Electrophysiology of the suprachiasmatic nucleus
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Citation for published version (APA):

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Chapter 5

Circadian modulation of GABA function in the rat suprachiasmatic nucleus

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Submitted
5. CIRCADIAN MODULATION OF GABA FUNCTION IN THE RAT SUPRACHIASMATIC NUCLEUS

5.1 Introduction

The biological clock of the mammalian brain is located in the suprachiasmatic nucleus (SCN) of the hypothalamus [2, 3, 6, 7, 65, 66]. SCN neurons exhibit a cell-autonomous circadian rhythm in spontaneous firing [24, 34–37, 67], which is transmitted to target areas and may impose a circadian rhythm on a wide range of physiological functions and behaviours. The neurons of the SCN form a complex network, consisting of several different cell types loaded with many different neuroactive substances [26–28, 61, 117]. The neurotransmitter γ-aminobutyric acid (GABA) is abundantly present in neurons and terminals within the SCN [60, 61, 118], and electrophysiological studies have revealed that SCN neurons frequently receive GABA<sub>A</sub> receptor-mediated postsynaptic inputs [26, 119]. These GABAergic postsynaptic responses are at least partly of intranuclear origin [62], suggesting an important role for GABA in neural integration within the SCN.

The circadian rhythm in spontaneous firing rate (SFR) of SCN neurons combined with the inhibitory nature of GABAergic neurotransmission in most areas of the matured mammalian central nervous system [120–122], led us to the initial hypothesis that GABA would effectively subdue the circadian rhythm in firing rate. That is, during the subjective day (when the SFR of SCN neurons is high) the neurons of the SCN should be subjected to GABAergic inhibition to a larger extent than during the night (when the SFR of the SCN neurons is low). However, Wagner and colleagues [123] presented evidence that GABA acts as an excitatory neurotransmitter during the day and switches to an inhibitory neurotransmitter during the night, indicating that GABA may boost the amplitude of the circadian rhythm in firing activity. These results are contradicted by other studies [64, 124–127], in which mainly inhibitory actions of GABA were observed. To elucidate this controversy and to unravel the underlying mechanism of GABAergic involvement in the circadian time-keeping system, we used the gramicidin-perforated patch-clamp technique in acutely prepared slices from the rat brain [94]. With this electrophysiological technique detailed information about GABA<sub>A</sub> receptor-mediated potentials and currents can be obtained without disturbing the transmembrane Cl⁻ gradient, a disruption that does occur in conventional whole-cell patch-clamp recordings [112].

5.2 Material and methods

5.2.1 Slice preparation

Male Wistar rats, weighing 150-300 g, were subjected to a 12:12h light:dark cycle for at least 4 weeks before use (lights on at CT 0). During the light period, rats were anaesthetized by intraperitoneal injection of either Nembutal (60 mg/kg sodium
pentobarbital) or chloralhydrate (350 mg/kg) followed by transcardial perfusion with 35 ml ice-cold artificial cerebrospinal fluid (ACSF). The ACSF contained (in mM): 124.0 NaCl, 3.5 KCl, 1.0 NaH₂PO₄, 26.2 NaHCO₃, 1.3 MgSO₄, 2.5 CaCl₂ and 11.0 D(+)-glucose, and was gassed with a mixture of 95% O₂ and 5% CO₂ (pH 7.4: osmolality 303-307 mOsm kg⁻¹). The rats were decapitated, their brains were rapidly removed and immersed in cold ACSF. The brains were trimmed to a block containing the hypothalamus. Transverse slices of 200 μm thickness were cut with a vibroslicer (Campden Instruments, UK). Slices containing the SCN were incubated in ACSF for at least one hour. After this period a slice was placed in the recording chamber through which ACSF (30°C) flowed at 2-3 ml/min. The delay between preparation and recording was approximately equal for day and night measurements. During the 3 years of full-time research that this study took to be completed, we usually alternated ‘day’ and ‘night’ experiments. The experiments were in accordance with the Dutch national guidelines on animal experiments.

Besides anaesthetizing the rats with Nembutal, we used Chloralhydrate to investigate whether pentobarbital would affect GABAergic synaptic transmission in SCN slice experiments. The decay time constants of electrically evoked IPSCs (measured at V_H=-103 mV) did not show significant differences between Nembutal and Chloralhydrate treatment (Mann-Whitney U-test, n=24). Furthermore, no differences in reversal potential were observed between the two treatments (Mann-Whitney U-test, n=19). These results indicate a lack of a lingering effect of Nembutal on GABAergic transmission in SCN slices.

