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Chapter 4

Circadian modulation of membrane properties in slices of rat suprachiasmatic nucleus

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4. CIRCADIAN MODULATION OF MEMBRANE PROPERTIES IN SLICES OF RAT SUPRACHIASMATIC NUCLEUS

4.1 Introduction

The suprachiasmatic nucleus (SCN) is commonly thought to contain pacemaker cells imposing circadian rhythmicity on patterns of hormone release, muscular activity and other physiological functions [6,7]. The circadian rhythm of SCN neurons is principally expressed by way of a slow modulation of their spontaneous firing rate (SFR), and rhythmic changes in peptide release and/or synthesis are associated with this circadian modulation [24,34,37,109]. Although recent studies in SCN slices have elucidated some of the membrane properties governing the excitability of SCN neurons [13,26,27,100,106,110], little progress has been made thus far in identifying day-night differences in these membrane properties as a basis for explaining the circadian rhythm in SFR. This problem can be considered to be of outstanding importance to this field of research [43], since changes in ionic conductance are likely to constitute a mechanism intermediate between the presumably intracellular, molecular machinery of clock neurons and clock output [13,26,27,37,42,100,106,110], consisting of spike trains propagated from SCN projection neurons towards target areas.

In a series of voltage-clamp recordings in the whole-cell configuration, Jiang et al. [26] described a day-night difference in current required to hold SCN cells at -60 mV. Their data also suggested a slow modulation of input resistance which was, however, out of phase with the changes in holding current. Our own experience with whole-cell recording in SCN has been that, apart from circadian aspects, membrane properties can be monitored quite well for a limited duration after membrane rupture (about 10-45 min.) [27,100,106] but that circadian rhythmicity is gradually lost possibly as a consequence of whole-cell dialysis. Therefore we examined circadian modulation of SCN membrane properties using a perforated patch technique that preserves SCN firing, and leaves levels of second messengers, enzymes and other intracellular regulatory factors intact during recording [111,112].

4.2 Material and methods

4.2.1 Slice preparation and perforated patch recording

Slices were always prepared during daytime, as described in Pennartz et al. (1997) [106]. Briefly, male Wistar rats (180-300 g) were subjected to a 12:12 hours light-dark regime in a room kept at 22-24°C for at least 3 weeks. They were anaesthetized with 60 mg/kg pentobarbital i.p. (Nembutal; Sanofi Sante, the Netherlands), subsequently perfused transcardially with 50 ml ice-cold ACSF and decapitated, in accordance with national guidelines on animal experiments. Coronal hypothalamic slices (200 μm) containing the SCN were cut on a vibroslicer (Campden, London, U.K.). Slices were transferred to the recording chamber after a recovery period of at least 45 min. at
room temperature (21-24°C). This chamber was mounted on an upright microscope supplied with a fixed stage (Axioskop, Carl Zeiss) and a water immersion objective (40X) with Hoffman modulation contrast. In this chamber, slices were fully submerged and superfused at a speed of 1.5-2.5 ml/min. with oxygenated (95% O₂/5% CO₂) ACSF containing (in mM): 124.0 NaCl, 3.5 KCl, 26.2 NaHCO₃, 1.0 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 10.0 D-Glucose (pH 7.3; 31-33°C). The majority of recordings was made from the dorsomedial region of the SCN. Measurements during subjective day were restricted to circadian time 4-8 (CT 4-8: 4 to 8 hours after ‘lights on’ in the animal facility) and those during subjective night to CT 13-19 (lights off at CT 12). The basic pipette solution contained (in mM): 135.0 Kgluconate, 10.0 KCl, 10.0 Hepes, 0.5 EGTA (pH 7.3; osmolality 270-280 mOsm). This basic medium was used to fill the pipette tip (4-8 MΩ) throughout all recordings. In one series of recordings, the remainder of the pipette was backfilled with this same medium but now supplemented with 5 µg/ml gramicidin (diluted 1000 times from a stock solution in DMSO) [112]. In another series of recordings, both gramicidin (5 µg/ml) and amphotericin B (250 µg/ml) [111] were used as perforating substances. The liquid junction potential was approximately -13 mV [80] and all membrane voltages in this study were corrected for this value. Gigaseals (3-30 GΩ) were formed under visual control. Maximal perforation was achieved 10-30 min. after gigaseal formation. Membrane integrity was monitored by regularly examining the series resistance estimated from the instantaneous current response to a voltage step (-20 mV, 5 ms) [113]. Furthermore, in current clamp mode membrane rupture could be easily recognized by a sudden increase in spike amplitude, due to the decrease in series resistance and in the associated filtering effect. The series resistance amounted to 78 ± 4 MΩ (N=39) and 47 ± 4 MΩ (N=27; mean ± s.e.m.) under gramicidin and gramicidin/amphotericin B conditions, respectively. Efforts to lower the series resistance below these values usually resulted in membrane rupture. The estimated mean voltage error (due to series resistance) caused by a current injection of -30 pA amounted to 2-3 mV [82]. Series resistances were about equal for the day and night phase of recording and therefore cannot account for the day-night differences in membrane properties.

