GABA Regulation of Circadian Responses to Light. I. Involvement of GABA_A-Benzodiazepine and GABA_B Receptors

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Light-induced phase shifts of the circadian locomotor rhythm of hamsters can be blocked by agents that alter GABA neurotransmission. The GABA antagonist bicuculline blocks phase delays induced by light and the benzodiazepine diazepam, which can potentiate GABA activity, blocks light-induced phase advances. In the experiments reported here, we found that the bicuculline blockade of phase delays was reduced by agents that mimic or potentiate GABA activity. Conversely, the diazepam blockade of phase advances was reduced by both competitive and noncompetitive antagonists of GABA. This indicates that the GABA-benzodiazepine receptor-ionophore complex is the most likely site of action for the effects of these drugs on circadian rhythms. However, competitive GABA agonists did not mimic the blocking effects of benzodiazepines, nor did the antagonist picrotoxin mimic the blocking effect of bicuculline. Therefore, the classic action of GABA, increased chloride conductance, may not be the effector mechanism in this case. We also found that the GABA_B agonist baclofen blocked both phase advances and delays and that the blockade of advances was reversed by the antagonist delta-aminovaleric acid. Taken together, these results indicate that GABA is involved in the regulation of circadian responses to light and that the regulation is mediated by both GABA_A and GABA_B receptors.

The entrainment of circadian rhythms by light cycles is thought to occur primarily through daily, light-induced phase advances and delays of endogenous circadian oscillations (Pittendrigh, 1960, 1965; DeCoursey, 1964). Single light pulses, given to animals otherwise held in constant dark, induce phase shifts in free-running circadian rhythms. Pulses given in the early subjective night induce phase delays, whereas pulses given in the late subjective night induce phase advances. For nocturnal rodents, subjective night is considered to be the 12 hr interval following the onset of running wheel activity in constant lighting conditions.

Recently, we have reported phase-dependent differences in the sensitivity of these light responses to agents that affect GABA

neurotransmission. In the golden hamster, *Mesocricetus auratus*, light-induced phase delays are blocked by the GABA antagonist, bicuculline, but responses are unaffected by this drug at times when light induces phase advances (Ralph and Menaker, 1985). Conversely, diazepam, a benzodiazepine (BZ) that potentiates GABA neurotransmission through action at GABA_A-BZ receptors, blocks light-induced phase advances but not delays (Ralph and Menaker, 1986). Taken together, these results suggest that GABA may be involved in the regulation of circadian responses to light. However, while bicuculline is thought to be a relatively selective antagonist of GABA, diazepam has numerous other effects in the CNS that may contribute to the blockade of the response to light. Such mechanisms include inhibition of adenosine uptake (Phillis et al., 1980, 1981; Wu et al., 1981), inhibition of phosphodiesterase activity (Beer et al., 1972), and blockade of calcium channels (Taft and DeLorenzo, 1984).

The experiments reported here were designed to determine whether, and in what specific ways, GABA is involved in the light responsiveness of the circadian system in mammals. If GABA neurotransmission is an important component of mammalian circadian systems, then agents that affect it should affect circadian rhythms in ways that reflect their known pharmacological actions and interactions.

We have tested the specific hypothesis that the effects of both bicuculline and diazepam on light-induced phase shifts are mediated by GABA_A-BZ receptors. The GABA_A receptor is part of a supramolecular receptor complex that includes, along with the GABA site, binding sites for BZs, barbiturates, and picrotoxin. The effects of the variety of drugs that are active at these sites have been reviewed extensively (see Olsen, 1982; Ticku, 1983; Haefely et al., 1985). The cellular effects of drugs at these sites are most often interpreted as being mediated through changes in the conductance of a chloride ionophore associated with the complex; however, recent reports have suggested that, in addition, the effects of drugs acting at the BZ site may be mediated through the inhibition of adenylate cyclase activity (Fung and Fillenz, 1984; Gray et al., 1984). We have tested the involvement of GABA_A-BZ receptors by examining numerous agents that affect GABA neurotransmission. If the hypothesis is correct, then these should alter the circadian responses to light in ways that reflect the known interactions of these drugs with GABA and the receptor complex.