5.2.2 Electrophysiological recordings

Gramicidin-perforated patch-clamp recordings were performed as described in De Jeu et al. (1998) [94]. The electrode solution contained (in mM): 135.0 KGlucurate, 10.0 KCl, 10.0 Hepes, 0.5 EGTA (pH adjusted to 7.2-7.4 with KOH; osmolality between 270-290 mOsm kg⁻¹). Gramicidin was dissolved in dimethylsulfoxide (5 mg/ml) and added to the electrode solution (final concentration 5 μg/ml). The tip of the patch pipette was pre-filled with a small amount of gramicidin-free electrode solution in order to prevent interference of gramicidin with seal formation. Patch pipettes (4-8 MΩ) were back-filled with the gramicidin containing electrode solution. The liquid junction potential was approximately -13 mV [80] and all the membrane voltages in this study were corrected for this value. Patch pipettes were positioned on SCN neurons under visual control, using an upright fixed-stage microscope (Axioskop, Zeiss) equipped with a 40x water-immersion lens (NA: 0.75) with Hoffman modulation contrast. In order to keep the patch pipette clean while penetrating the slice, a constant positive pressure was applied. Formation of a gigaseal (>3 GΩ) was accomplished by mouth suction. After gigaseal formation, membrane integrity and the progress of perforation were monitored by regularly measuring the capacitive current (filtered at 10 kHz) evoked by a -20 mV voltage step. Membrane rupture could easily be recognized by a sudden increase in capacitive current or, in current-clamp mode, by a sudden increase in spike amplitude due to the decrease in series resistance and in the associated filtering effect. Perforated patches with stable series resistance values were usually obtained after 30 minutes. Cells were accepted for this study when the series
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Resistance remained at a stable value below 125 Ω (75 ± 5 Ω, n=55). Current and voltage traces were acquired using an Axopatch-1D amplifier, and were relayed by a Digidata 1200A Interface to a personal computer equipped with pClamp 6.0.2 and Axotape 2.0.2 (all from Axon Instruments). The estimated mean voltage error was 0.3 - 0.7 mV [82]. To further reduce this error and the low-pass filtering of spikes, numerous attempts were made to lower the access resistance. However, enhancing the gramicidin concentration, enlarging the pipette tip or applying other variations invariably resulted in membrane rupture. Thus it proved technically unfeasible to achieve an access resistance similar to values found in most whole-cell studies (cf. [128]). This technical detail also explains why absolute spike amplitudes are somewhat lower than in whole-cell studies (cf. [106,129]); membrane rupture in perforated-patch mode invariably resulted in spikes reaching amplitudes of 80-100 mV with respect to baseline. Action potential waveforms recorded in perforated-patch mode with an Axopatch 1D and an Axoclamp 2B amplifier did not reveal systematic differences in action potential amplitude or shape, arguing against substantial spike deformation induced by current absorption of the Axopatch 1D amplifier, as suggested by Magistretti et al. (1998) [114] (see also [27,94]).

For focal electrical stimulation a tungsten bipolar electrode (Frederick Haer & Co, Bowdoinham, USA) was placed along the dorsal or ventrolateral borders of the SCN. Stimulation pulses were bipolar and biphasic (duration: 0.2 ms; stimulus intensity: < 0.5 mA).

Neurons in this study (primarily cluster I cells; [27]) clearly exhibited a circadian rhythm in firing rate (Fig. 5.3C) and the firing rate values were similar to the values measured with the extracellular cell-attached technique and the values found immediately after the membrane rupture using the whole-cell patch-clamp technique [129]. Furthermore, initial and final spontaneous firing rates were similar, revealing that the spontaneous firing rate is well preserved under perforated-patch conditions even after long periods of recording [94]. Occasionally, a membrane rupture occurred during a perforated-patch recording, which resulted always in an increment of the spike amplitude toward a value of 80-100 mV; similar to the spike amplitude values measured with the whole-cell patch-clamp technique. All these observations indicate that our measurements were performed from healthy neurons.