Current and voltage traces were acquired using an Axopatch 1D amplifier and a Digitel data 1200 interface. The pClamp 6.02 suite of programs and Axotape 2.0 (all from Axon Instruments) were employed for data analysis. Sampling rates varied from 5 to 10 kHz. Action potential waveforms recorded in perforated patch mode with the Axopatch 1D and the Axoclamp 2B amplifier were compared and did not reveal significant differences. This finding argues against substantial spike deformation induced by current absorption by the Axopatch 1D amplifier, as described by Magistretti et al. (1998) [114].

As pointed out by Kyrozis and Reichling (1995) [112], membrane potential measurements obtained with the use of the Cl⁻ impermeant antibiotic gramicidin are not biased by a Donnan potential. Because recordings using gramicidin and gramicidin/amphotericin B yielded highly similar membrane potential values under the same conditions (data not shown), we did not correct for a potential Donnan effect in the latter experiments even though amphotericin B forms Cl⁻ permeant pores.
4.2.2 Drugs

Tetrodotoxin was obtained from Alomone Labs (Jerusalem, Israel) and bicuculline methochloride from Tocris Cookson (Bristol, U.K).

4.2.3 Quantification of membrane properties

Cells were accepted for a detailed analysis of their membrane properties when the series resistance remained at a stable value below 125 MΩ. The general viability of the cells was assessed from their membrane potential, spike amplitude and input resistance (Table 4.1.). That the spike amplitudes did not reach the same levels as is usual in whole-cell recordings [27,100,106] can be ascribed to the relatively high series resistance. Namely, when during a perforated patch recording membrane rupture occurred, spike amplitudes were seen to increase to 70-90 mV.

Membrane properties were quantified as pointed out in Pennartz et al. (1998) [27]. In short, the membrane potential was determined from the low-pass readout of the amplifier. The initial voltage deflections to hyperpolarizing current pulses (1 sec. duration, up to -30 pA) were fitted with a monoexponential function in order to estimate the input resistance and time constant. The presence of low-threshold Ca\(^{2+}\) spikes was assessed using current pulses from -3 to -60 pA (1 sec.). The spike amplitude was determined as the difference between peak voltage and basal membrane potential and the spike width was quantified at half-amplitude. Current pulses of +5 to +40 pA (1 sec.) were used to elicit spike trains. The coefficient of variation (CV) of spike intervals was computed from patterns of spontaneous firing recorded for at least 1 min. The capacitance was determined from the time constant of a monoexponential fit to the decaying capacitive transient elicited in voltage-clamp mode by a voltage step of -20 mV and 5 ms duration, divided by the series resistance [113]. Numerical values in the text refer to mean ± s.e.m.

