GABA Receptor-Mediated Inhibition of Neuronal Activity in Rat SCN
In Vitro: Pharmacology and Influence of Circadian Phase

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GABA receptor-mediated inhibition of neuronal activity in rat SCN in vitro: pharmacology and influence of circadian phase. J Neurophysiol 90: 1438–1448, 2003. First published May 15, 2003; 10.1152/jn.01082.2002. The effect of γ-aminobutyric acid (GABA) on neuronal firing rate in rat suprachiasmatic nucleus (SCN) slices was examined using continuous recording methods. GABA inhibited neuronal discharge during both the subjective day and the subjective night in a concentration-dependent manner characterized by two apparent affinity states. The GABA_A receptor agonist muscimol caused potent inhibition regardless of circadian time; repeated applications of the agonist did not reverse the direction of effect. The GABA_A receptor antagonists bicuculline and picrotoxin increased excitability when applied during either subjective day or subjective night. A significant increase in GABA_A receptor–mediated inhibition, as well as endogenous GABAergic tone, was observed on the second day after slice preparation. The GABA_B receptor agonist baclofen inhibited cell firing during subjective day and night, but the GABA_B antagonist phaclofen had no significant effect. These data provide additional strong support for a predominantly inhibitory role of GABA in the rat SCN, regardless of the time of application in relation to the circadian rhythm, and demonstrate an important level of plasticity of this system in vitro.

INTRODUCTION

The neurons of the mammalian suprachiasmatic nucleus discharge in a circadian pattern linked to the light/dark cycle, entraining circadian behaviors and homeostatic mechanisms (Ralph et al. 1990; Rusak and Zucker 1979; van den Pol and Dudek 1993). The neurons of the suprachiasmatic nucleus (SCN) maintain a circadian discharge rhythm in vivo and in vitro in the absence of external entraining stimuli such as light (Bouskila and Dudek 1993; Gillette and Reppert 1987; Gribkoff et al. 1998; Miche and Colwell 2001). Individual dissociated SCN neurons, and even immortalized SCN cell lines, also display intrinsic circadian rhythms (Earnest et al. 1999; Liu and Reppert 2000; Shirakawa et al. 2000; Welsh et al. 1995). These data suggest that intrinsic neuronal properties are responsible for the ability to discharge rhythmically, and that synchronized rhythms are maintained for long periods in vitro. Recently several genetic components of the mammalian intrinsic clock mechanism were identified based on homology with clock genes in simpler systems (Baey et al. 2001; Chang and Reppert 2001; Dunlap 1999; Herzog et al. 1998; Panda et al. 2002). Little is known, however, about the mechanisms whereby clock genes ultimately determine circadian rhythm-dependent changes in neuronal firing rates. Conceivably, these changes could result from changes in the expression of ion channels and transporters, or changes in the degree or nature of local circuit interactions, among many possibilities. The identification of contributors to circadian rhythm generation is a key question in circadian rhythm research.

Neurotransmitters may shape the response of SCN neurons to timing cues, modulate intrinsic clock properties, alter neuronal synchrony, or even contribute to rhythm generation. The primary extrinsic regulator of the precise timing of these rhythms in vivo is the influence of the retina-hypothalamic tract transmitting visual information to the SCN, a pathway that releases glutamate, pituitary adenylate cyclase activating polypeptide, and substance P as neurotransmitters or neuromodulators (Abe et al. 1996; De Vries et al. 1993; Hannibal et al. 2000; Kim et al. 1999). In addition, several other intranuclear neurotransmitters and peptides can alter the timing of circadian discharge rhythms in the SCN. These include the pineal hormone melatonin, as well as serotonin and neuropeptide Y (Cassone 1998; Ehlen et al. 2001; Gribkoff et al. 1998; Liu et al. 1997). The most abundant transmitter in the SCN, originating primarily within the component neurons of the SCN itself, is γ-aminobutyric acid (GABA) (Bujs et al. 1994; Castel and Moore 2000; Moore and Speh 1993; Moore et al. 2002; O’Hara et al. 1995; van den Pol 1986; van den Pol and Gorcs 1986). The actions of GABA in the SCN were previously investigated, yet there is considerable recent controversy concerning the degree and direction of its regulation of discharge patterns, and hence its role(s), in this important nucleus (De Jeu and Pennartz 2002; Gribkoff et al. 1997; Liu and Reppert 2000; Shimura et al. 2002; Shirakawa et al. 2000; Wagner et al. 1997, 2001).