5.2.3 Drugs

Receptor antagonists used in the present study were applied to brain slices by bath perfusion. Bicuculline methochloride (GABA_A receptor antagonist), D-2-amino-5-phosphonopentanoic acid (D-AP5: NMDA receptor antagonist), 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX: non-NMDA receptor antagonist; all from Tocris Cookson, Bristol, UK) and CGP 55845A (GABA_B receptor antagonist; gift from Ciba-Geigy, Switzerland) were dissolved in distilled water for preparation of stock solutions and diluted in ACSF to their final concentrations.

For experiments with GABA pulses, GABA (Tocris Cookson, Bristol, UK) was dissolved in ACSF containing D-AP5 (50 μM), NBQX (5 μM) and CGP 55845A (1 μM). The pH was adjusted to 7.2-7.4 with HCl. GABA was locally applied by using a BPS-8 system equipped with a micromanifold (inner diameter 100 μm), and the
temperature of the applied solution was kept at 30°C by a PRT-2000 system (all from ALA Scientific Instruments, New York, USA). It should be noted that the GABA concentration at the cell is lower than in the application system because of limited GABA diffusion through the superficial layers of the slice. In pilot experiments responses to lower GABA concentrations were smaller or absent.

5.3 Results

5.3.1 Excitatory GABA<sub>A</sub>-mediated responses during the night

We activated GABA<sub>A</sub> receptors in the SCN by focal electrical stimulation of GABAAergic fibers, providing a short endogenous GABA pulse, or alternatively by pulse application of (exogenous) GABA. Focal electrical stimulation of the SCN, in the presence of NMDA, AMPA/kainate and GABAB-receptor antagonists (D-AP5, 50 μM; NBQX, 5 μM; CGP 55845A, 1 μM, respectively), evoked postsynaptic responses, which were completely and reversibly blocked by 12.5 μM bicuculline during both day and night (Fig. 5.1A and C). During the day period, electrically evoked GABA<sub>A</sub> receptor-mediated responses (Fig. 5.1A) were hyperpolarizing in 10 out of 11 neurons, whereas only one neuron exhibited depolarizing responses. Likewise, pulse application of (exogenous) GABA (1 mM) during the day in the presence of D-AP5 (50 μM), NBQX (5 μM) and CGP 55845A (1 μM) elicited hyperpolarizing responses (Fig. 5.1B) in 9 out of 10 neurons and depolarizing responses in only one neuron.

In contrast, during the night period electrically evoked GABA<sub>A</sub> receptor-mediated responses were hyperpolarizing in 7 out of 15 and depolarizing in 8 out of 15 neurons. In 6 out of 8 neurons these depolarizing GABA<sub>A</sub> receptor-mediated responses were capable of triggering action potentials and thus appeared to be excitatory (Fig. 5.1C). Similarly, pulse application of GABA during the night period resulted in hyperpolarizing response patterns in 7 out of 12 and depolarizing response patterns (Fig. 5.1D) in 5 out of 12 neurons. In these night neurons, the membrane potential was not significantly different between neurons with a depolarizing and hyperpolarizing GABA response (Student’s t-test for independent samples and Mann-Whitney U-test: all p>0.05; n<sub>el stim</sub>=15, n<sub>appl</sub>=12), indicating that the switch in polarity was not caused by a change in membrane potential. Focal electrical stimulation and GABA application experiments (in this order) were often performed on the same neuron (n=12) and in all these neurons, taken individually, the polarity of the GABA<sub>A</sub>-mediated response was identical for both technical approaches, showing that the obtained results were independent of the technical procedure.

Both during day and night the membrane potential changes induced by exogenous GABA were strongly reduced by bicuculline (100 μM; this dose was selected because the relatively high dose of GABA is likely to compete with bicuculline for the receptor binding site). An interesting observation was that in all neurons, whether displaying hyperpolarizing or depolarizing GABA response patterns, the firing activity was completely arrested during exogenously applied GABA except for the initial response phase when GABA was depolarizing (see Fig. 5.1D, arrow). This initial depolarizing response gave rise to one or several spikes, compatible with an excitatory action of
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GABA. With respect to the prolonged phase of the depolarizations, the cessation of firing can be explained by the occurrence of a massive shunting effect (130; Fig. 5.1D, asterisks) in conjunction with Na\(^+\) channel inactivation. It should be stressed that the generation of action potentials was not prevented by these artifactual processes during evoked or spontaneous synaptic GABA\(_A\)-mediated depolarizing responses, which occur on a much faster time-scale. In conclusion, results obtained with focal electrical stimulation of GABAergic fibers and pulse application of GABA indicate that the polarity and functional effect of the GABA\(_A\) receptor-mediated response vary in a circadian manner in a large subpopulation of SCN neurons.

