>sf_r, V_M$</td>
</tr>
<tr>
<td></td>
<td>GABAₐ</td>
<td>G-protein K⁺-ion channel</td>
<td>Non-photic-like PRC</td>
<td>$</td>
<td>sf_r V_M$</td>
</tr>
<tr>
<td>Substance P</td>
<td>NK</td>
<td>G-protein PLC</td>
<td>Photic-like-PRC</td>
<td>$</td>
<td>sf_r$</td>
</tr>
<tr>
<td>PACAP</td>
<td>PACAP-type I, II</td>
<td>G-protein PLC/AC</td>
<td>Photic-like-PRC</td>
<td>$</td>
<td>sf_r$</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Y1, Y2, Y5</td>
<td>G-protein</td>
<td>Non-photic-like PRC</td>
<td>$</td>
<td>sf_r$</td>
</tr>
<tr>
<td>Enkephalin</td>
<td>µ, δ-, κ-opioid</td>
<td>G protein</td>
<td>Non-photic-like PRC</td>
<td>$=sf_r$</td>
<td>[182–185]</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT1C, 2</td>
<td>G-protein</td>
<td>?</td>
<td>?</td>
<td>[186]</td>
</tr>
<tr>
<td></td>
<td>5-HT1A/7</td>
<td>G-protein AC/PKA/K⁺ channel</td>
<td>Non-photic-like PRC</td>
<td>$</td>
<td>sf_r V_M$</td>
</tr>
<tr>
<td>Melatonin</td>
<td>Mel1a</td>
<td>G-protein</td>
<td>?</td>
<td>$</td>
<td>sf_r V_M$</td>
</tr>
<tr>
<td></td>
<td>Mel1b</td>
<td>G-protein</td>
<td>Phase-advance</td>
<td>$=sf_r$</td>
<td>[195–197]</td>
</tr>
<tr>
<td>GRP</td>
<td>BB2</td>
<td>G-protein PKA, PKC, PLC, MAPK</td>
<td>Photic-like-PRC</td>
<td>$</td>
<td>sf_r V_M$</td>
</tr>
<tr>
<td>VP</td>
<td>V1</td>
<td>G-protein</td>
<td>No</td>
<td>$</td>
<td>sf_r$</td>
</tr>
<tr>
<td>VIP</td>
<td>PACAP-type II</td>
<td>G protein AC</td>
<td>Photic-like-PRC</td>
<td>$</td>
<td>sf_r$</td>
</tr>
<tr>
<td>PHI</td>
<td>PACAP-type II</td>
<td>G-protein AC</td>
<td>Photic-like-PRC</td>
<td>$</td>
<td>sf_r$</td>
</tr>
</tbody>
</table>

Abbrev.: PKC: Protein kinase C, AC: Adenylyl cyclase, PLC: Phospholipase C, PKA: Protein kinase A, MAPK: Mitogen-activated protein kinase, PRC: Phase response curve, sf_r: spontaneous firing rate, $V_M$: membrane potential, $|$: increase or depolarization, $\|$: decrease or hyperpolarization, ?: unknown
6.3.5 Hormones

Besides classical neurotransmitters and peptides, several hormones affect the circadian physiology of the SCN. In the SCN, hormone receptors are present for melatonin [250,251], estrogen [252], prolactin [253] and leptin [254]. Melatonin has been shown to affect circadian rhythms, whereas the effect of other hormones on the circadian clock still needs to be demonstrated. In a specific circadian time domain (from CT 6 to 18 h), melatonin can induce phase advances of the circadian rhythm in firing activity by directly acting on SCN pacemaker neurons, as was shown by extracellular recordings in SCN slices [194,255]. Whole-cell patch-clamp studies revealed that melatonin hyperpolarizes the membrane of SCN neurons, resulting in an inhibition of firing activity [69,256]. This melatonin-induced hyperpolarization is caused by activation of a K\(^+\) conductance [69,256] and is thought to be mediated by the Mel\(_{1A}\) receptor [193]. However, the phase-shifting properties of melatonin are not ascribed to its action on the Mel\(_{1A}\) receptor but on the Mel\(_{1B}\) receptor [193]. However, a contribution of the Mel\(_{1A}\) receptor to phase-shifting can as yet not be excluded, because melatonin-induced phase-shifts (10 pM) in Mel\(_{1A}\) receptor knock-out mice were significantly smaller than those in wild-type mice [193]. The elevated levels of melatonin at night suggest that the Mel\(_{1A}\) receptor will contribute to the reduction of membrane excitability of SCN neurons at night.

