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A Reexamination of the Role of GABA in the Mammalian Suprachiasmatic Nucleus

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Abstract Three independent electrophysiological approaches in hypothalamic slices were used to test the hypothesis that γ-amino butyric acid $(GABA)_A$ receptor activation excites suprachiasmatic nucleus (SCN) neurons during the subjective day, consistent with a recent report. First, multiple-unit recordings during either the subjective day or night showed that GABA or muscimol inhibited firing activity of the SCN population in a dose-dependent manner. Second, cell-attached recordings during the subjective day demonstrated an inhibitory effect of bath- or microapplied GABA on action currents of single SCN neurons. Third, gramicidin perforated-patch recordings showed that bicuculline increased the spontaneous firing rate during the subjective day. Therefore, electrophysiological data obtained by three different experimental methods provide evidence that GABA is inhibitory rather than excitatory during the subjective day.

Key words circadian, $GABA_A$, hypothalamus, muscimol, bicuculline

INTRODUCTION

The neurotransmitter γ-amino butyric acid (GABA) is present in most neurons and terminals within the mammalian suprachiasmatic nucleus (SCN) (van den Pol, 1986), a hypothalamic nucleus known to regulate behavioral and homeostatic circadian rhythmicity. Several studies have shown fast synaptic events in SCN neurons mediated by $GABA_A$ receptors (Kim and Dudek, 1992; Jiang et al., 1997), and recent electrophysiological experiments have provided evidence that SCN neurons are interconnected by GABAergic synapses (Strecker et al., 1997). These and other data suggest an important role for GABA in neural integration within the SCN (Strecker et al., 1997). Recently, a study by Wagner and colleagues (1997) presented evidence that GABA acts as an inhibitory neurotransmitter (its usual role in the adult central nervous system) during the subjective night (when firing rate is low) and switches to an excitatory neurotransmitter during the subjective day (when firing rate normally is high). Those authors suggested that synaptic activation of $GABA_A$ receptors might act in a novel way to amplify and synchronize the well-known endogenous circadian rhythm in firing rate of SCN neurons (as discussed further by Colwell, 1997). To test this hypothesis, our three laboratories independently conducted electrophysiological experiments to determine the actions of GABA in the SCN. Our data, however, support the hypothesis that GABA is predominantly inhibitory—not excitatory—during the subjective day.

METHODS

Multiple-Unit Recordings

Male Long-Evans hooded rats (Harlan, Indianapolis, IN) were housed with an ambient 12-h light, 12-h dark cycle. The rats were euthanized by decapitation

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between Circadian Time (CT) 2.0 and CT 4.5, and the brain was rapidly dissected from the skull. A block of tissue containing the hypothalamus was manually dissected from the brain and transferred to a manual chopper, where coronal hypothalamic slices (500 μm in thickness) containing the SCN were prepared. Slices were placed in a Haas-type brain slice chamber (Medical Systems, Greenvale, NY) (Haas et al., 1979) and continuously superfused with artificial cerebrospinal fluid (ACSF) containing 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 116.3 mM NaCl, 1.0 mM NaH_2PO_4, 24.6 mM glucose, 26.2 mM $NaHCO_3$, and 5 mg/l gentamycin sulphate and warmed to 37°C (pH 7.4, gassed with 95% O_2 and 5% CO_2). Neurons in the SCN slices remained viable for more than 72 h under these conditions, although most experiments were terminated at the end of the second day following slice preparation. To record the rhythm of SCN electrical activity, a 76 μm-diameter, Teflon-coated, platinum-iridium wire electrode was lowered into the SCN using MM33 mechanical manipulators (Stoelting, Wood Dale, IL) (Bouskila and Dudek, 1993). The electrical activity was amplified, and the number of electrical events was counted with a window discriminator (Fintronics, New Haven, CT; Cambridge Electronic Design, Cambridge, UK) and collected by a computer (Data Wave Technologies, Longmont, CO; Spike2 software, Cambridge Electronic Design). Occasionally, raw data were simultaneously collected for display (see Fig. 1) using Axotape software (Axon Instruments, Foster City, CA). The magnitude of changes in spontaneous electrical activity with drug treatment during the subjective day and night were determined by calculating the average electrical activity in successive 5-min intervals during drug application. These were then compared to the average electrical activity in the 5-min period prior to drug application. Maximum effects are reported relative to the effects of their corresponding vehicle, determined in separate slices. Slices were not used if their viability was compromised at any point in the experiment. Viability was confirmed by the presence of a peak discharge rate on Day 2 at least 70% of that recorded or observed on Day 1 and the presence of a firing rate during the subjective night between Days 2 and 3 at least 50% of that recorded during the subjective night between Days 1 and 2. The putative inhibitory neurotransmitter γ-amino-*N*-butyric acid (GABA) and the $GABA_A$ receptor antagonist (–)-bicuculline methiodide were purchased from Sigma (St. Louis, MO) and were dissolved directly in ACSF. The $GABA_A$ receptor agonist muscimol HBr was purchased from Research Biochemicals (Natick, MA) and dissolved directly in ACSF. Picrotoxin was purchased from Research Biochemicals, and a stock solution was prepared in ethanol; the final experimental vehicle concentration was 0.1% ethanol.