4.3 Results

A total of 38 SCN neurons were recorded in perforated patch mode using gramicidin and satisfied the acceptance criteria (Table 4.1.). These experiments were conducted in normal ACSF and will be presented first. In addition, 27 cells were patched using a mixture of amphotericin B and gramicidin and these cells were examined under conditions where spikes and GABA\(_A\) receptor mediated transmission were blocked.

In agreement with previous results obtained in the dorsomedial region of SCN [27], most neurons could be classified as cluster I cells because of their irregular firing behaviour and monophasic spike afterhyperpolarization (28 out of 38 cells, 74%). Cluster II and III comprised only small fractions of the total population (cluster II: 3 out of 38 cells, 8%; cluster III: 3 out of 38 cells, 8%). The remaining 4 cells (11%) could not be classified because the neurons exhibited characteristics intermediate to two clusters, or not all data were available to classify them. The size of these fractions did not notably differ between subjective day and night.

Table 4.1. summarizes the membrane properties obtained from all cells recorded with gramicidin in normal ACSF (highly similar values were obtained when only cluster I
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Tab. 4.1: Overview of membrane properties of SCN neurons recorded by the gramicidin perforated patch method in normal ACSF.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>Night</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFR (Hz)</td>
<td>5.6 ± 0.7</td>
<td>2.0 ± 0.5</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>0.33 ± 0.05</td>
<td>0.50 ± 0.10</td>
<td>n.s.</td>
</tr>
<tr>
<td>Membrane potential (mV)</td>
<td>-52.9 ± 0.9</td>
<td>-57.4 ± 0.7</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Input Resistance (GΩ)</td>
<td>1.5 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Time constant (ms)</td>
<td>24 ± 3</td>
<td>21 ± 2</td>
<td>n.s.</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>8.2 ± 0.8</td>
<td>8.2 ± 0.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>52 ± 2</td>
<td>59 ± 2</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Spike threshold (mV)</td>
<td>-39 ± 1</td>
<td>-41 ± 1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Spike Width (mV)</td>
<td>1.8 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

For quantification, see methods. The SFR was determined from traces measured 30-45 min. after seal formation. The coefficient of variation was computed from the same traces used to determine the final SFR (additional criterion: SFR > 1 Hz) [27]. P-values for day-night differences were computed using Mann-Whitney’s U-test; Student’s t-test for independent samples gave similar results. The values for day and night recordings were based on groups of 19 cells each. It should be noted that spike amplitudes are lower than is usual in whole-cell recordings [27,100,106] because of low-pass filtering effects caused by the relatively high series resistance. Furthermore, values given for membrane potentials are similar to those reported in whole-cell recordings [27,100,106]. Values are mean ± s.e.m.

cells were selected). When comparing day and night values, the following observations are of particular interest. First, a highly significant day-night difference was found in the SFR (e.g. Fig. 4.1A), determined after maximal perforation of the patch was achieved. This result demonstrates functional preservation of the circadian rhythm using the gramicidin perforated patch technique in thin slices. We also compared this steady-state SFR value to the initial SFR of the same groups of cells, measured within 5 min after seal formation (when disruption of the intracellular ionic milieu is still minimal) [112]. These initial SFR values were highly similar to steady-state values (day: 5.0 ± 1.2 Hz, N=9; night: 2.1 ± 0.8 Hz, N=11; not significantly different from the corresponding steady-state values according to the Wilcoxon’s matched-pairs signed-rank test). Second, perforated patch clamp recordings revealed a significant difference in basal membrane potential between day and night, with the day phase being more depolarized. Third, associated with the depolarized state was a significantly higher input resistance during the day (e.g. Fig. 1B). Although the time constants showed a parallel trend with higher values during daytime, the difference was not significant. Finally, the spike amplitude was significantly lower during the day.