5.3.2 Day-night difference in GABA\(_A\) reversal potential

These results predict that a circadian shift in GABA\(_A\) reversal potential (most likely by changes in intracellular Cl\(^-\) concentration) should occur in approximately 50\% of the SCN neurons. To test this, we investigated the reversal potential of GABA\(_A\) receptor-mediated currents during the subjective day and night period. Both electrical stimulation of GABAergic fibers (Fig. 5.2.) and pulse application of 1 mM GABA (both in the presence of D-AP5 (50 \(\mu\)M), NBQX (5 \(\mu\)M) and CGP 55845A (1 \(\mu\)M)) were used to determine the GABA\(_A\) reversal potential. On several SCN neurons both experimental procedures were performed; the GABA pulse application experiments were always preceded by the focal electrical stimulation experiments. For electrically evoked GABA\(_A\) receptor-mediated currents during the subjective day, the GABA\(_A\) reversal potential was \(-70 \pm 2\) mV (n=17), in agreement with the inhibitory role of GABA in this circadian time domain. With application of exogenous GABA this value was \(-67 \pm 3\) mV (n=17), which is not significantly different. Activating the GABAergic receptors with electrical stimulation during the subjective night period revealed a more depolarized reversal potential: \(-59 \pm 4\) mV (n=18). Pulse application of GABA during the night period gave a similar result (-57 \pm 3 mV, n=15). In both experimental approaches, a significant day-night difference in GABA\(_A\) reversal potential was found (Student's t-test for independent samples and Mann-Whitney U-test; all p<0.05). Normality tests (Shapiro-Wilks' W test) revealed a normal distribution of GABA\(_A\) reversal potential of SCN neurons during the day (p>0.05), whereas GABA\(_A\) reversal potential of SCN neurons during the night was not normally distributed (p<0.05). In conclusion, the distributions of GABA\(_A\) reversal potentials revealed a significant day-night difference, which can be ascribed to a large subgroup of SCN neurons with markedly depolarized GABA\(_A\) reversal potentials in the night phase.