GABA, acting primarily through interaction with GABA_A and GABA_B receptors, produces neuronal inhibition in most brain regions through membrane hyperpolarization and increased membrane conductance, effectively shutting transmembrane voltage shifts. However, under some circumstances, such as early in development, GABA can be depolarizing and potentially excitatory (Chen et al. 1996; Cherubini et al. 1991; Han et al. 2002; Obrietan and van den Pol 1995; Staley et al.

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In the SCN most cells and a large proportion of synapses are immunopositive for the presence of GABA, and several GABA receptors and receptor subunits are expressed in the SCN (Castel and Morris 2000; Decavel and van den Pol 1990; Gao et al. 1995; Moore and Speh 1993; Naum et al. 2001; O’Hara et al. 1995; Okamura et al. 1989; van den Pol 1993; van den Pol and Gorcs 1986), suggesting that GABA plays an important role in this nucleus.

Initial electrophysiological studies found intrinsic GABA or extrinsic GABA application to be predominately inhibitory (Bos and Mirmiran 1993; Burgoo and Boullant 1998; Liou et al. 1990; Mason et al. 1991; Shibata et al. 1983a,b; Tominaga et al. 1994). In a more recent study of SCN cells in culture, virtually every cell tested was inhibited by GABA or GABA agonists (Liu and Reppert 2000), although this has not been universal (Shirakawa et al. 2000). When the relationship of the effect of GABA and circadian cycle were directly explored using single-cell recording techniques, GABA was found to be predominantly inhibitory during both subjective day and subjective night (Liou and Albers 1990; Liu and Reppert 2000; Mason 1986; Mason et al. 1991). There is evidence that the GABA system is influenced by circadian rhythms, although these appear to be changes in degree rather than direction (Aguilar-Roblero et al. 1993; Cardinali and Golombek 1998; Huhman et al. 1996; Naum et al. 2001; Trachsel et al. 1996). These studies suggested that circadian variations in the GABA system were attributed to changes in GABA level or receptor expression. Several recent studies, however, have suggested that the nature of GABA’s actions in the SCN may be phase-dependent. Specifically, GABA was reported to be inhibitory during subjective night, but excitatory during subjective day by one group (Wagner et al. 1997). This has been attributed to a possible diurnal increase in intracellular chloride (Cl\(^{-}\)) concentration resulting in a shift in the Cl\(^{-}\) reversal potential (Shimura et al. 2002; Wagner et al. 1997, 2001). This required a new appraisal of GABA’s role in circadian regulation, given that these data suggested that GABA released locally in the SCN contributes to the generation of circadian discharge rhythms, or at least significantly influences their phasic amplitudes by dampening activity during subjective night and increasing activity during subjective day. Initial attempts to replicate these findings failed to confirm a circadian difference in GABA’s effects (Gribkoff et al. 1999). Curiously, results of another recent study suggest an opposite circadian phase-dependency for GABA’s effects (inhibitory during subjective day, excitatory during subjective night) (de Jeu and Pennartz 2002). The results of the latter study strongly suggest a technical origin of these contradictory results.

The current study represents an entirely new set of experiments, in relation to a previous short collaborative report dealing with GABA in the SCN (Gribkoff et al. 1999). We examined the effects of GABA, and agonists and antagonists of GABA receptors, to more fully characterize the pharmacology and phase-dependent actions of this important neurotransmitter on the cells of the SCN, and delineate possible contributions to circadian rhythmicity. Specifically, using multunit activity (MUA) recording techniques, we determined the concentration–response relationships of GABAergic agents in the SCN during subjective day and night, examined the effects of repeated applications of GABA agonists, and studied the effects of GABA receptor ligands on the timing and amplitude of circadian discharge rhythms in the SCN of the rat in vitro.

The techniques used in these experiments, although not designed to ascertain response profiles of individual SCN neurons, have distinct advantages over both episodic single-unit and whole cell patch recordings. MUA recording is the only technique that involves recording from the same groups of cells over complete circadian cycles in the relatively intact slice system, and demonstrates directly the response of cells during different components of the circadian rhythm (Bouskila and Dudek 1993; Gribkoff et al. 1998). In addition it is relatively noninvasive and removes any opportunity for unintentional experimenter bias in cell sampling after initial electrode placement; the electrode is placed in the SCN and recording optimized, and then is untouched for the remainder of the experiment (as long as 72 h).