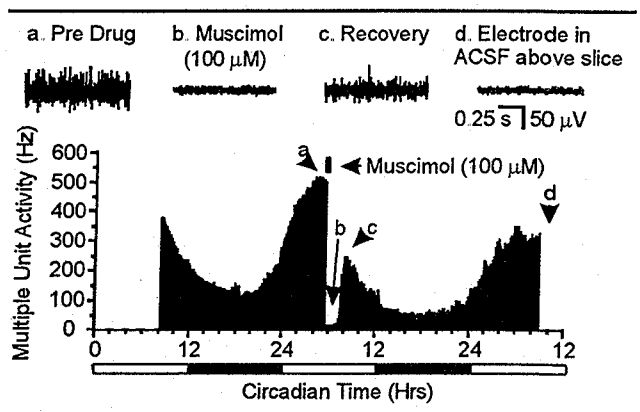

Figure 1. Inhibitory effect of muscimol on multiple-unit activity. Averaged discharge rate from an SCN slice is shown over a 2-day period. Bath application of the γ-amino butyric acid $(GABA)_A$ agonist muscimol (100 μM) during the subjective day (CTs 5.50-5.75) produced profound and reversible inhibition. Similar results were obtained with GABA, which was less potent. Examples of multiple-unit recordings obtained at the indicated times are shown above the graph (recorded at CT 5.42 (a), CT 5.83 (b), and CT 7.83 (c). ACSF = artificial cerebrospinal fluid.

Cell-Attached Recordings

Sprague-Dawley rats (Harlan) were housed under a 12-h light, 12-h dark cycle, and recordings were made during the light phase between CT 5.75 and CT 11.75. Coronal slices (175-225 μm) of hypothalamus were cut on a Vibratome and maintained in ACSF containing 125 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, and 10 mM glucose, bubbled with 5% CO_2 and 95% O_2 (pH 7.4) at 30 to 32°C. Recordings were made either at room temperature (20-22°C) or at 30 to 32°C on rats ages 21 to 47 days. Patch pipettes had tip diameters of 1 to 2 μm for both recording and drug microapplication (3-5 MΩ). Recording pipettes contained 140 mM K-gluconate or KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaCl, 5 mM EGTA, 10 mM HEPES, and 2 mM Mg-ATP. Experiments also were conducted with pipettes filled with ACSF or with a solution containing 140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose. GABA (100 μM dissolved in ACSF) was applied by Picospritzer (General Valve, Fairfield, NJ) onto the recorded cell. Pulse duration was approximately 100 ms, although sometimes it was adjusted depending on the amplitude of the response.

Gramicidin Perforated-Patch Recordings

Male Wistar rats (180-300 g) were kept in a 12-h light, 12-h dark regime, and slices were prepared during the day (Pennartz et al., 1997, 1998). Coronal hypothalamic slices (200 μm) containing the SCN were cut on a Vibroslicer (Campden, London) and were transferred to the recording chamber after at least 45 min at 21 to 24°C. Slices were submerged and superfused at 1.5 to 2.5 ml/min with oxygenated (95% O_2/5% CO_2) ACSF containing 124 mM NaCl, 3.5 mM KCl, 26.2 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose (pH 7.3, 31-33°C). Most recordings were made from the dorsomedial region of the SCN. Measurements during the subjective day were restricted to CT 4 to CT 9 (4-9 h after lights on), and those during the subjective night were restricted to CT 13 to CT 19 (lights off at CT 12). Patch pipette tips contained 135 mM K-gluconate, 10 mM KCl, 10 mM HEPES, and 0.5 mM EGTA (pH 7.3, osmolality 270-280 mOsm, 4-8 MΩ). The remainder of the pipette was backfilled with this same medium supplemented with 5 mg/ml gramicidin. Gigaseals (3-30 GΩ) were formed under visual control. Maximal perforation was achieved 10 to 30 min after gigaseal formation. Membrane integrity was monitored by regular examination of the series resistance estimated from the instantaneous current response to a voltage step (–20 mV, 5 ms) (Kyrozis and Reichling, 1995). 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. Series resistance was 78 ± 4 MΩ (mean ± *SEM*, n = 39). 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 to 3 mV (Armstrong and Gilly, 1992). Series resistances were approximately equal for the day and night phases of recording. Current and voltage traces were acquired using an Axopatch 1D amplifier with pClamp 6.02 and Axotape software (Axon Instruments).