5.3.3 Spontaneous GABA\(_A\)-mediated inputs attenuate firing activity during the day

We next investigated the contribution of spontaneous GABA\(_A\) receptor-mediated neurotransmission to the circadian rhythm in SFR by measuring the effect of the GABA\(_A\) receptor antagonist bicuculline on the SFR during the subjective day and night period. Bicuculline (12.5 \(\mu\)M) blocked virtually all fast spontaneous synaptic inputs recorded in SCN neurons and also affected their SFRs (Fig. 5.3A and B).
Fig. 5.1: Activation of GABA<sub>A</sub> receptors by focal electrical stimulation and by pulse application of GABA. A. Example of electrically evoked hyperpolarizing postsynaptic potentials, which were completely blocked by bicuculline (12.5 μM). These current-clamp recordings were obtained from a SCN neuron in the subjective day period. Arrows mark electrical stimulus artifacts. B. Example of a membrane hyperpolarization evoked by pulse application of GABA (1 mM, 2 s; day). Rectangular current pulses (200 ms) of -15 pA were applied every 1200 ms to observe changes in input resistance (*). C. Example of electrically evoked depolarizing postsynaptic potentials which were capable of triggering an action potential. The depolarizing postsynaptic potentials and associated action potentials were blocked in the presence of bicuculline (12.5 μM). These current-clamp recordings were obtained from a SCN neuron in the subjective night period. Arrows mark the electrical stimulus artifacts. D. Example of a membrane depolarization evoked by pulse application of GABA (1 mM, 2.5 s; night). At the initial stage of the depolarization, two action potentials were generated (see inset graph, arrow). In association with the strong decrease in input resistance, generation of any subsequent spikes was arrested. Current pulses (200 ms) of -10 pA were applied every 1200 ms. All of these experiments were performed in the presence of D-AP5 (50 μM), NBQX (5 μM) and CGP 55845A (1 μM). Scale bars: 40 mV, 600 ms (A); 20 mV, 2500 ms (B); 30 mV, 600 ms (C); 20 mV, 2500 ms (D).
Fig. 5.2: Reversal potential measurements of GABA_A receptor-mediated currents in SCN neurons during the subjective day and night period. A. GABA_A receptor-mediated current responses, evoked by focal electrical stimulation at the ventrolateral border of the SCN, were recorded in voltage-clamp mode at different holding potentials (V_H). These voltage-clamp recordings were obtained from a SCN neuron in the subjective day period (CT 5.5) and from a SCN neuron in the subjective night period (CT 15.5). B. Determination of the GABA_A reversal potential of the neurons in (A.). Peak amplitudes of current responses (I_Peak) in (A.) are plotted against the holding potential; linear regression was used to fit the data points. The intersection of the regression line with the abscissa (-75 mV and -44 mV in these examples) was taken as the GABA_A reversal potential. C. GABA_A reversal potentials (determined by evoked IPSC's) during the subjective day and night period. Reversal potentials are plotted against the circadian time (CT) of recording. Average reversal potentials and SEMs are represented by solid horizontal lines and grey bars, respectively. Scale bars: 100 pA, 50 ms (left panel; A); 100 pA, 83 ms (right panel; A).
During the subjective day period, bicuculline increased the SFR of all SCN neurons (mean increment: 2.9 ± 0.8 Hz, mean ± SEM; Wilcoxon’s matched-pairs signed-rank test: p<.01, n=10; Fig. 5.3C), suggesting that GABA_A receptor-mediated postsynaptic potentials inhibited SCN neurons during this period. During the subjective night, the effects of bicuculline were variable and the mean change was not significant (mean increment: 1.4 ± 1.5 Hz, n=10; Fig. 5.3C). The bicuculline-induced changes in SFR were significantly larger during the day than during the night (Mann-Whitney U-test: p<.01, n=20). Removal of bicuculline generally resulted in recovery of the SFR to baseline levels. Moreover, the effect of bicuculline on the firing activity of SCN neurons could not be ascribed to a blocking effect on I_{AHP}. This was suggested by the observation that bicuculline did not enhance or prolong the rebound depolarization. Such rebound depolarizations are evoked upon a release of a negative rectangular current and are curtailed by I_{AHP} [27,131]. Taken together, these results suggest that GABA moderately attenuates the circadian peak in spontaneous firing at daytime but has no strong effect on low-level spontaneous firing at night.

Furthermore, the membrane was not significantly depolarized or hyperpolarized by bicuculline either during day or night (Wilcoxon’s matched-pairs signed-rank test: ns; day: n=9, night: n=10;) and no correlation was observed between bicuculline-induced changes in firing rate and small fluctuations in membrane potential (linear regression: R=.31, n=19). Likewise, the input resistance and time constant were not affected by bicuculline (n=9; day: n=4, night: n=5). These results strongly suggest that the SFR was affected via discrete synaptic events and not by tonic activation of GABA_A receptors by ambient GABA.

Upon closer inspection of the night data, three neurons showed a bicuculline-induced increase in SFR, three neurons showed a bicuculline-induced decrease in SFR, and four silent cells did not start firing after the application of bicuculline. This mixture of effects was clearly related to the value of the GABA_A-mediated reversal potential of these SCN neurons (hyperpolarized of depolarized with respect to rest). Neurons displaying a bicuculline-induced decrease in SFR exhibited spontaneous depolarizing postsynaptic potentials that were capable of triggering spikes at rest (e.g. Fig. 5.3B, asterisks). And in contrast, neurons displaying a bicuculline-induced increase in SFR exhibited spontaneous hyperpolarizing postsynaptic potentials at rest. Both types of spontaneous postsynaptic potentials were blocked by bicuculline. Thus, these results are in agreement with the day-night difference in electrically evoked GABA_A-mediated responses and with the day-night difference in GABA_A-mediated reversal potentials.

