

Circadian Modulation of GABA Function in the Rat Suprachiasmatic Nucleus: Excitatory Effects During the Night Phase

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de Jeu, Marcel and Cyriel Pennartz. Circadian modulation of GABA function in the rat suprachiasmatic nucleus: excitatory effects during the night phase. *J Neurophysiol* 87: 834–844, 2002; 10.1152/jn.00241.2001. Gramicidin-perforated patch-clamp recordings were made from slices of the suprachiasmatic nucleus (SCN) of adult rats to characterize the role of γ -amino butyric acid (GABA) in the circadian timing system. During the day, activation of GABA_A receptors hyperpolarized the membrane of SCN neurons. During the night, however, activation of GABA_A receptors either hyperpolarized or depolarized the membrane. These night-restricted depolarizations in a large subset of SCN neurons were capable of triggering spikes and thus appeared to be excitatory. The GABA_A reversal potentials of SCN neurons revealed a significant day-night difference with more depolarized GABA_A reversal potentials during the night than during the day. **The emergence of depolarizing GABA_A-mediated responses in a subset of SCN neurons at night can be ascribed to a depolarizing shift in GABA_A reversal potential.** The GABA_A receptor antagonist bicuculline (12.5 μ M) increased the spontaneous firing rate of all SCN neurons during the day, indicating that spontaneous GABA_A-mediated inputs inhibited the SCN neurons during this period. The effect of bicuculline (12.5 μ M) on the spontaneous firing rate of SCN neurons during the night was heterogeneous due to the mixture of depolarizing and hyperpolarizing GABA_A-mediated inputs during this period. We conclude that GABA uniformly acts as an inhibitory transmitter during the day but excites a large subset of SCN neurons at night. This day-night modulation of GABAergic neurotransmission provides the SCN with a time-dependent gating mechanism that may counteract propagation of excitatory signals throughout the biological clock at day but promotes it at night.

INTRODUCTION

The biological clock of the mammalian brain is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Lehman et al. 1987; Moore and Eichler 1972; Ralph et al. 1990; Rusak and Zucker 1979; Stephan and Zucker 1972; Takahashi and Zatz 1982). SCN neurons exhibit a cell-autonomous circadian rhythm in spontaneous firing (Bos and Mirmiran 1990; Bouskila and Dudek 1993; Green and Gillette 1982; Groos and Hendriks 1982; Inouye and Kawamura 1979; Welsh et al. 1995), which is transmitted to target areas and may impose a circadian rhythm on a wide range of physiological functions and behaviors. The neurons of the SCN form a complex network consisting of several different cell types loaded with many different neuroactive substances (Buijs et al. 1994; Jiang

et al. 1997; Pennartz et al. 1998a; van den Pol and Dudek 1993; van den Pol and Tsujimoto 1985). The neurotransmitter γ -amino butyric acid (GABA) is abundantly present in neurons and terminals within the SCN (Buijs et al. 1994; Moore 1993; van den Pol 1986), and electrophysiological studies have revealed that SCN neurons frequently receive GABA_A receptor-mediated postsynaptic inputs (Jiang et al. 1997; Kim and Dudek 1992). These GABAergic postsynaptic responses originate at least partly within the nucleus itself (Stecker et al. 1997), 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 CNS (Kaila 1994; Krnjevic 1976; Mody et al. 1994) 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 (1997) presented evidence that GABA acts as an excitatory neurotransmitter during the day but assumes an inhibitory role during the night, indicating that GABA may boost the amplitude of the circadian rhythm in firing activity. These results are contradicted by other studies (Bos and Mirmiran 1993; Gribkoff et al. 1999; Liou and Albers 1990; Liou et al. 1990; Liu and Reppert 2000) in which mainly inhibitory actions of GABA were observed. To resolve 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 (de Jeu et al. 1998). With this electrophysiological technique, detailed information about GABA_A 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 (Kyrois and Reichling 1995).

The main new finding presented in this study is the experimental evidence arguing in favor of an excitatory GABA effect in a subset of SCN neurons recorded in the subjective night. A small part of our results were already mentioned in a brief communication (Gribkoff et al. 1999), but this was restricted to

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bicuculline effects in the day phase. These results are recapitulated here to permit comparisons to the night phase.

METHODS

Slice preparation

Male Wistar rats, weighing 150–300 g, were subjected to a 12:12 h light:dark cycle for ≥ 4 wk before use (lights on at CT 0). During the light period, rats were anesthetized by intraperitoneal injection of either pentobarbital sodium (Nembutal, 60 mg/kg) 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 NaH_2PO_4 , 26.2 NaHCO_3 , 1.3 MgSO_4 , 2.5 CaCl_2 , and 11.0 D(+)-glucose and was gassed with a mixture of 95% O_2 –5% CO_2 (pH 7.4; osmolality, 303–307 mOsm). The rats were decapitated, and 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). Slices containing the SCN were incubated in ACSF for ≥ 1 h. 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 yr 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 anesthetizing 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 inhibitory postsynaptic currents (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 lack of a lingering effect of Nembutal on GABAergic transmission in SCN slices.