6.3.6 Difficulties in interpreting the role of neuroactive substances in the circadian clock

Thus far, scientists investigating signal integration mechanisms in the SCN used mainly (long-term) extracellular recording techniques \textit{in vitro} to determine phase-shift properties of neuroactive substances. This was accomplished by exposing SCN neurons to neuroactive substances for a limited amount of time at different circadian timepoints and examining the effect of this exposure on the timing of the firing activity peak during the next subjective day. In SCN neurons, glutamate-mediated Ca\(^{2+}\) influx (via NMDA receptors) has been argued to be the initial step of a signal transduction cascade by which light can phase-shift the circadian clock [54,55]. An increment of intracellular Ca\(^{2+}\) concentration due to Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores may also play a role in resetting of the circadian clock [155]. Furthermore, Colwell (2000) [149] showed that Ca\(^{2+}\) can enter SCN neurons via high-threshold Ca\(^{2+}\) channels and that the electrical activity of SCN neurons contributes to the intracellular level of Ca\(^{2+}\) via these channels. There is evidence that an increase in intracellular Ca\(^{2+}\) concentration can affect the molecular clock mechanism directly by regulation of the basic helix-loop-helix transcription factors [156], like CLOCK and BMAL. From the combination of all of these results it can be concluded that manipulations that alter the intracellular Ca\(^{2+}\) concentration can induce phase-shifts. The intracellular Ca\(^{2+}\) concentration of SCN neurons can be easily changed by membrane manipulations that activate or prevent the activation of high-threshold Ca\(^{2+}\) channels, depolarize the cell to allow NMDA receptor activation or by manipulating Ca\(^{2+}\) release from intracellular stores. Therefore, phase-shifts ascribed to non-Ca\(^{2+}\)-mediated mechanisms (like G-protein-, adenylyl cyclase-, guanylate cyclase-,protein kinase A-,
protein kinase C-, phospholipase A- and phospholipase C-mediated mechanisms [56]) could very well be mediated by a Ca\(^{2+}\) mechanism. An additional problem of the above mentioned research strategy is that "side-effects" (e.g. shunting) of most of the neuroactive substances tested to date may induce phase-shifts by affecting the intracellular Ca\(^{2+}\) concentration. Earnest and Sladek (1987) [257], Schwartz (1991) [258] and Prosser (1994) [190] revealed that high K\(^{+}\) pulses and K\(^{+}\) ionophores can induce phase-shifts, suggesting that membrane de- and hyperpolarizations can cause phase-shifts. This methodological problem concerning entrainment research leads to our proposal that current evidence is compatible with the hypothesis that transmitter-induced phase-shifts can be generally explained by effects on [Ca\(^{2+}\)] of SCN clock neurons.

The rationale for this proposal can be clarified as follows. NMDA, AMPA/kianate, Substance P, PACAP, GRP, VIP and PHI all increase the membrane excitability of SCN neurons and also induce photic-like PRCs, whereas GABA (A and B), neuropeptide Y (Y2), Serotonin (5HT1A/7) all reduce the firing rate and all induce non-photic like phase shifts. Membrane depolarizations (evoked by K\(^{+}\) pulses), NMDA, and Kainate induce a rise in [Ca\(^{2+}\)] by opening high-threshold Ca\(^{2+}\) channels [160]. PACAP induces an increment of [Ca\(^{2+}\)] most likely by releasing Ca\(^{2+}\) from intracellular stores [240]. Both substance P and PACAP induced phase-shifts can partially be blocked by the NMDA receptor antagonist MK-801, indicating an additional pathway that contributes to the rise of [Ca\(^{2+}\)] [165,259]. GABA, neuropeptide Y and Serotonin can all depress different types of intracellular Ca\(^{2+}\) transients [171,260–262]. These data suggest that a reduction of [Ca\(^{2+}\)] may result in non-photic like phase-shifts (this reduction would then only be effective during the subjective day), whereas an increment of [Ca\(^{2+}\)] results in photic like phase-shifts (this increment would only be effective during the subjective night: see Fig. 6.2.). Exceptions to this rule seem to be enkephalin, melatonin (when acting on the Mel\(_1\)B receptor) and Vasopressin. Activation of opioid and Mel\(_1\)B receptors induces phase-shifts, but do not change the membrane excitability. However, enkephalin-induced phase-shifts (systemic injections) might be caused by indirect (via IGL) activation of the SCN [182–184]. Melatonin (Mel\(_1\)B receptor) uses a different signal transduction pathway to induce phase-shifts, since it does not seem to affect [Ca\(^{2+}\)] of SCN neurons [240]. Activation of the Mel\(_1\)B receptor inhibits adenylyl cyclase via a G-protein and the resulting decrease in cAMP might affect the circadian clock via protein kinase A [263].

Vasopressin does not induce phase-shifts, while it does increase the membrane excitability of SCN neurons [203,206] and has been known to activate the phosphoinositol signal transduction pathway [264,265]. This exception to our hypothesis could be explained by postulating that the vasopressin V1 receptor is located on non-pacemaker SCN output neurons. The effects of somatostatin and 5-HT\(_{1}\)B receptor activation on membrane potential and excitability of SCN neurons are unknown and therefore they cannot be addressed in this hypothesis.