5.3.4 Alterations of GABA_A-mediated response during long-lasting activation

We decided to test SCN responses to long-lasting GABA pulses (> 10s) in more detail, because some of the existing discrepancies in the literature may be related to the long duration of GABA pulses used in many previous studies. During long-lasting GABA pulses (1 mM) a partial recovery of the change in membrane potential was observed, suggesting a shift in reversal potential and/or desensitization of GABA_A receptor/channels [132,133]. In voltage-clamp mode (V_H=-103 mV), the GABA_A receptor-mediated inward current decreased in all cells (n=13) already during the
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Fig. 5.3: Effect of GABA_A receptor-mediated postsynaptic inputs on the spontaneous firing rate of SCN neurons. A. Current-clamp recordings in perforated-patch mode showing the effect of bicuculline (12.5 μM) on the spontaneous firing behaviour and spontaneous postsynaptic potentials of an SCN neuron recorded at circadian time 8 (CT 8; subjective day). Lower traces (-103 mV) were obtained by injecting a tonic hyperpolarizing current. Bicuculline-sensitive hyperpolarizing postsynaptic potentials were observed in this neuron at rest (-58 mV; *). The blocking effect of bicuculline on spontaneous postsynaptic potentials indicates that these potentials are mediated by GABA_A receptors. B. Current-clamp recordings in perforated-patch mode showing the effect of bicuculline (12.5 μM) on the spontaneous firing behaviour and spontaneous postsynaptic potentials of a SCN neuron recorded at CT 16 (subjective night). Lower traces (-103 mV) were obtained by injecting a tonic hyperpolarizing current. Bicuculline-sensitive depolarizing postsynaptic potentials with superimposed spikes were observed in this neuron at rest (-58 mV; *). C. Effect of bicuculline on the spontaneous firing rate of SCN neurons (mean ± SEM) during their subjective day (CT 4-9, n=10) and night period (CT 14-19, n=10). * p<.01, Wilcoxon’s match pairs signed-rank test. Under control conditions, the spontaneous firing rates were significantly different between day and night (Mann-Whitney U-test, p< .01, n=20; see also [94]). Scale bars: 30 mV, 2 s (top; A); 30 mV, 12 s (top; B); 30 mV, 400 ms (middle and bottom; A, B).
initial phase of such long-lasting pulses (> 10 s). The reduction in GABA_A receptor-mediated current can also be observed by application of two consecutive GABA pulses while the neuron is clamped at a constant holding potential not too close to the GABA_A reversal potential (Fig. 5.4A and B, left panels). Attenuation of the second GABA response may be caused either by a shift in GABA_A reversal potential or by desensitization of GABA_A receptor/channels. These processes can be separated by applying a second protocol, in which the neuron is clamped near the GABA_A reversal potential during the first GABA pulse but at the same holding potential of the previous protocol during the second pulse. If the change in holding potential can prevent the attenuation of the second current response, the reduction of this second response can be attributed to a shift in GABA_A reversal potential (Fig. 5.4B): if not, it can be ascribed to desensitization of GABA_A receptor/channels (Figure 5.4A). To ensure the stability of our system, we always executed a third protocol (which was identical to the first protocol) in order to show “recovery” of our system. These recovery protocols indicated that there was no run-down or desensitization during the entire experiment. These double-pulse experiments suggested that desensitization of GABA_A receptor/channels occurred in 38% of the neurons (5 out of 13; e.g. Fig. 5.4A), whereas a shift in GABA_A reversal potential was found in 85% of the neurons (10 out of 13; e.g. Fig. 5.4B). Investigation of the GABA_A conductance (g_GABA) during long-lasting applications showed that the onset of the decay in g_GABA was delayed compared to the decay in GABA_A receptor-mediated current response (I_GABA), and that g_GABA decreased more slowly than I_GABA (e.g. Fig. 5.4C and D, n=4). This result suggests that the initial decline in I_GABA was primarily caused by a shift in reversal potential (cf. [133]).

5.4 Discussion

Our data indicate that the GABA_A reversal potential, and thus probably the [Cl⁻], is subjected to a circadian modulation in a subpopulation of SCN neurons (Fig. 5.2C), resulting in a polarity shift of the GABA_A receptor-mediated postsynaptic response from hyperpolarization during the subjective day to depolarization during the subjective night. The net effect of spontaneous GABA_A receptor-mediated postsynaptic potentials on the circadian rhythm in firing rate was to moderately subdue the peak at daytime. During the day, GABAergic inputs attenuated the spontaneous firing of almost all SCN neurons, whereas during the night these inputs inhibited some SCN neurons but promoted firing in others by way of depolarizing GABA_A receptor-mediated postsynaptic potentials (Fig. 5.1. and 5.3.). These GABA potentials at night were excitatory because spikes were triggered on top of them, and bicuculline blocked these GABAergic inputs as well as their associated spikes. Furthermore, our results do not contradict the study of Gribkoff et al. (1999) [127], because the conclusions of this study were limited to GABA function in the day phase (being inhibitory in agreement with our results), and did not pertain to the night phase.