We found that GABA and GABA receptor agonists were inhibitory regardless of phase and GABA\(_A\) antagonists were excitatory regardless of phase. We also found that GABA-mediated inhibition of spontaneous activity in SCN slice preparations increased after long periods of incubation in vitro. Finally, we altered the ionic composition of the bathing medium to match the extracellular concentration of potassium (K\(^{+}\)) in previous studies (Wagner et al. 1997, 2001) to determine the role of this component of the ionic milieu on the regulation of GABA’s effects in the nucleus. We found that it had small but phase-independent effects on the response to GABA receptor activation.

Our data demonstrate that the predominant role for GABA in the SCN is inhibition throughout the circadian cycle, and that this inhibitory influence increases with incubation in vitro.

METHODS

Slice preparation

Male Long-Evans hooded rats (Harlan Sprague Dawley, Indianapolis, IN) were housed in a colony room with an ambient 12:12 light–dark cycle (lights on at 0700 h, lights off at 1900 h) for ≥3 wk before experimentation to ensure that their circadian systems were entrained. The rats were housed in stainless steel wire cages, 5 animals per cage with unrestricted access to food and water. All procedures were in accordance with animal care and use guidelines and were approved by the institutional Animal Care and Use Committee.

After the adaptation period, rats were killed by decapitation between 0900 and 1130 h (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 brain slices (500 μm in thickness) containing the SCN were prepared (1–2 slices/brain). Slices were placed in a Haas-type brain slice chamber (Haas et al. 1979; Harvard Apparatus, Holliston, MA) and continuously superfused with (in most experiments) medium containing (in mM) CaCl\(_2\) 1.8, KCl 5.4, MgSO\(_4\) 0.8, NaCl 116.3, Na\(_2\)HPO\(_4\) 1.0, dextrose 24.6, NaHCO\(_3\) 26.2, and 5 mg/l gentamicin sulfate (Sigma, St. Louis, MO) and warmed to 37°C. In a small number of experiments examining the effects of extracellular K\(^{+}\) concentration on responses to GABA, potassium gluconate was added to the normal incubation medium (0.8 mM) to bring the final K\(^{+}\) concentration to 6.2 mM. Experiments were terminated at the end of the second day after slice preparation. A total of 319 slices from approximately 240 animals were used in this study.
Continuous MUA recording

To record multiunit SCN electrical activity, a 76-μm-diameter, Teflon-coated platinum-iridium wire electrode was lowered under visual inspection into the SCN using MM33 mechanical manipulators (Stoelting, Wood Dale, IL; Bouskila and Dudek 1993). The recording/ incubation chamber and manipulators were mounted on an air floatation table to reduce room vibrations. The electrical activity was amplified, and the number of electrical events was counted with a window discriminator (Cambridge Electronic Design, Cambridge, UK) and collected by a computer (BrainWave software, DataWave Technologies, Longmont, CO; Spike2 software, Cambridge Electronic Design, Cambridge, UK). The average number of discriminated electrical events in successive 1-min periods was determined and plotted against the circadian time of recording. The magnitude of changes in spontaneous electrical activity with drug treatment during subjective day and night were determined by calculating the average electrical activity in successive 5-min recording intervals during drug application. These were then compared with the average electrical activity in the 5-min period before 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 clear circadian discharge rhythm over ≥2 days with a peak discharge rate on day 2 ≥60% of that recorded on day 1. The results of the present experiments (see RESULTS below) suggest that reductions in peak discharge rate on day 2 may not reflect viability as much as an increase in the level of endogenous GABA-mediated inhibition. Nevertheless, all slices used in these experiments involved these criteria to allow for direct comparison, particularly the presence of a circadian discharge rhythm for ≥2 days.

In some experiments the peak of the neuronal activity rhythm on day 2 was determined by fitting several hours of data on either side of CT 6 in the subjective day as described previously (Gribkoff et al. 1998) using KaleidoGraph software (Synergy Software, Reading PA). Although this was not the primary focus of these experiments, the fitted activity peak was used to determine whether the neuronal activity rhythm during subjective day 2 was phase-shifted by prior drug treatment.