RESULTS

Multiple-Unit Recordings

Using multiple-unit extracellular recording (Bouskila and Dudek, 1993; Liu et al., 1997; Gribkoff et al., 1998), application of GABA (10 μM-10 mM, n = 33 slices) or the $GABA_A$ receptor agonist muscimol (10 nM-100 μM, n = 31) (Fig. 1) produced consistent and concentration-dependent inhibition of cell firing in rat SCN slices when applied during the subjective night (near CT 18) or day (near CT 6). Consistent with this result, the $GABA_A$ receptor antagonists bicuculline (25 μM, n = 10) and picrotoxin (25 μM, n = 9) produced significant excitation during the subjective day and night.

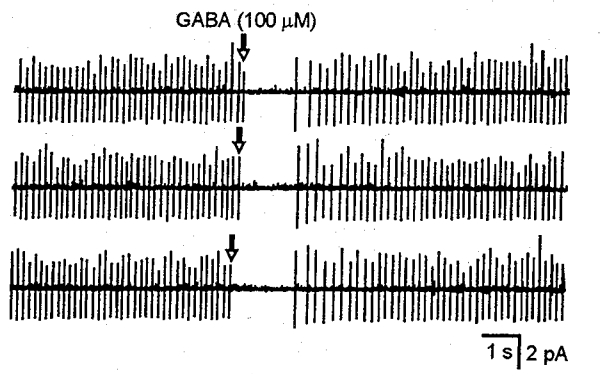

Figure 2. Inhibitory effect of γ-amino butyric acid (GABA) on action currents recorded in the cell-attached mode with a patch pipette. Spontaneous firing was inhibited by brief, focal applications of GABA. Extracellular cell-attached patch recording shows transient currents reflecting action potential activity in an SCN neuron recorded between CT 7.5 and CT 8.0. GABA (100 μM) was applied approximately every 15 sec by 75-ms-long pressure pulses through a second pipette, resulting in a transient inhibition of firing. Consecutive 15-sec records are shown. Sustained bath application of GABA (100 μM) inhibited firing completely in this cell (data not shown).

Cell-Attached Recordings

Cell-attached patch recordings of firing rate from rat SCN neurons were made during the subjective day (CTs 5.75-11.75). Brief pressure-induced microapplications of GABA (100 μM) reversibly inhibited 44 of 46 spontaneously firing SCN neurons (Fig. 2), had no effect on 8 nonfiring cells, and resulted in transient excitation or excitation followed by inhibition in only 2 SCN neurons. About half of the cells were recorded in whole-cell mode to confirm viability after the on-cell experiments. Cells with resting potentials less negative than –40 mV were not included in these data. The average resting potential of the neurons was –47 mV and did not differ between spontaneously firing and nonfiring cells. No relation was apparent between resting potential and tendency to be inhibited by GABA.