Electrophysiological recordings

Gramicidin perforated patch-clamp recordings were performed as described in de Jeu et al. (1998). The electrode solution contained (in mM) 135.0 Kgluconate, 10.0 KCl, 10.0 HEPES, and 0.5 EGTA (pH adjusted to 7.2–7.4 with KOH; osmolality between 270 and 290 mOsm/kg). Gramicidin was dissolved in dimethylsulfoxide (5 mg/ml) and added to the electrode solution (final concentration, 5 $\mu\text{g}/\text{ml}$). The tip of the patch pipette was prefilled with a small amount of gramicidin-free electrode solution to prevent interference of gramicidin with seal formation. Patch pipettes (4–8 M Ω) were back-filled with the gramicidin containing electrode solution. The liquid junction potential was approximately -13 mV (Neher 1992), 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 $\times 40$ water-immersion lens (NA: 0.75) with Hoffman modulation contrast. To keep the patch pipette clean while penetrating the slice, a constant positive pressure was applied. Formation of a gigaseal (>3 G Ω) 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.

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). 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) (see also de Jeu et al. 1998; Pennartz et al. 1998b).

Perforated patches with stable series resistance values were usually obtained after 30 min. At this time the series resistance remained at a stable average value of 75 ± 5 (SE) M Ω ($n = 55$).

This relatively high series resistance posed a point of special concern in this study. First we note that other groups have attempted to apply gramicidin-perforated patch-clamp recordings to the SCN but concluded it was technically unfeasible (Colwell 1997). Second, we made numerous attempts 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. It is of note that this technical limitation has also been encountered in other gramicidin-perforated patch-clamp studies (Kyrozi and Reichling 1995; Ulrich and Huguenard 1997; Vale and Sanes 2000). The high series resistance also explains why absolute spike amplitudes are somewhat lower than in whole cell studies (cf. Pennartz et al. 1997; Schaap et al. 1999); membrane rupture in perforated-patch mode invariably resulted in an increment of spikes reaching amplitudes of 80–100 mV with respect to baseline.

Given the high series resistance, we asked whether the resulting voltage errors would prevent us from drawing strong conclusions. The voltage error can be calculated by

$$V_{\text{err}} = I_h R_s$$

where I_h is the holding current and R_s is the series resistance (Armstrong and Gilly 1992). Notably, the extremely high-input resistance of SCN neurons (range 1–3 G Ω) (de Jeu et al. 1997; Pennartz et al. 1998b) results in very small holding currents. The mean voltage error was thus estimated to lie in the range of 0.3–0.7 mV, which is generally considered acceptable. Furthermore, the series resistance values for the day and night phase were similar (Mann-Whitney U test; $P > 0.05$; day: $n = 27$, night: $n = 28$) and cannot explain the day-night difference in reversal potential. Finally, the values for the reversal potential and series resistance were not significantly correlated for day, night, or both taken together (for all correlations; $P > 0.05$).

For focal electrical stimulation a tungsten bipolar electrode (Fredrick Haer, Bowdoinham, ME) 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) (Pennartz et al. 1998b) clearly exhibited a circadian rhythm in firing rate (Fig. 4C), 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 (Schaap et al. 1999). 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 (de Jeu et al. 1998). Membrane rupture resulted in an increment of spike amplitude toward a value of 80–100 mV, similar to the spike amplitude measured with the whole cell patch-clamp technique. All these observations indicate that our measurements were performed from healthy neurons.

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: *N*-methyl-D-aspartate (NMDA) receptor antagonist], 6-nitro-7-sulfamoylbenzo-(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, Basel, 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) was dissolved in ACSF containing D-AP5 (50 μ M), NBQX (5 μ M), and CGP 55845A (1 μ M). 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 (100 μ M ID), and the temperature of the applied solution was kept at 30°C by a PRT-2000 system (all from ALA Scientific Instruments, New York). 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.

RESULTS

Excitatory GABA_A-mediated responses during the night

We activated GABA_A receptors in the SCN by focal electrical stimulation of GABAergic 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 GABA_B-receptor antagonists (D-AP5, 50 μ M; NBQX, 5 μ M; CGP 55845A, 1 μ M, respectively), evoked postsynaptic responses, which were completely and reversibly blocked by 12.5 μ M bicuculline during both day and night (Figs. 1A and 2A). During the day period, electrically evoked GABA_A receptor-mediated responses (Fig. 1A) were hyperpolarizing in 10 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 μ M), NBQX (5 μ M), and CGP 55845A (1 μ M) elicited hyperpo-

larizing responses (Fig. 1B) in 9 of 10 neurons and depolarizing responses in only one neuron.

In contrast, during the night period electrically evoked GABA_A receptor-mediated responses were hyperpolarizing in 7 of 15 and depolarizing in 8 of 15 neurons. In six of eight neurons these depolarizing GABA_A receptor-mediated responses were capable of triggering action potentials and *thus appeared to be excitatory* (Fig. 2A). Similarly, pulse application of GABA during the night period resulted in hyperpolarizing response patterns in 7 of 12 and depolarizing response patterns (Fig. 2B) in 5 of 12 neurons. In these night neurons, the membrane potential was not significantly different between neurons with a depolarizing and hyperpolarizing GABA responses (Mann-Whitney *U* test; all *P* > 0.05; $n_{\text{el.stim.}}$ = 15, $n_{\text{appl.}}$ = 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; n_{day} = 5, n_{night} = 7) and in all these neurons, taken individually, the polarity of the GABA_A-mediated response was identical for both technical approaches, indicating that the 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 μ M; 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. 2B). This *initial* depolarizing response was accompanied by one or several spikes, compatible with an excitatory action of GABA. With respect to the *prolonged* phase of the depolarizations, the cessation of firing can be explained by the