No significant day-night differences were found in the membrane capacitance, spike width and spike threshold (Table 4.1.). Judged on a subjective basis, we also observed no pronounced day-night differences in frequency adaptation (e.g. Fig. 4.1C), in the strength and threshold of time-dependent inward rectification (e.g. Fig. 4.1B) or in
Fig. 4.1: Membrane properties recorded in current clamp mode during the subjective day and night period. A, spontaneous firing activity of an SCN neuron recorded during the day phase (left panel) and a silent SCN neuron recorded during the night phase (right panel). Basal membrane potentials were -53 and -59 mV, respectively. B, hyperpolarizing current pulses (left and right panel: -15 and -30 pA for 1 s) were applied to estimate the input resistance and time constant (see text). The input resistances of the day (left panel) and night (right panel) neuron were 1.5 and 1.2 GΩ and the time constants amounted to 30 and 18 ms, respectively. Note the slowly developing depolarizing sag (time-dependent inward rectification; indicated by arrow) reflecting activation of the H-current. No significant rebound depolarizations were seen. C, spike train evoked by a current injection of +30 pA showed a similar frequency adaptation in the SCN neuron recorded in the day phase (left panel) as compared to the neuron recorded in the night phase (right panel). Scale bars: A, 25 mV, 1000 ms; B, 25 mV, 325 ms; C, 20 mV, 300 ms.
Tab. 4.2: Overview of membrane properties of SCN neurons recorded by perforated patch clamp using both amphotericin B and gramicidin and in the presence of tetrodotoxin and bicuculline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>Night</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential (mV)</td>
<td>-45.0 ± 2.1</td>
<td>-56.9 ± 2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Input Resistance (Ω)</td>
<td>2.0 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Time constant (ms)</td>
<td>29 ± 2</td>
<td>23 ± 3</td>
<td>n.s.</td>
</tr>
<tr>
<td>Capacitance (pF)</td>
<td>11 ± 1</td>
<td>11 ± 3</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

See Table 4.1. for explanations. The values for day and night recordings were based on 15 en 12 cells, respectively.

the size of rebound depolarizations (low-threshold Ca$^{2+}$ spikes).

In perforated patch recordings using a mixture of amphotericin and gramicidin, 15 day and 12 night cells were treated with tetrodotoxin (TTX; 0.5 mM) and bicuculline methochloride (BIC; 12.5 mM) in order to examine whether the circadian modulation of membrane properties was maintained when spikes and GABA$_A$ receptor mediated transmission were abolished (Table 4.2.). Under these conditions the day-night difference in membrane potential was maintained and turned out even larger than when cells were capable of generating spikes (i.e. under control and TTX/BIC conditions the differences were 4 and 12 mV, respectively). This enlargement was primarily due to the fact that cells firing at high rates during daytime were depolarized upon TTX/BIC application, while the membrane potential of night cells was generally not affected. Furthermore, the day-night difference in input resistance was also maintained under TTX/BIC conditions. The time constant and capacitance did not differ significantly between day and night (Table 4.2.).

4.4 Discussion

Before the main findings presented in this paper are discussed in detail, it should be emphasized that our conclusions pertain mainly to cluster I cells in the dorsomedial region of the SCN. Cluster I cells were previously reported to constitute a majority of neurons recorded with visual patch clamp in SCN [27]. The number of cluster II and III cells was too small to allow meaningful conclusions about their participation in the expression of circadian rhythmicity.