Drugs

All drugs used in these experiments were purchased from Sigma (St. Louis, MO). GABA, the GABA_A receptor antagonist (−)-bicuculline methiodide, the GABA_A receptor agonist muscimol HBr, and the GABA_B receptor antagonist phaclofen were dissolved directly in mM stock solution in EtOH; the final experimental vehicle concentration was 0.1% EtOH. The GABA_A receptor agonist (+)-baclofen HCl was prepared as a 5 mM stock solution in distilled (DI) water. The final concentration of baclofen was either 0.2% DI H_2O or 0.8% DI H_2O depending on the concentration of baclofen. Effects of drug solutions were generally expressed as percentage of vehicle control response, as defined above. Where concentration–response relationships were generated, EC_{50} values were estimated using logistic fits of resulting curves using KaleidoGraph software (Synergy Software).

RESULTS

Inhibitory effects of GABA in the SCN

GABA application at any of the tested time points in the diurnal discharge cycle produced a concentration-dependent decrease in neuronal firing rates (Fig. 1, A and B). Although no detailed attempt was made to determine the degree to which longer GABA applications could result in tachyphylaxis, it was observed that inhibitory responses could be maintained for periods in excess of 1 h duringcontinuous GABA application without decrement. GABA-mediated inhibition was reversible even after prolonged administration. Given the much higher average discharge rates observed during the subjective day, when inhibition was expressed as percentage of control discharge rate, the net effect was usually larger in subjective night (Fig. 1B).

The concentration–response curves for GABA applied during subjective day and night were best fit using a 2-site model, although the apparent contribution of the higher affinity site to the concentration–response to GABA was reduced during the subjective day. The estimated EC_{50} values for GABA effects during the subjective day were 44.4 μM for the higher affinity site, and 1.31 mM for the lower affinity site. The EC_{50} values for GABA effects during subjective night were 54.0 μM for the higher affinity site, and 2.31 mM for the low affinity site. Although these values were obtained in the absence of pharmacological manipulation of GABA reuptake, they suggested that at least two GABA receptor subtypes were present and functional in the SCN, and contributed to these responses. In these experimental conditions (ionic composition as described in METHODS for “normal” incubation medium), the only multiunit response to GABA application was inhibition. Repeated application of GABA at a concentration near the apparent EC_{50} within a subjective day resulted in inhibition that was similar in amplitude from application to application (Fig. 2).

GABA receptor pharmacology

AGONISTS. To determine the degree to which different GABA receptors contributed to the inhibitory effects of GABA application, specific agonists for GABA_A and GABA_B receptors, muscimol and baclofen, respectively, were applied at a range of concentrations during subjective day and night. Muscimol application during subjective day or night produced potent and profound inhibition (Figs. 3, A and B). There was no significant difference in EC_{50} estimates (1.4 μM subjective day; 0.76 μM subjective night) between curves generated during subjective day and subjective night. The GABA_B agonist baclofen, however, although equally effective when applied during either subjective day or subjective night (Fig. 4, A and B), had significantly greater potency when applied during the subjective night (EC_{50} 5.2 μM subjective day; 36 nM subjective night).

ANTAGONISTS. Application of the GABA_A receptor antagonist bicuculline (BIC) (Fig. 5A) or picrotoxin (Fig. 5B) always produced increases in neuronal activity when applied to SCN slices at mid-subjective night or mid-subjective day. In some cases, in the presence of the antagonist, the level of discharge during subjective night was almost as great as the peak of the spontaneous activity observed in the previous subjective day. However, application of the antagonist at or near the peak on the following subjective day produced a similar level of increase in the discharge rate (Fig. 5). This suggests that the level of GABA-mediated inhibition, although clearly important in determining the maximum discharge rates at any particular time point in the rhythm, had only a relatively small contribution to the generation of the rhythm per se. Long-term application of either antagonist did not remove circadian rhythm-
FIG. 1. A: two panels display examples of GABA-mediated inhibition of neuronal firing in SCN slices maintained over multiple 24-h cycles. GABA-mediated inhibition was observed during both subjective day (CT 0–12) and subjective night (CT 12–24). Under these conditions response to GABA application was always inhibition. B: concentration–response curves for GABA-mediated inhibition in groups of SCN slices (n ≥ 6 for each concentration, total slices = 72; n = 6 slices for vehicle control), expressed as percentage inhibition of neuronal firing relative to vehicle.