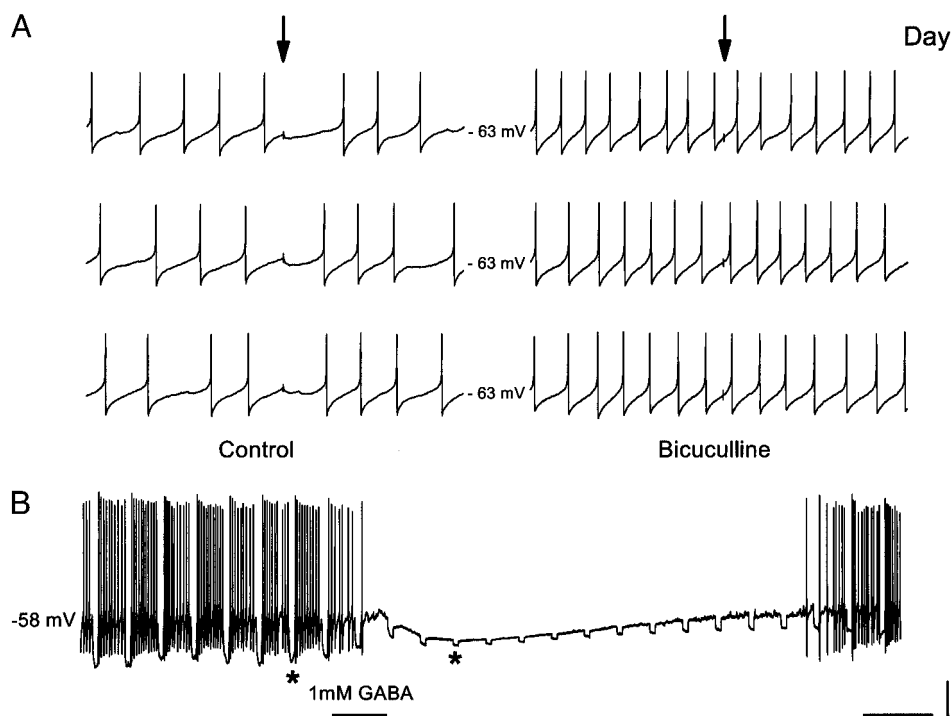


FIG. 1. Activation of GABA_A receptors of a suprachiasmatic nucleus (SCN) neuron during the subjective day period by focal electrical stimulation and by pulse application of GABA. A: example of electrically evoked hyperpolarizing postsynaptic potentials, which were completely blocked by bicuculline (12.5 μ M). \downarrow , electrical stimulus artifacts. B: example of a membrane hyperpolarization evoked by pulse application of GABA (1 mM, 2 s). Rectangular current pulses (200 ms) of -15 pA were applied every 1,200 ms to observe changes in input resistance (*). All of these experiments were performed in the presence of D-2-amino-5-phosphonopentanoic acid (D-AP5, 50 μ M), 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX, 5 μ M), and CGP 55845A (1 μ M). Scale bars: 40 mV, 600 ms (A); 20 mV, 2,500 ms (B).