Our results argue against the hypothesis that the GABAergic network of the SCN reinforces the circadian rhythm in SFR by exciting SCN neurons during the day period and inhibiting them during the night, as proposed by Wagner et al. (1997) [123].
Fig. 5.4: Reversal potential shift and desensitization of GABA<sub>A</sub> receptor/channels in SCN neurons. A. and B. Voltage-clamp recordings showing GABA<sub>A</sub> receptor-mediated current responses using double pulse application of GABA (1 mM; pulse duration: 2 s, pulse interval: 15 s). Two different voltage command protocols were used to examine reversal potential shifts and/or desensitization of the GABA<sub>A</sub> receptor/channels. First, two GABA<sub>A</sub> receptor-mediated current responses were measured at a constant holding potential, revealing the attenuation of the current response to the second GABA pulse. Second, double GABA<sub>A</sub> receptor-mediated current responses were evoked in a protocol where the holding potential during the first GABA pulse was approximately kept at the GABA<sub>A</sub> reversal potential and at the same holding potential of the previous protocol during the second pulse. A. Attenuation of the second GABA response could not be prevented by holding the potential at the reversal potential during the first GABA pulse (arrows); this result indicates that the GABA<sub>A</sub>-receptor/channels were subject to a desensitization process. B. In another cell, the attenuation of the second current response could be largely prevented by holding the potential close to the reversal potential during the first GABA pulse (arrows), indicating that the attenuation of the second GABA pulse response was caused by a shift in reversal potential. C. Recording of a GABA<sub>A</sub> receptor-mediated current response evoked by long-lasting GABA application (1 mM) at a holding potential of -103 mV. Voltage steps of -30 mV (200 ms) were applied every 1200 ms. D. Relative changes in GABA<sub>A</sub> receptor-mediated current (I<sub>GABA</sub>; closed squares) and in GABA<sub>A</sub> receptor-mediated conductance (g<sub>GABA</sub>; open squares) of the response shown in C. \[ g_{GABA} = (\Delta I_{GABA} - \Delta I_{BASELINE}) / V_S; \] where \[ \Delta I_{GABA} \] is the current response to the voltage step during the GABA application, \[ \Delta I_{BASELINE} \] the current response before the GABA application, and \[ V_S \] the magnitude of the voltage step. Scale bars: 15 pA, 5 s (A); 30 pA, 5 s (B); 100 pA, 5 s (C).
An important source of discrepancy could be the use of long-lasting GABA applications (> 10 s; [123], but also [64,124–127]). Long-lasting GABA applications appear to cause shifting of the reversal potential and/or desensitization of GABA_A receptor/channels (Fig. 5.4.), which may confound functional interpretation of GABA effects on SCN excitability. If prolonged GABA pulses induce a shift in GABA_A reversal potential, the driving force for Cl^- will be reduced. Therefore the mean amplitude of spontaneous GABA_A receptor-mediated currents will decrease and, consequently, a reduction in GABAergic inhibition or excitation is likely to occur. Desensitization of GABA_A receptor/channels may also result in an attenuated postsynaptic response to spontaneous GABAergic inputs. Consequently, the results obtained with long-lasting applications of GABA may paradoxically come to resemble results obtained with GABA_A receptor antagonists. The validity of this explanation remains unknown because we did not use very long-lasting GABA applications, but in any case our results demonstrate that results obtained with such application are confounded by multiple factors. In addition, application of exogenous GABA can cause massive shunting ([130]; Fig. 5.1.: asterisks), which further complicates the interpretation of results, in particular because it partially occludes the excitatory effect of GABA at night (Fig. 5.1D: firing activity is arrested after an initial spike-triggering depolarization). Such massive shunting may explain why several extracellular recording studies found uniformly inhibitory GABA effects, regardless of circadian phase [64,124–126] (cf. Fig. 5.1D). Functional interpretations of bicuculline effects (Fig. 5.3.), electrically evoked GABA_A receptor-mediated potentials/currents (Fig. 5.1A, C; 2A) and the initial effect of exogenous GABA pulses (Fig. 5.1B, D) are much less confounded by these factors. A further discrepancy between our study and that of Wagner et al. (1997) [123] concerns the day-night difference in GABA_A reversal potential. Wagner et al. (1997) [123] found a more hyperpolarized reversal potential during the night than during the day, whereas the present results indicate a more depolarized mean reversal potential at night. Estimation of the GABA_A reversal potential by studying the voltage-dependence of the standard deviation in membrane potential in whole-cell recordings [123] may be confounded by the fact that this parameter can be influenced by intrinsic ionic currents (eg. a slow low-threshold component of Na^+ current [106]). Direct measurement of the reversal potential of GABA_A receptor-mediated postsynaptic currents (Fig. 5.2.) evoked by electrical stimulation or exogenous GABA in gramicidin-perforated patch voltage-clamp mode is not susceptible to this problem.