FIG. 2. Repeated application of GABA (5 mM) produced consistent inhibition of neuronal discharge during subjective day. In slices exhibiting even modestly decreased peak firing rates on day 2, such as was seen with this slice, response to GABA was significantly enhanced during second diurnal phase (see text and Fig. 6).
ity. The GABA<sub>B</sub> receptor antagonist phaclofen had no significant effect on basal firing rates at any time point.

Evidence for a time-dependent increase in GABA transmission in SCN in vitro

As discussed above, repeated application of either GABA or the GABA<sub>A</sub> agonist muscimol resulted in consistent inhibition of neuronal firing, as did prolonged application of either compound. However, it was noted during the course of these experiments that the day of application greatly affected the degree of inhibition produced by GABA. In most experiments, experimental compounds were applied either during the subjective night after the day of slice preparation (day 1), or on the following subjective day (day 2). However, in a separate group of experiments, the effect of application during subjective days 1 and 2 were compared, and it was found that responses to GABA agonists were greater during subjective day 2, compared with responses generated on the day of slice preparation. There were very large increases in the responses to both GABA (see Fig. 2) and muscimol (Fig. 6A) during successive subjective days, although the only response to either compound on either day was inhibition.

In many SCN slices, peak spontaneous neuronal activity levels were greater on the day of slice preparation than on subsequent days. We assumed that this reflected a decline in slice viability. However, an alternative explanation could be that lower control firing rates on day 2 reflect higher levels of endogenous tonic GABA neurotransmission, rather than decreased viability. To test this hypothesis, tonic inhibition was removed by a brief (1–2 h) application of 25 μM BIC near CT 1442.
6 on day 1 and day 2. The response to BIC application on both days was excitation. The absolute peak firing levels reached on both days was very similar in the presence of the antagonist, regardless of the amplitude of the pretreatment discharge level (Fig. 6B), suggesting that, although more quiescent on day 2, the neurons were fully capable of firing at a rate comparable to that seen during the previous peak when tonic inhibition was removed. This strongly suggests that at least over this 2-day period neuronal firing rates are increasingly affected by a higher level of tonic GABAergic inhibition. The data for day-dependent inhibition and excitation for muscimol and BIC, respectively, are summarized in Fig. 6C. A modest (r = 0.81) but significant linear correlation was fitted between the ratio of day 1 to day 2 spontaneous firing rate and the ratio of the effect of BIC on day 2 versus day 1 (Fig. 6D).

Phase modulation by GABA receptor antagonists

Although phase-modulation by GABA receptor ligands was not a focus of this study, the effects of some of the pharmacological manipulations in this study on phase timing were evaluated. The time of drug application was CT 18–19 for antagonists and CT 5.5–6.5 for agonists, and the time of peak discharge rate on day 2 was recorded. The results are summarized in Fig. 7. Significant phase shifts were observed only when either a combination of GABA antagonists or picrotoxin was applied at CT 18–19 on the subjective night after slice preparation. These data confirm that endogenous GABA has an influence on the timing of rhythms.

Effects of altering the concentration of extracellular K+ on GABA inhibition

We performed experiments to determine whether changes in ionic conditions could affect the direction of GABA-mediated discharge modulation. In particular, the extracellular concentration of K+ used by Wagner and colleagues (1997, 2001) was 6.2 mM compared with our usual K+ concentration of 5.4 mM. In this modified medium, the predominant effect of GABA application in our preparations was inhibition during subjective day and night. However, in 3 slices, a transient excitation was observed early in the period of GABA application, followed by inhibition (Fig. 8). In 2 of these slices, wherein excitation was followed by inhibition during the subjective night, the inhibition was followed by rebound excitation after cessation of drug application. Although interesting, this transient excitation was observed when GABA was applied during either the subjective day or the subjective night, and was not limited to applications during the subjective day.