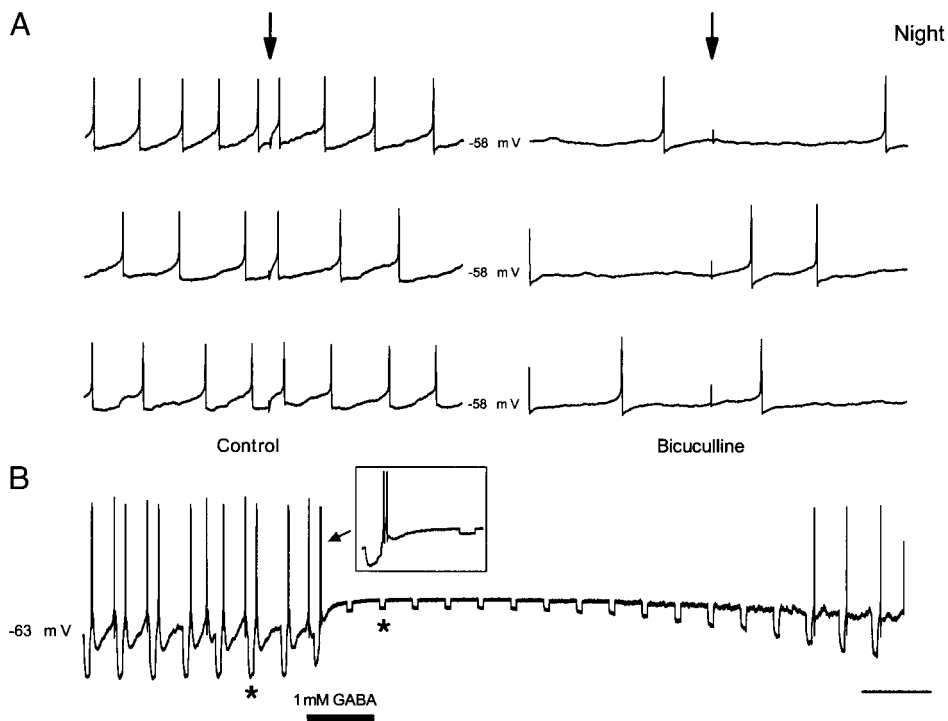


FIG. 2. Activation of GABA_A receptors of an SCN neuron during the subjective night period by focal electrical stimulation and by pulse application of GABA. *A*: example of electrically evoked depolarizing postsynaptic potentials that were capable of triggering an action potential. The depolarizing postsynaptic potentials and associated action potentials were blocked in the presence of bicuculline (12.5 μ M). Note that this neuron is not representative of the entire population of night neurons because many of them also showed hyperpolarizing GABA responses. \downarrow , the electrical stimulus artifact. *B*: example of a membrane depolarization evoked by pulse application of GABA (1 mM, 2.5 s). Current pulses (200 ms) of -10 pA were applied every 1,200 ms. Note that during the initial stage of the depolarization action potentials were still generated (see inset graph, \leftarrow) but that later stages (associated with a strong decrease in input resistance) were marked by an arrest of firing activity. All of these experiments were performed in the presence of D-AP5 (50 μ M), NBQX (5 μ M), and CGP 55845A (1 μ M). Scale bars: 30 mV, 600 ms (*A*); 20 mV, 2,500 ms (*B*).

occurrence of a massive shunting effect (Torre and Poggio 1978) (Fig. 2*B*, $*$) in conjunction with Na⁺ channel inactivation (see DISCUSSION). 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 (Figs. 2*A* and 4*B*). 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.

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 $\sim 50\%$ 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. 3) and pulse application of 1 mM GABA [both in the presence of D-AP5 (50 μ M), NBQX (5 μ M), and CGP 55845A (1 μ 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 ± 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 ± 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 ± 4 mV ($n = 18$). Pulse application of GABA during the night period

gave a similar result (-57 ± 3 mV, $n = 15$). In both experimental approaches, a significant day-night difference in GABA_A reversal potential was found (Mann-Whitney *U* test; all $P < 0.05$). Normality tests (Shapiro-Wilks' *W* test) suggested a normal distribution of GABA_A reversal potential of SCN neurons during the day ($P > 0.05$), whereas the 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.

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 μ M) blocked virtually all fast spontaneous synaptic inputs recorded in SCN neurons and also affected their SFRs (Fig. 4, *A* and *B*). During the subjective day period, bicuculline increased the SFR of all SCN neurons (mean increment: 2.9 ± 0.8 Hz, mean \pm SE; Wilcoxon's matched-pairs signed-rank test; $P < 0.01$, $n = 10$; Fig. 4*C*), suggesting that GABA_A receptor-mediated postsynaptic potentials inhibited SCN neurons during this period (cf. Gribkoff et al. 1999). 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 on release of a negative rectangular current and are curtailed by I_{AHP} (Debarbieux et al. 1998; Pennartz et al. 1998b).