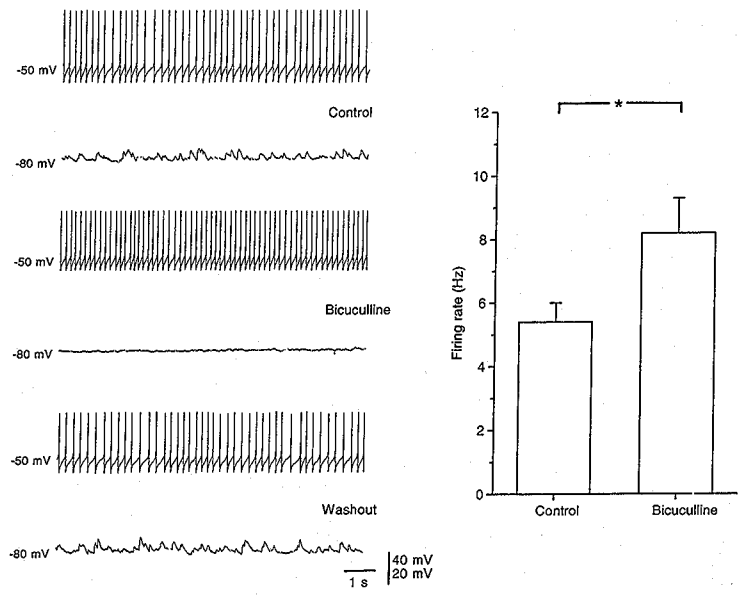

Figure 3. Excitatory effect of bicuculline on the spontaneous firing rate in the perforated-patch mode. The traces on the left show the effect of bicuculline (12.5 μM) on the spontaneous firing behavior and postsynaptic potentials of an SCN neuron recorded at CT 9. Lower records (–80 mV) were obtained by injecting a tonic hyperpolarizing current (40- and 20-mV scale bar values refer to upper and lower records, respectively). The histogram on the right shows the effect of bicuculline (12.5 μM) on the spontaneous firing rate of SCN neurons (means ± *SEM*) recorded during the subjective day (CTs 4-9, $N = 10$). Under control conditions, the spontaneous firing rates in this preparation were significantly different between day and night (Mann-Whitney U test, $p < .01$, $N = 20$). *$p < .01$, Wilcoxon's match pairs signed-rank test.

The average firing rate of spontaneously active cells was 5.6 Hz.

Gramicidin Perforated-Patch Recordings

Perforated-patch recordings with gramicidin, which produces Cl^--impermeant membrane perforations (Kyrozis and Reichling, 1995), were used to obtain intracellular recordings without altering the internal Cl^- concentration and, therefore, without affecting the polarity of the $GABA_A$ receptor-mediated responses. Series resistance was monitored to confirm membrane integrity. Perforated-patch recordings from rat SCN neurons during the subjective day (CTs 4-9) indicated that, as expected, the spontaneous firing rate was high (5.9 ± 0.5 Hz). Bicuculline (12.5 μM) induced a significant and reversible increase in firing rate at resting potential (mean increase in firing rate = 2.8 ± 0.8 Hz, $n = 10$) and blocked depolarizing postsynaptic potentials when cells were current-clamped near –80 mV (Fig. 3). During the subjective night, bicuculline also induced a net increase in firing rate, as seen during the daytime, although the responses of individual cells were more varied. This disinhibition of firing by bicuculline was similar to that observed using long-term multiple-unit recording and indicates that $GABA_A$ receptor activation by endogenous GABA during the subjective day attenuated rather than enhanced spontaneous firing.

DISCUSSION

Our findings, from three independent laboratories using three appropriate but different recording techniques, show a pronounced inhibitory role for GABA during the subjective day in the SCN. In other studies, GABA responses have been shown to shift in magnitude and polarity, and physiological and developmental factors have been identified that determine whether GABA is depolarizing or hyperpolarizing (Chen et al., 1996; Cherubini et al., 1991; Staley et al., 1995). Previous studies in the SCN have reported conditions where GABA occasionally can be excitatory (Liou and Albers, 1990) or depolarizing (Zidichouski et al., 1995, 1997), but no significant day-night difference in response to GABA has been observed previously (Liou and Albers, 1990). It could be argued that high concentrations of GABA or muscimol could shunt the firing of SCN neurons even during a hypo-

thetical depolarization, but inhibitory effects consistently were seen with low agonist concentrations (including a complete concentration range from subthreshold to supramaximal in the multiple-unit studies), similar to concentrations reported by Wagner and colleagues (1997). Neither would this explain the consistent excitation observed during the subjective day and night to application of $GABA_A$ antagonists. In the multiple-unit studies, we have continuously recorded from the same population of SCN neurons over at least a complete circadian cycle, guaranteeing that the same neurons were participating in the experimental manipulations at the two time points. Another concern might be that our slices were somehow "unhealthy," but we consistently have recorded from SCN neurons for many hours and even up to several days (Bouskila and Dudek, 1993; Gribkoff et al., 1998). Further-more, patch-clamp recordings in the perforated-patch mode showed that the recorded neurons were healthy in terms of spike amplitude, membrane potential, and input resistance and that they had a significant circadian rhythm in firing rate (see also De Jeu et al., 1998). Additional research is needed to understand the specific factors that can produce the differences between our results and those of Wagner and colleagues (1997). Nevertheless, these data from three laboratories demonstrate that the most consistent and robust effect of $GABA_A$ receptor activation throughout the diurnal cycle is inhibition.

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