Discussion

These results both confirm that GABA is an important regulator of SCN function and demonstrate that SCN neurons display significant temporal plasticity in terms of GABAergic tone and responsivity under these experimental conditions. We obtained no evidence from these studies to support the hypothesis of Wagner and colleagues (1997, 2001) and others (de Jeu and Pennartz 2002; Shimura et al. 2002) that GABA has differential actions during subjective day and night.
All of these studies, ours included, used in vitro preparations, either slices or dissociated neurons, all of which have caveats associated with the applicability of their results to the physiology of the SCN in vivo. In the present experiments we used MUA recording in SCN slices. This technique is relatively noninvasive, has no effect on intracellular ionic regulation per se, and is uniquely capable of recording neuronal activity continuously for several days over successive circadian rhythms in the slice preparation (Bouskila and Dudek 1993; Gribkoff et al. 1998, 1999). This technique allowed us to measure the effects of GABA and GABA-receptor subtype-specific ligands on the same populations of SCN neurons over successive phases of the circadian cycle. In all cases, regardless of the time of drug application, GABA, the GABAA agonist muscimol, and the GABA B agonist baclofen produced concentration-dependent inhibition of the discharge of groups of SCN neurons. At maximally effective concentrations of each agonist, the degree of inhibition was profound. These data indicate that effects of exogenous GABA were mediated by more than one receptor, likely a combination of GABAA and GABA B receptors, given the response profiles of the specific receptor agonists. No attempt was made to further pharmacologically characterize the subtype composition of SCN GABA A receptors. The only potentially significant day–night difference in GABAergic pharmacology observed in the study was an increase in baclofen potency during subjective night. The reasons for this are unknown.

These data, because of the use of MUA recording techniques, cannot exclude the possibility that some cells were excited by GABA. Only some form of single-cell recording, usually an episodic technique not capable of monitoring the activity of neurons for the entire circadian cycle, could directly address the question of whether some cells during subjective day or night are excited by GABA. Some previous single-cell studies examined this using episodic recording. Even though the proportion of cells inhibited by GABA and GABA agonists varied from study to study, the majority were inhibited regardless of phase, and where examined the proportions were similar during subjective day and night (Liou and Albers 1990; Mason 1986; Mason et al. 1991). A single-cell recording technique that is nonepisodic is the multielectrode array long-term recording of cultured SCN neurons (Liu and Reppert 2000; Shirakawa et al. 2000; Welsh et al. 1995). Using this technique, all cells were inhibited by GABA and muscimol regardless of circadian time in one study (Liu and Reppert 2000), whereas in another the majority of cells were excited by BIC, indicating that endogenous GABA was inhibitory (Shirakawa et al. 2000). A distribution of GABA sensitivity was also seen in the study by Wagner and colleagues (1997). However, in their study a small majority of cells sampled individually were inhibited during the subjective night (56%), whereas a large majority were excited during the subjective day (71%) by GABA.

In our study the mean effect of GABA and GABA receptor subtype-specific agonist application was always inhibition, and the profound degree of maximal inhibition for GABA (Fig. 1), muscimol (Fig. 3), and baclofen (Fig. 4) left little room for a
FIG. 6. Some decrement in peak neuronal discharge levels was observed in most slices from subjective day 1 (day of slice preparation) to subjective day 2. It was also noted that responses to GABA\textsubscript{A} receptor agonists and antagonists were significantly greater in those slices wherein a decrement was observed. In examples shown agonist muscimol (A) and the antagonist bicuculline (B) produced much greater inhibition and excitation, respectively, when applied near peak of neuronal firing on day 2, when compared with day 1. Level of neuronal activity depicted in presence of bicuculline on day 2 in B is nearly equal to that observed on day 1 in presence of antagonist, despite starting from lower baseline. This is quantified in C, where differences in responses to antagonist (n = 9) and agonist (n = 10) were significantly greater on day 2 (P < 0.05). D: correlation between ratios of effects of antagonist bicuculline on day 2 vs. day 1, and peak spontaneous firing rates on day 1 vs. day 2 before addition of antagonist.
significant proportion of cells to have been excited at any time point. Nevertheless, some cells may have been excited by GABA at either time point. In our experiments inhibition continued for the duration of the longest applications of all of the agonists (H11022 1 h at the highest tested concentrations) and was reversible. The only exception we noted was when extracellular K+ was increased modestly to match that used by Wagner et al. (1997, 2001). The transient excitations seen in a small proportion of slices occurred during either the subjective night or the subjective day. Although we cannot rule out that GABA may have a differential effect in a subset of cells that were not adequately sampled by our extracellular recording techniques, we find this unlikely. The data presented represent group means of recordings of groups of cells, and as such present the response of large numbers of SCN neurons. The neurons discharged in a predictable circadian pattern and remained viable throughout the course of the experiments.