Attempts to understand the putative actions of GABA in regulating the effects of light on circadian responses are complicated by the fact that the effects of GABA in the CNS are known to be mediated by at least 2 receptor types. The GABA_B receptor is distinct from GABA_A and does not appear to be associated with a BZ receptor site nor a chloride ionophore

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(Bowery et al., 1983). Moreover, it is relatively insensitive to GABA_A agonists and to bicuculline (Bowery et al., 1981, 1983). This receptor, therefore, cannot account for the effects of diazepam and bicuculline on circadian rhythms. On the other hand, because the GABA_B agonist baclofen decreases synaptic transmission in the suprachiasmatic nucleus (Shibata et al., 1986), the only identified site of rhythm generation in mammals (Rusak and Zucker, 1979; Turek, 1985), it is possible that GABA may influence circadian rhythms or their responses to light via GABA_B receptors, as well as via GABA_A-BZ receptors. To test this hypothesis, we have examined the effects of baclofen and a GABA_B antagonist, delta-aminovaleric acid (DAVA).

Materials and Methods

Male golden hamsters 8–12 weeks of age (Lakeview, Charles River), were housed individually, and their locomotor rhythms (wheel-running activity) were recorded on Esterline-Angus event recorders as previously described (see Pittendrigh, 1965; Takahashi et al., 1984). Animals were initially exposed to a light cycle of 14 hr of light at 100 lux and 10 hr of dark (LD 14:10) to which the animals were allowed to entrain before being released into constant dark (DD). After free-running for 7 d in DD, each animal was given either an intraperitoneal injection of a drug or combination of drugs specific for GABA_A-BZ or GABA_B sites, or a drug injection followed by a 15 min pulse of 515 nm monochromatic light at an intensity of $0.23 \mu\text{W cm}^{-2} \text{sr}^{-1}$. Injections were made in the dark with the aid of an infrared viewer (FIJW Industries). Experiments were conducted at 2 circadian time (CT) points: at CT 13.5 (1.5 hr after activity onset) when light normally induces phase delays, and at CT 18 (6 hr after activity onset) when light normally induces phase advances. The total fluence delivered during a light pulse (3×10^{14} photons $\text{cm}^{-2} \text{sr}^{-1}$) was calculated to produce approximately half-maximal phase shifts in control animals at either time point (see Takahashi et al., 1984).

Involvement of GABA_A-BZ receptors. Experiments conducted at CT 13.5 were designed to test whether the effects of bicuculline could be mimicked by other GABA_A-BZ antagonists or reduced by GABA_A-BZ agonists. Experiments conducted at CT 18 were designed to test whether the effects of diazepam could be mimicked by other GABA_A-BZ agonists or reduced by GABA_A-BZ antagonists. All drugs were administered in 100% DMSO (0.2 ml). Experimental animals were given an i.p. injection of a drug or drug combination prior to a light pulse (at 1 of the 2 time points). Control animals (at both time points) were given either an injection of the vehicle prior to a light pulse or an injection of one of the drugs or combinations with no light pulse. Because the drugs used have different rates of absorption and metabolism, the interval between the injection time and the light pulse was adjusted so that the peak effectiveness was most likely to occur during the pulse. Bicuculline was administered 2 min prior to the light pulse; diazepam, 45 min prior to the pulse; and all others, 30 min prior to the pulse. The timing of administration was determined prior to the experiment from behavioral observations on test animals and from behavioral observations reported in the literature (for examples and other references, see Meldrum, 1981; Braestrup et al., 1982; Clody et al., 1982; File, 1982; Polc et al., 1982; Thiébot and Soubrié, 1983; Greenblatt and Shader, 1985). In order to reduce the possibility of false negatives, the timing and dosage were adjusted in cases where effects on rhythms were small.

Diazepam, clonazepam, flunitrazepam, midazolam, and RO15-1788 were gifts from Hoffmann-LaRoche (Nutley, NJ). Triazolam was a gift from Upjohn (Kalamazoo, MI). THIP [4,5,6,7-tetrahydroisoxazolo-(4,5-c)-pyridin-4-ol] was a gift from Lundbeck AG (Copenhagen, Denmark).

Involvement of GABA_B receptors. Experiments were conducted at both CT 13.5 and CT 18. The experimental protocol was as described for the GABA_A-BZ experiments. Thirty minutes prior to the light pulse, experimental animals were given an i.p. injection of baclofen (*Lioresal*, Ciba-Geigy, NJ) or vehicle. Baclofen was dissolved in 0.1% lactic acid, which was then titrated to pH 7.4 using NaOH. Control groups were given drug or vehicle injections without the light pulse.