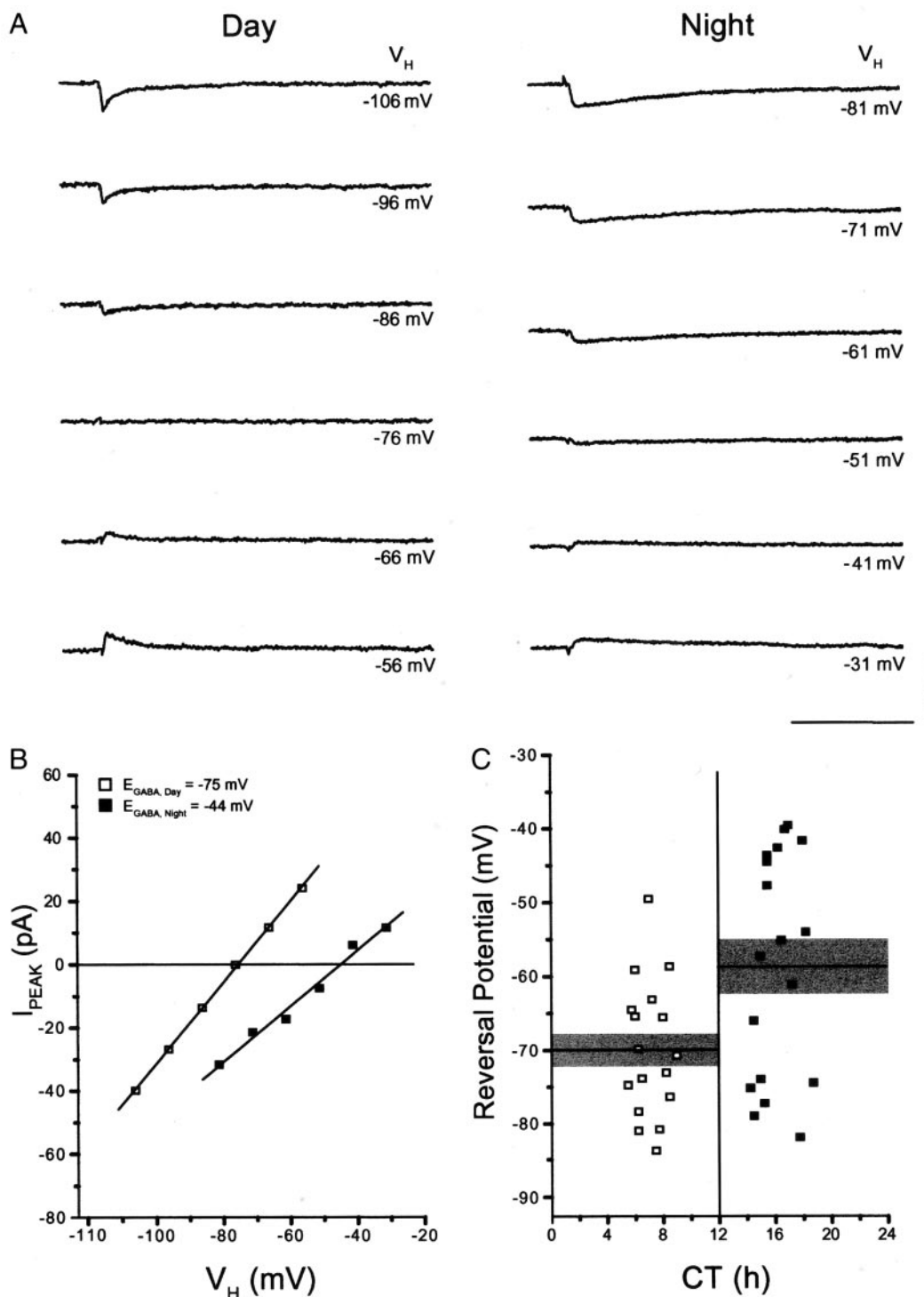


FIG. 3. Reversal potential measurements of GABA_A receptor-mediated currents in SCN neurons during the subjective day and night period. **A:** GABA_A receptor-mediated current responses, evoked by focal electrical stimulation at the ventrolateral border of the SCN, were recorded in voltage-clamp mode 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**. The difference in decay kinetics between (**A**) and (**B**) was not consistently found across the 2 populations studied. 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 and -44 mV in these examples) was taken as the GABA_A reversal potential. **C:** GABA_A reversal potentials (determined by evoked IPSCs) during the subjective day and night period. Reversal potentials are plotted against the circadian time (CT) of recording. Average reversal potentials and SEs are represented by solid horizontal lines and gray bars, respectively. When the total population of night neurons was divided in 2 subsets representing the cells with reversal potentials above ($n = 10$) and below ($n = 8$) the mean, no difference between these subsets was found in the series resistance (Mann-Whitney U test; $P > 0.05$), confirming that this was not a confounding factor. Scale bars: 100 pA, 50 ms (left; **A**); 100 pA, 83 ms (right; **A**).