GABA_A receptor antagonism by BIC or picrotoxin resulted in excitation during subjective day or night, supporting the conclusion that endogenous GABA is likewise inhibitory. GABA therefore has a significant inhibitory influence in the nucleus under these recording conditions throughout the circadian cycle. We found no evidence to support the hypothesis that endogenous GABA, by inhibiting at night and exciting during the day, magnified the day–night differences in spontaneous activity that result in circadian rhythms in the SCN. In 2 slices, prolonged application of BIC from CT18 during subjective night to CT6 during subjective day resulted in an overall increase in neuronal excitability, although the shape of the rhythm remained relatively unchanged.

The question of why our data, and the conclusions we have drawn from them, are so different from the results of other recent studies (de Jeu and Pennartz 2002; Wagner et al. 1997) was not directly approached in our study. We did not use standard whole cell or perforated patch-clamp techniques, and therefore do not know whether we would have observed circadian changes in GABA effects under those conditions. Patch-clamp recording is an invasive technique that could alter intracellular chloride levels. In relation to studies wherein gramicidin was used, SCN cells are apparently problematic for perforated patch recordings [as pointed out by de Jeu and Pennartz (2002)]. We therefore suspect that the results of Wagner et al. (1997) and de Jeu and Pennartz (2002), similar in their description of a phase-dependency of GABA action but opposite in direction, are a result of the technical challenges of
standard whole cell and perforated patch-clamp recording in SCN. No other technique has revealed GABA-mediated excitation in a majority of cells, regardless of circadian time.

The modest phase advance seen with antagonist combinations or with picrotoxin applied during the subjective night extend previous findings that endogenous GABA can affect timing of rhythms and their response to timing cues (Mintz et al. 2002; Ralph and Menaker 1985, 1989; Rangarajan et al. 1994). The reasons for the greater effect of picrotoxin relative to that of BIC in these experiments are not understood. It is also unclear why muscimol did not have a significant effect on phase timing, as previously reported (Tominaga et al. 1994). In our experiments muscimol was applied at or near the peak of the discharge rhythm on day 1 (CT 5.5–6.5). A larger phase advance may have been observed if the agonist was applied earlier in the subjective day (Liu and Reppert 2000; Tominaga et al. 1994). Note, however, that muscimol and GABA have also been shown to induce phase delays in individual clock cells, and the responses to exogenously applied GABA appear to act in a manner that is at least partly independent of the effects of endogenous GABA. However, although exogenously applied baclofen can profoundly inhibit SCN cells, and the responses to exogenously applied GABA appear to be composed of multireceptor components, endogenous GABA exerts its effects predominantly by interaction with GABA_A receptors or other phacolofen-insensitive receptors.

The finding that endogenous GABA inhibition during the subjective day was increased as a function of incubation time after slice preparation was both novel and unexpected. Although cells appeared to be viable under these conditions for the entire recording period (as indicated by their maximal firing rates in the presence of bicuculline), their spontaneous discharge rates during the subjective day appeared to be greatly affected by increasing levels of tonic inhibition. The finding that the effects of both exogenously applied GABA_A agonists (including muscimol) and the antagonist bicuculline are significantly increased on the second day in vitro strongly suggests an increase in tonic inhibition resulting (at least) from an increase in receptor density. Whether there is a concomitant increase in tonic endogenous GABA release cannot be determined from these experiments. In addition we did not examine whether GABA_A receptor–mediated responses are likewise affected by time of incubation. We do not yet know whether this increase in inhibitory tone affects other pharmacological responses at any time point during prolonged experiments, but these data do suggest that care must be exercised to take into account changing levels of inhibition on such factors as rhythm shifts and the amplitudes of direct pharmacological responses in vitro. One advantage of the multiunit recording method is the ability to detect phenomena such as the one described here. With episodic recording techniques peak aggregate firing rates cannot be easily compared between comparable points in successive rhythms.

In conclusion, our data continue to strongly support the hypothesis that GABA is an inhibitory neurotransmitter in the mature rat SCN throughout the circadian cycle in vitro. Employing long-term and relatively noninvasive recording techniques we never observed a circadian rhythm-dependent change in the direction of the effects of GABA receptor ligands. We do not understand the reasons for circadian time-dependent reversals of the direction of GABAergic influence observed first by Wagner and colleagues (1997), and later observed in the opposite direction by de Jeu and Pennartz (2001), but suggest that the differences may result from the invasiveness of the techniques used and/or the need for continuous sampling to construct groups of cells at different circadian times.

**DISCLOSURES**

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