In another experiment, DAVA was coadministered with baclofen at CT 18. At low concentrations, this drug has been reported to act as an antagonist at GABA_B sites (Muhyaddin et al., 1982; Nakahiro et al., 1985). In these experiments, baclofen was administered via i.p. injection

Figure 1. Reduction of the bicuculline blockade of light-induced phase delays by GABA_A-BZ agonists. *Injection control*, animals given either vehicle only (0.2 ml), vehicle + THIP, or vehicle + diazepam, respectively, at CT 13.5. *Light group*, animals given the same injections as controls prior to a 15 min phase-delaying light pulse; *Light/bicuculline group*, animals given the same injections as controls along with 3.5 mg/kg bicuculline prior to a 15 min phase-delaying light pulse. The *open histogram* in the last set shows the bicuculline blockade of the light-induced phase shift. * $p < 0.05$, ** $p < 0.01$ compared with bicuculline plus light; and *** $p < 0.01$ compared with vehicle control. $n = 6$ per group. Error bars represent SEM.

30 min prior to the light pulse, and DAVA was administered 5 min prior to the light pulse via bilateral intracranial cannulae aimed at a point 0.5 mm dorsal to the SCN. The placement of cannula guides, implanted 2–3 weeks prior to the experiment, was later verified histologically.

Results

Interactions of GABA_A-BZ drugs at CT 13.5

Bicuculline, 4.0 mg/kg, significantly blocked the phase-delaying effect of the light pulse at CT 13.5. The GABA agonist THIP, 10 mg/kg, reduced this effect of bicuculline (Fig. 1). THIP by itself had no effect on the response to light, nor did the drug affect the phase of the free-running rhythm when administered alone at this time.

The effect of bicuculline was also reduced by diazepam (Fig. 1). Phase delays for animals that received both drugs prior to the light pulse were smaller than for controls that received the vehicle prior to the pulse ($p < 0.05$) but did not differ from animals that received diazepam alone prior to the pulse. Diazepam induced small phase delays in free-running rhythms (-0.19 ± 0.04 hr) in the absence of light ($p < 0.05$ vs -0.02 ± 0.05 hr for vehicle injected controls; $n = 5$ per group).

Picrotoxin, at doses from 0.5 to 6 mg/kg, had no effect either on the magnitude of light-induced phase delays or on free-running rhythms in the absence of light (Table 1). At the highest dose, picrotoxin induced convulsions in all of the animals used. For animals given light pulses, convulsions were observed prior to the pulse and for up to 20 min after the end of the pulse. The inverse agonist FG 7142 (Ferrosan, Denmark) likewise had no effect on rhythmicity (Table 1).

Interactions of GABA_A-BZ drugs at CT 18

At 5 mg/kg, diazepam significantly reduced and at 12.5 mg/kg, it completely blocked the phase-advancing effects of light pulses

Figure 2. Dose–response curves for the reduction of the diazepam blockade of phase-advancing light pulses by GABA and benzodiazepine antagonists. *Closed circles* represent groups that received 5 mg/kg diazepam; *open circles* represent groups that received 12.5 mg/kg diazepam ($\pm$ SEM). Control animals that received light pulses only are included for each antagonist; *horizontal arrows* and *dashed lines* represent mean values ($\pm$ SEM) for each light only control group. Effects of diazepam alone on the light response are shown at 0 mg/kg for each antagonist. *Asterisks* indicate the lowest dose of each antagonist that significantly reduced the effect of diazepam ($p < 0.01$). ($n = 4$ for bicuculline at 8 mg/kg; $n = 5$ for all other points.)

at CT 18. Bicuculline, picrotoxin, and RO15-1788 (a competitive antagonist of diazepam) all blocked the effect of 5 mg/kg diazepam (Fig. 2). The effects of 12.5 mg/kg diazepam were almost completely blocked by picrotoxin and RO15-1788; however, only a partial reduction could be obtained using bicuculline at the highest dose that did not produce severe toxic effects. Neither bicuculline, picrotoxin, nor RO15-1788 had any effect on light-induced phase shifts when given without diazepam, and none had any effect on the phase of the free-running rhythm when given without light or diazepam (Table 1).

The GABA_A site-specific agonists tested in these experiments had little effect on light-induced phase advances or delays when used alone (Table 2). One agonist, THIP, induced small phase advances of the free-running rhythm (no light) at this time point. The highest dose of each GABA_A agonist used was sufficient to produce sedation and an interruption of wheel-running activity for up to 4 hr. There was no transient delay of the onset of activity on the days immediately following the injections, as is

common after injections of diazepam (see Ralph and Menaker, 1986).