The circadian variation of the GABA_A reversal potential in approximately 50% of SCN neurons may be important, not so much for regulating the absolute magnitude of the circadian rhythm in spontaneous firing rate, but rather for the integration of environmental or internal timing cues in the circadian system. For instance, photic inputs primarily enhance the firing rate of SCN neurons by way of the glutamatergic fibers of the retinohypothalamic tract [9–12]. The inhibitory nature of GABAergic transmission during the day period may counteract the integration of timing cues by arresting the propagation of these signals throughout the SCN (Fig. 5.5.). In contrast, during the night the excitatory nature of GABAergic transmission in a subset of cells may promote signal dissemination throughout a restricted domain of the SCN network and beyond (Fig. 5.5.). Thus, the GABAergic network may behave
Circadian modulation of GABA function in the SCN

Fig. 5.5: Schematic representation of a novel hypothesis on the function of the GABAergic network in the SCN. Retinal inputs reach the SCN via a glutamatergic pathway. Glutamate (GLU) release activates SCN neurons (increment of firing activity: circles filled with black). Propagation of light information is counteracted during the day by an inhibitory GABAergic network (decrease of firing activity: circles filled with grey). The excitatory nature of GABAergic transmission in a subset of cells during the night (increment of firing activity: circles filled with black) promotes the propagation and integration of light information throughout and beyond the SCN.

as an active filter that passes excitatory inputs during the night but cuts them off during the day. This idea is in line with the enhanced light-responsiveness of SCN neurons at night compared to day [23]. Propagation of light information throughout the SCN was also revealed by spatio-temporal SCN profiles of clock gene expression (e.g. mper1) and activity markers (eg. c-fos [48]).

In order to induce a phase-shift a perturbation of the molecular clock is necessary [52, 53]. GABA_A-mediated depolarizations may serve to disseminate phase-shifting signals throughout the SCN network and support the activation of the ensuing signal transduction cascade leading to clock resetting. It is tempting to speculate that depolarizing GABA_A receptor-mediated responses may increase intracellular Ca^{2+} levels by opening voltage-activated Ca^{2+} channels [134]. Subsequently, activation of Ca^{2+}-dependent signal transduction pathways (e.g. involving ERK/MAPK and CREB; [55,56,135]) may cause a resetting of the molecular clock. In a similar way, the GABAergic network may synchronize the ‘clock cells’ of the SCN [136,137].
Recently, Liu and Reppert (2000) [64] showed that cultured SCN clock cells could be synchronized by prolonged application of GABA. Whether the depolarizing and excitatory action of GABA described here mediates this synchronizing effect remains to be investigated, because a maximal phase-shift was induced around the subjective day-to-night transition, not throughout the subjective night.

The circadian modulation of the GABA_A reversal potential has broader implications than for understanding SCN network functioning. First, it indicates that in the adult brain, GABA can switch between an excitatory and inhibitory function in a time-dependent, cyclic manner. Previously, only an irreversible unidirectional switch from excitatory to inhibitory function was shown to occur during embryonic development [138,139]. Second, combining our results with molecular studies may prompt research on the unresolved issue as to how cyclic expression of clock genes leads to modulation of mechanisms regulating membrane excitability, including ionic transporters [140].