In order to test the Ca\(^{2+}\) hypothesis, experiments should be performed that elucidate whether or not direct manipulations of [Ca\(^{2+}\)] can induce phase-shifts and which PRC waveform is obtained by these manipulations. Such [Ca\(^{2+}\)] manipulations can be obtained by using Bapta-AM or by photolytically releasing Ca\(^{2+}\) from a caged
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Fig. 6.2: Ca\(^{2+}\) hypothesis on resetting of the circadian clock. Changes in \([Ca^{2+}]_i\) are only effective within the concentration range of the peak and the trough of the circadian rhythm in \([Ca^{2+}]_i\) (indicated, here, by the dashed lines). Reduction of \([Ca^{2+}]_i\) during the day will result in a non-photic-like phase shift, whereas an increment of \([Ca^{2+}]_i\) during the night will result in a photic-like phase shift.

compound.

In conclusion, transmitter side-effects on membrane physiology and \([Ca^{2+}]_i\) are an underestimated problem in research on phase-shifting mechanisms, and therefore phase-shift results obtained should be interpreted with caution. To solve this problem, long-term application of agonists in high concentrations should be limited and supplemented with other methods. Electrical stimulation of the innervating pathways or SCN cell clusters can be used to mimic the inputs in a more physiological manner and some effects on membrane excitability can be prevented by specific receptor subtype antagonists.

6.4 What can electrophysiology contribute to the research on SCN functioning: future perspectives

In the quest for ion channels that mediate the circadian rhythm in firing activity, further use of the perforated-patch clamp technique holds many promises. Comparison of perforated-patch clamp recordings from SCN pacemaker neurons (selected: see section 2.2) between the subjective day and night phase may lead to the identification of channels which are subject to circadian modulation and affect firing rate. A practically more difficult approach is to perform stable long-term perforated-patch clamp recordings (> 4h) during the circadian rise and decay period of the firing rate. The advantage of this approach would be that the firing rate and membrane properties of one cell could be directly correlated, preventing "contamination" by interneuronal heterogeneity of SCN neurons. Alternatively, the signal transduction pathway between the genetic clock and neuronal excitability of SCN neurons can be
studied by intracellular (timed) over- or underexpression of clock related elements. This approach may also lead to the characterization and identification of the involved ion channels.

Knowledge about the integration of inputs in the SCN needs to be further refined and specified (see above), because the traditional bath application of agonists and antagonists often affects the entire SCN network. Electrical stimulation of innervating pathways or SCN cell clusters combined with perforated-patch clamp recordings and the use of antagonists (for characterization of receptor function only) may further elucidate the integration mechanisms of inputs (e.g. [174]). Intranuclear communication and signal processing can be explored by dual intracellular recordings [266,267] or by exciting local circuitry in a combined approach of perforated patch-clamp and flash photolysis of cage compounds (cf. [62]). Concerning this subject, it is worthwhile to follow the developments on voltage-sensitive dyes, because they may provide a powerful tool to study the information processing of the SCN network as soon as this technology allows single-cell measurements in brain slices. Research of these processes in a more behavioural setting can be performed by \textit{in vivo} multi-electrode recordings (i.e. tetrode recordings).

The results described in chapter 5 indicate that GABAergic transmission within the SCN varies in a circadian manner and we proposed that the GABAergic network prevents the propagation of signals throughout the SCN network during the day, but not during the night. Our results indicate that the circadian change in GABAergic transmission is due to circadian rhythmicity in intracellular chloride concentration. However, these results were obtained by using a day/night sampling strategy. Recently, a genetically encoded indicator for intracellular chloride has been developed, which allows long-term monitoring of the $[\text{Cl}^-]$ [268]. With the use of this novel technique the circadian rhythm in $[\text{Cl}^-]$ can be monitored directly and may provide additional evidence for this circadian rhythm. Furthermore, this technique might elucidate the cell-type that exhibits this circadian rhythm and could be used to investigate the mechanism underlying this circadian rhythm. Although, there is no direct evidence how the circadian rhythm in $[\text{Cl}^-]$ is generated, the involvement of the K$^+$-Cl$^-$ cotransporter has been suggested [140]. Therefore, it would be interesting (in order to test our GABA hypothesis) to investigate the circadian behaviour and entrainment capacity of K$^+$-Cl$^-$ cotransporter knock-out mice (e.g. [269]). However, it should be noted that there could be interspecies difference between the mouse and the rat.

The growing amount and refinement of technological tools announces promising and exciting times for circadian research. However, electrophysiology will remain a necessary tool for circadian research, because electrical signals form the link between the circadian clock and the environment.