An important methodological result in the present study was that the initial and final SFR, as recorded under perforated patch conditions, were similar and both showed a day-night difference. These findings imply, first, that the expression of a circadian rhythm in SFR is excellently preserved under perforated patch conditions, even after prolonged periods of recording. In this respect, perforated patch recording is a method preferable above conventional whole-cell recording, which does allow to study SCN membrane properties [27,100] but also shows a gradual loss of rhythmicity. Secondly, in perforated patch recordings of cultured spinal cord neurons it has been reported that ionic exchange between pipette medium and cytoplasm takes about 15-30 min.
before a new ionic equilibrium is reached [112]. Because the final SFR values in our study were generally assessed after 30 - 45 min. of recording, when intracellular Na\(^+\), K\(^+\) and (in the case of the amphotericin B/gramicidin-recordings) Cl\(^-\) concentrations were probably clamped to a large extent to uniform pipette concentrations, it seems likely that day-night changes in the transmembrane gradients of Na\(^+\), K\(^+\) and Cl\(^-\) are not a major factor in determining the day-night difference in membrane potential. The circadian difference in tonic membrane potential was highly significant and became even more pronounced when spikes and GABA\(_A\) receptor mediated transmission were abolished by TTX and BIC. Although the cause of this enlargement is not fully clear at present, the result shows that this circadian modulation is not dependent on spike generation in the recorded neuron itself, on TTX-sensitive Na\(^+\) current supporting spontaneous firing [106] or on spike-dependent synaptic input to the neuron, in particular GABA\(_A\) receptor mediated synaptic transmission. The most parsimonious explanation for this day-night difference thus holds that it is maintained by a cell-autonomous regulation of TTX-insensitive, tonically active ionic conductance(s), although intercellular regulation via gap junctions in a restricted subpopulation of SCN neurons cannot be excluded [115]. Because the depolarized state of the cells recorded during daytime is accompanied by an increase in input resistance, it is logical to propose that the circadian modulation of ionic conductance would involve closure of hyperpolarizing (K\(^+\) or Cl\(^-\)) channels during mid-day and opening during the night. However, as yet this proposal must be considered with caution because the time constants, showing a parallel trend with higher values during daytime, were not significantly different between day and night. This lack of significance might be attributable to inaccuracies in estimating time constant \(\tau_m\) from a monoeponential fit to voltage responses of multi-compartment neurons [116], especially in the presence of spontaneous synaptic input and given the relatively high series resistance. In a whole-cell patch-clamp study, Jiang et al. (1997) [26] reported a significant circadian fluctuation in the holding current required to keep SCN cells at -60 mV in voltage clamp mode. They also found a weaker variation in input resistance that was, however, out of phase with the day-night difference in holding current. The presently reported day-night difference in membrane potential constitutes a novel result, and it is compatible with the difference in holding current proposed by Jiang et al. (1997) [26]. The two studies clearly diverge in that we found a day-night difference in input resistance roughly in phase with the membrane potential difference. It should be added, however, that an exact phase relationship could not be determined from the present results. The cause of this discrepancy is not exactly clear at present, although a few suggestions can be given. First, an important methodological difference is that Jiang et al. (1997) [26] quantified membrane properties in whole-cell mode, which in our hands gives rise to a gradual loss of rhythmicity. Even when measurements are restricted to the first 1-3 minutes after membrane rupture, washout of intracellular modulators may already have blurred a circadian modulation of input resistance. Second, the estimated input resistance reported by Jiang et al. [26] was about half the values presented in this study. This low value could indicate the presence of substantial leak current, which may mask a physiological variation in input resistance. Third, the two studies may have sampled from different subpopulations
of SCN neurons exhibiting different circadian characteristics. Apart from the membrane potential and input resistance, the spike amplitude was significantly different between day and night. However, this difference does not necessarily imply a circadian modulation of Na$^+$ channel kinetics because it may also be a direct consequence of the day-night difference in membrane potential. No marked circadian modulation was observed in a number of active membrane properties, such as the spike threshold, spike width, frequency adaptation and time-dependent inward rectification. Nonetheless, it will be worthwhile to examine especially the latter two properties in more detail by systematic quantification and voltage-clamp experiments. This study demonstrates a robust day-night difference in the membrane potential of SCN neurons that is accompanied by a difference in input resistance and is maintained when spike-dependent synaptic transmission is blocked. The combination of results suggests that hyperpolarizing ionic (K$^+$ or Cl$^-$) conductances would be open at night and closed during mid-day, when spontaneous firing reaches peak levels in the SCN. Interestingly, this suggestion bears a crude resemblance to the ionic mechanism underlying the circadian rhythm in basal retinal cells of the mollusc *Bulla gouldiana* [43]. However, the precise nature of the ionic conductances subjected to circadian modulation in SCN, and their causal involvement in determining the rhythm in firing rate, remain to be elucidated in future investigations.