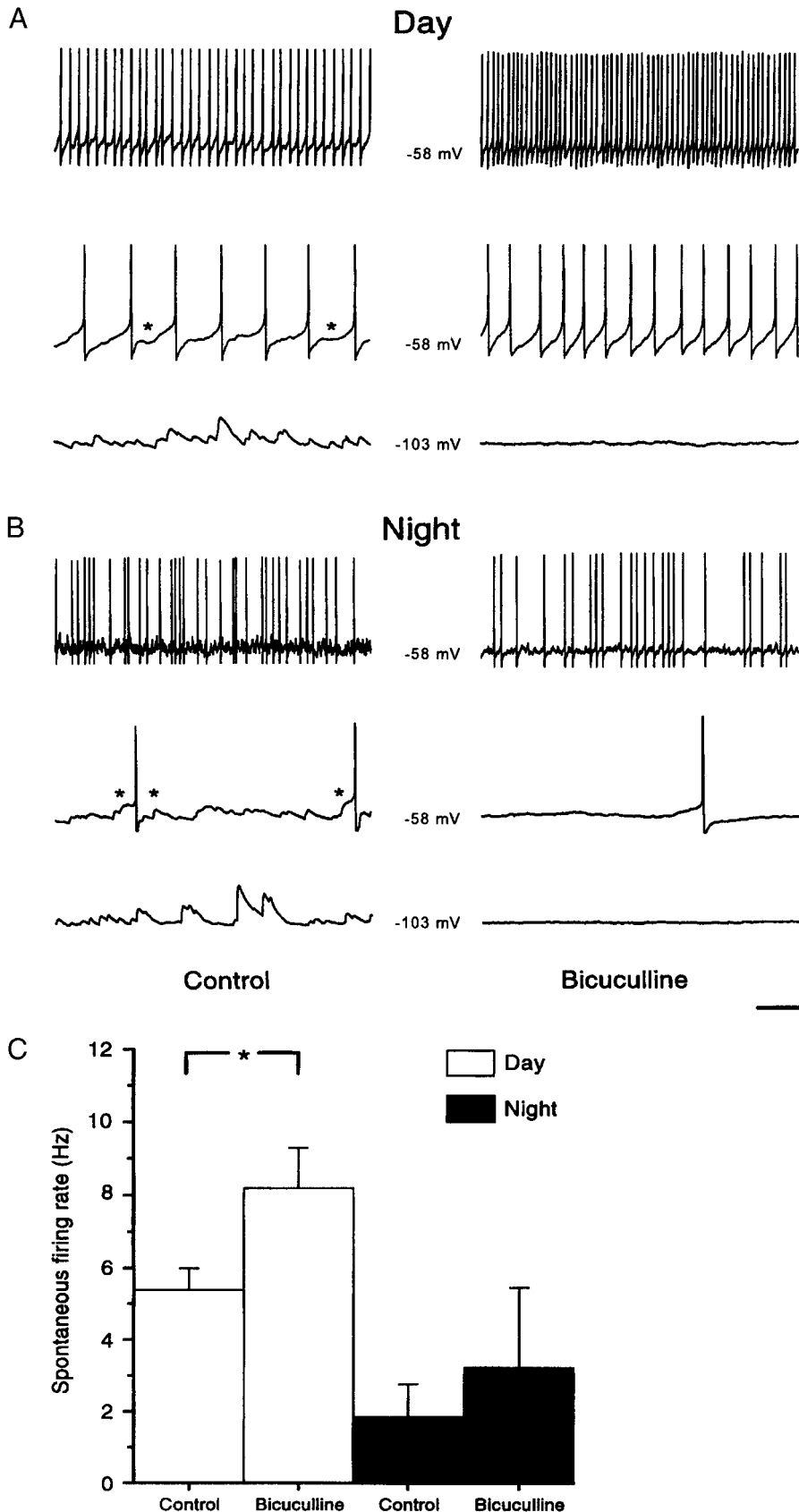


FIG. 4. Effect of GABA_A receptor-mediated postsynaptic inputs on the spontaneous firing rate of SCN neurons. *A*: current-clamp recordings in perforated-patch mode showing the effect of bicuculline (12.5 μ M) on the spontaneous firing behavior and spontaneous postsynaptic potentials of an SCN neuron recorded at circadian time 8 (CT 8; subjective day). *Bottom 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 (*right panel*) 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 μ M) on the spontaneous firing behavior and spontaneous postsynaptic potentials of an SCN neuron recorded at CT 16 (subjective night). *Bottom 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 \pm SE) during their subjective day (CT 4–9, $n = 10$) and night period (CT 14–19, $n = 10$). * $P < 0.01$, 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, $P < 0.01$, $n = 20$) (see also de Jeu et al. 1998). Data from the subjective day phase were also reported in Gribkoff et al. (1999) and are shown here to permit a comparison to the night phase. Scale bars: 30 mV, 2 s (*top*; *A*); 30 mV, 12 s (*top*; *B*); 30 mV, 400 ms (*middle and bottom*; *A, B*).

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. 4C). The bicuculline-induced changes in SFR were significantly larger during the day than during the night (Mann-Whitney U test; $P < 0.01$, $n = 20$). Removal of bicuculline generally resulted in recovery of the SFR to baseline levels. 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 tonic changes in membrane potential, which occasionally occurred (linear regression: $R = 0.31$, $n = 19$). 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.

On closer inspection of the night data, three neurons showed a bicuculline-induced increase in SFR, three neurons showed a bicuculline-induced decrease in SFR (Fig. 4B), and four silent cells did not start firing after the application of bicuculline. This mixture of effects was clearly related to the polarity of the GABA_A-mediated response of these SCN neurons (hyperpolarized or depolarized with respect to rest; Figs. 2 and 4B). Neurons displaying a bicuculline-induced decrease in SFR exhibited spontaneous depolarizing postsynaptic potentials that were capable of triggering spikes at rest (e.g., Fig. 4B, *). 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 our results on electrically evoked GABA_A-mediated responses and GABA_A-mediated reversal potentials during the night.

Alterations of GABA_A-mediated response during long-lasting activation

We decided to test SCN responses to long-lasting (>10 s) GABA pulses 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 (Adams and Brown 1975; Huguenard and Alger 1986). In voltage-clamp mode ($V_H = -103$ mV), the GABA_A receptor-mediated inward current decreased in all cells ($n = 13$) already during the initial phase of such long-lasting pulses (>10 s; e.g., Fig. 5C). 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, A 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. 5B); if not, it can be ascribed to desensitization of GABA_A receptor/channels (Fig. 5A). To ensure the stability of our system, we always executed a third protocol (which was identical to the first protocol) 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 of 13; e.g., Fig. 5A), whereas a shift in GABA_A reversal potential was found in 85% of the neurons (10 of 13; e.g., Fig. 5B; note that some cells exhibited both phenomena). 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 with 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, C and D, $n = 4$). This result suggests that the initial decline in I_{GABA} was primarily caused by a shift in reversal potential (cf. Huguenard and Alger 1986).

DISCUSSION

Our data indicate that the GABA_A reversal potential, and thus probably the $[\text{Cl}^-]_i$, is subjected to a circadian modulation in a subpopulation of SCN neurons (Fig. 3C), 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. In the night phase, the reversal potentials were not normally distributed, in agreement with the functional heterogeneity of GABA effects observed in current clamp mode. However, considering the widespread distribution of values, our data do not permit to decide whether there exist one, two or more subpopulations in the night phase. Post hoc staining of recorded cells was not possible because the perforated membrane under the pipette tip could not be ruptured to allow cell labeling with a marker substance (cf. Pennartz et al. 1998a,b). 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 as indicated by bicuculline effects. In contrast, no significant effect of bicuculline was found during the night (Fig. 4C). During the day, electrically evoked or spontaneous 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 (Figs. 1, 2, and 4). 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. Although the results obtained with exogenous GABA are liable to criticisms set out below, they were compatible with the results on electrically evoked or spontaneous GABA potentials, in that they revealed

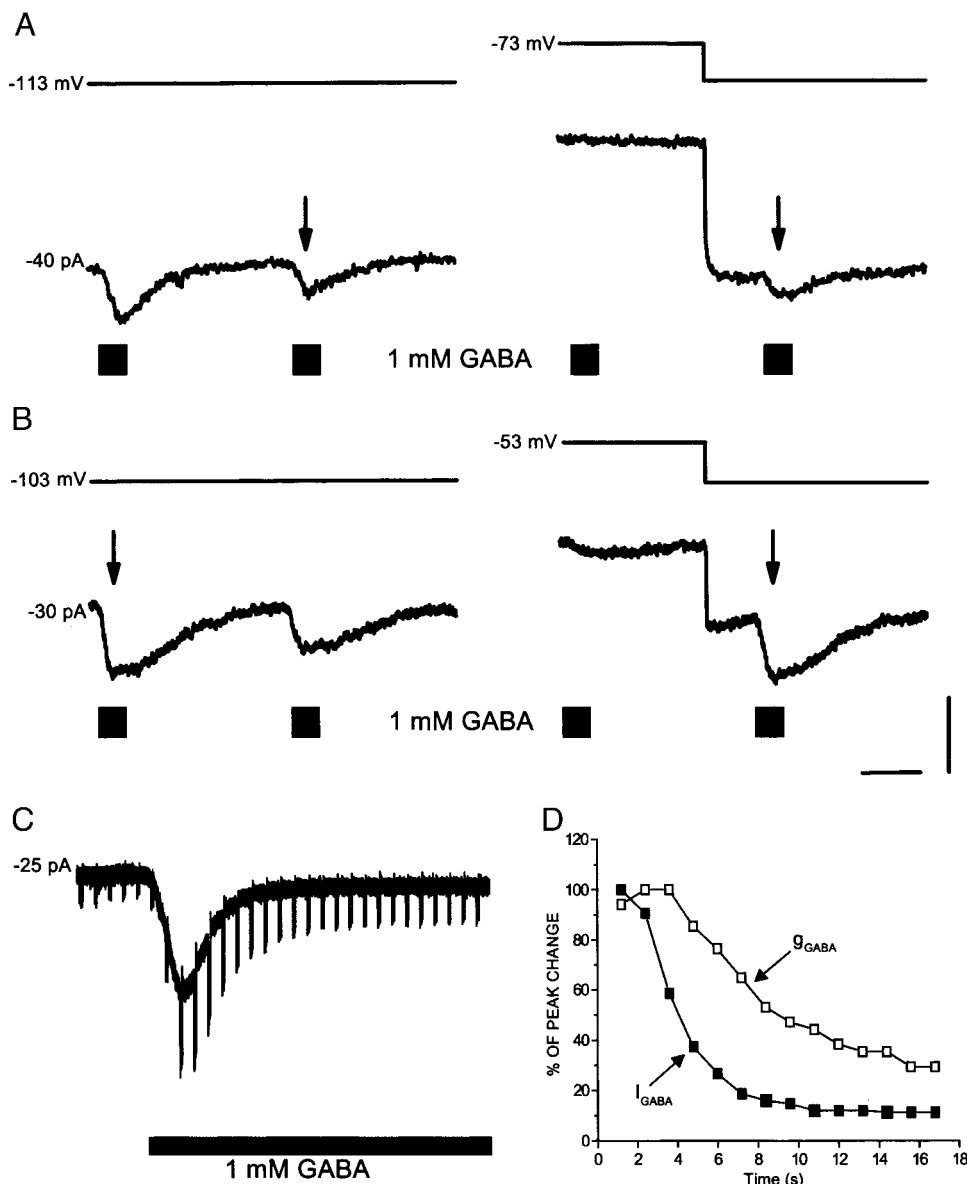


FIG. 5. Reversal potential shift and desensitization of GABA_A receptor/channels in SCN neurons. **A** and **B**: voltage-clamp recordings showing GABA_A receptor-mediated current responses using double-pulse application of GABA (1 mM; pulse duration: 2 s, pulse interval: 15 s). Two different voltage command protocols were used to examine reversal potential shifts and/or desensitization of the GABA_A receptor/channels. First, two GABA_A 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_A receptor-mediated current responses were evoked in a protocol where the holding potential during the first GABA pulse was approximately kept at the GABA_A reversal potential and at the same holding potential of the previous protocol during the second pulse. **A**: attenuation of the 2nd GABA response could not be prevented by holding the potential at the reversal potential during the first GABA pulse (\downarrow); this result indicates that the GABA_A-receptor/channels were subject to a desensitization process. **B**: in another cell, the attenuation of the 2nd current response could be largely prevented by holding the potential close to the reversal potential during the 1st GABA pulse (\downarrow), indicating that the attenuation of the second GABA pulse response was caused by a shift in reversal potential. **C**: recording of a GABA_A 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 1,200 ms. **D**: relative changes in GABA_A receptor-mediated current (I_{GABA} , \blacksquare) and in GABA_A receptor-mediated conductance (g_{GABA} , \square) of the response shown in **C**. $g_{GABA} = (\Delta I_{GABA} - \Delta I_{BASELINE})/V_S$; where ΔI_{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**).

both de- and hyperpolarizing GABA_A receptor-mediated responses during the night phase.

In a previous study, Gribkoff et al. (1999) concluded that GABA assumes a pronounced inhibitory role in SCN during the subjective day. Our results confirm this conclusion because we found GABA to hyperpolarize SCN neurons and inhibit firing during the same circadian phase. Furthermore, the multi-unit and cell-attached recordings in Gribkoff et al. (1999) showed a uniform cessation of firing induced by application of exogenous GABA or the GABA_A agonist muscimol, regardless of circadian phase. At first glance, the excitatory, night-restricted effect of GABA reported here would appear to contradict this result. However, in contrast to perforated-patch recordings, GABA-induced changes in membrane potential and input resistance cannot be assessed in multi-unit and cell-attached recordings. As further explained below, the possibility that the inhibitory effects of GABA observed during the night were caused by massive shunting cannot be ruled out with these techniques. A further difference between the results of

Gribkoff et al. (1999) and the present results is that GABA_A antagonists produced a significant excitation in multi-unit activity during both night and day, whereas in our perforated-patch recordings, a significant elevation of firing rate was found during the day but not night. A likely explanation of this difference is that multi-unit recordings evaluate population effects in which changes from many individual cells are lumped together, whereas our recordings reveal a large biological variability in a comparatively small sample of neurons. The overall population effect of GABA_A receptor/channel blockade, causing excitation in some individual cells and inhibition in others, may well be a net increase in firing (cf. Fig. 4C).

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). An important source of discrepancy could be the use of long-lasting GABA applications (>10 s) (Wagner et al. 1997; but

also Bos and Mirmiran 1993; Gribkoff et al. 1999; Liou and Albers 1990; Liou et al. 1990; Liu and Reppert 2000). Long-lasting GABA applications appear to cause shifting of the reversal potential and/or desensitization of GABA_A receptor/channels (Fig. 5), 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 and did not intend to replicate the experiments of Wagner et al. (1997), 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 (Torre and Poggio 1978) (Figs. 1*B* and 2*B*, *), which further complicates the interpretation of results, in particular because it partially occludes the excitatory effect of GABA at night. In contrast to the excitatory effects of short-lasting, electrically stimulated release of GABA (Fig. 2*A*), longer-lasting pulses of exogenous GABA resulted in an arrest of firing activity after the initial stage of the depolarization (Fig. 2*B*). In brief, such a shunting effect involves the massive opening of GABA_A receptor Cl⁻ channels, resulting in a strongly increased conductance and accompanying depolarizing GABA current. This results in an effective "clamping" of the membrane at the Cl⁻ reversal potential. Should any spike occur in this situation, such massive depolarizing shunting will curtail the ensuing spike repolarization, preventing Na⁺ channels from becoming deinactivated. This effect is essentially the same as the well-known depolarizing spike block observed during long-lasting application of conventional excitatory neurotransmitters like glutamate. Such massive shunting may explain why several extracellular recording studies found uniformly inhibitory GABA effects, regardless of circadian phase (Bos and Mirmiran 1993; Gribkoff et al. 1999; Liou and Albers 1990; Liou et al. 1990; Liu and Reppert 2000) (cf. Fig. 2*B*). Functional interpretations of bicuculline effects (Fig. 4), electrically evoked GABA_A receptor-mediated potentials/currents (Figs. 1*A*, 2*A*, and 3*A*), and the initial effect of exogenous GABA pulses (Figs. 1*B* and 2*B*) are much less confounded by these factors. A further discrepancy between our study and that of Wagner et al. (1997) concerns the day-night difference in GABA_A reversal potential. Wagner et al. (1997) 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 SD in membrane potential in whole cell recordings (Wagner et al. 1997) may be confounded by the fact that this parameter can be influenced by intrinsic ionic currents (e.g., a slow low-threshold component of Na⁺ current) (Pennartz et al. 1997). Direct measurement of the reversal potential of GABA_A receptor-mediated postsynaptic currents (Fig. 3) 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 ~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 (Colwell and Menaker 1992; Groos and Mason 1980; Liou et al. 1986; Moore 1973). 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. 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. Thus the GABAergic network may behave 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 with day (Meijer et al. 1998). Propagation of light information throughout the SCN was also indicated by spatiotemporal SCN profiles of clock gene expression (e.g., *mper1*) and activity markers (e.g., *c-fos*) (Albrecht et al. 1997).

To induce a phase shift, a perturbation of the molecular clock is necessary (Leloup and Goldbeter 1998; Olde-Schepers et al. 1999). 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 Ca²⁺ levels by opening voltage-activated Ca²⁺ channels (Obrietan and van den Pol 1995). Subsequently, activation of Ca²⁺-dependent signal transduction pathways (e.g., involving ERK/MAPK and CREB) (Gillette 1997; Ginty et al. 1993; Obrietan et al. 1998) may cause a resetting of the molecular clock. In a similar way, the GABAergic network may synchronize the "clock cells" of the SCN (Shigeyoshi et al. 1997; Yan et al. 1999). Recently, Liu and Reppert (2000) 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 (Cherubini et al. 1991; Rivera et al. 1999). 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 (Miles 1999).

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