Unlike the GABA_A agonists, however, all of the BZs tested reduced the phase-advancing response to light (Table 2). Diazepam induced small phase delays in the absence of light and the short-acting BZs triazolam and midazolam induced somewhat larger phase delays.

Effects of baclofen and DAVA at CT 13.5 and CT 18

The GABA_B agonist baclofen significantly reduced the effect of both phase-delaying and phase-advancing responses to light. The blockade of phase advances was dose dependent with an ED₅₀ of 7.5 mg/kg. Baclofen produced a maximum blockade of this response at 15 mg/kg and was perhaps less effective at higher doses (Fig. 3). The blockade of light-induced phase delays required slightly higher doses than were required to block phase advances. In some control animals (no light), baclofen induced early activity onsets on the day following the injection; however,

Table 1. Lack of effect of GABA_A-BZ antagonists on light-induced phase shifts of hamster locomotor rhythms

Treatment	Phase shift (hr $\pm$ SEM)			
	Drug only	p^a	Drug + light	p^b
CT 13.5				
Vehicle (0.2 ml)	-0.02 ± 0.03	—	-0.56 ± 0.08	—
Picrotoxin (0.5 mg/kg)	-0.04 ± 0.03	n.s.	-0.47 ± 0.10	n.s.
Picrotoxin (4 mg/kg)	$+0.01 \pm 0.03$	n.s.	-0.50 ± 0.11	n.s.
Picrotoxin (6 mg/kg)	$+0.01 \pm 0.03$	n.s.	-0.37 ± 0.13	n.s.
FG 7142 (40 mg/kg)	-0.13 ± 0.15	n.s.	-0.49 ± 0.13	n.s.
CT 18				
Vehicle (0.2 ml)	-0.03 ± 0.03	—	—	—
Bicuculline (4 mg/kg)	-0.05 ± 0.04	n.s.	$+1.21 \pm 0.13$	n.s.
RO15-1788 (50 mg/kg)	-0.04 ± 0.02	n.s.	$+1.11 \pm 0.15$	n.s.
Picrotoxin (4 mg/kg)	-0.04 ± 0.04	n.s.	$+1.14 \pm 0.14$	n.s.

Drugs were administered via i.p. injection at CT 13.5 or CT 18 (drug only) or prior to a light pulse at CT 13.5 or CT 18 (drug + light). Significance was tested by ANOVA; $n = 5$ per group. +, hours of phase advance; —, hours of phase delay.

^a Compared with vehicle control group.

^b Compared with vehicle + light control groups shown in Fig. 2.

Figure 3. Dose-response curves for the blockade of light-induced phase shifts by baclofen. Closed circles, blockade of phase delays at CT 13.5; open circles, blockade of phase advances at CT 18. $n = 5$ for all points. Open and closed triangles, repeats of experiments at 10 mg/kg ($n = 4$ /group at both time points). Asterisks indicate the lowest dose of baclofen that significantly reduced the effect of light ($p < 0.01$ by ANOVA and Scheffé test).

the drug did not have a significant effect on the steady-state free-running rhythm at either time point.

The effect of baclofen at CT 18 was reduced by the GABA_B antagonist DAVA but not by bicuculline (4 mg/kg) nor by RO15-

Figure 4. Effects of GABA_A-BZ and GABA_B antagonists on the baclofen blockade of light-induced phase advances. All animals were given a 15 min pulse of light (see Materials and Methods) at CT 18. Control intracranial injections of ACSF and DAVA are shown in the left histogram. Groups shown in the right histogram were given baclofen (15 mg/kg) prior to the light pulse, along with the antagonist indicated. * $p < 0.01$ compared with the ACSF control group; ** $p < 0.05$ compared with the baclofen group ($n = 4$ per group). ACSF (artificial cerebrospinal fluid) contained 133 mM NaCl, 5.0 mM KCl, 1.0 mM MgSO₄, 2.5 mM CaCl₂, 10 mM HEPES, 10 mM glucose, 0.1 mg/ml streptomycin sulfate, and approximately 5 mM NaOH to bring the pH up to 7.35 (Cahill and Menaker, 1987).

Table 2. Effects of GABA_A-BZ agonists on phase and light-induced phase advances of hamster locomotor rhythms

Treatment	Phase shift (hr $\pm$ SEM)			
	Drug only	p^a	Drug + light	p^b
CT 13.5				
Vehicle (0.2 ml)	+0.05 $\pm$ 0.05	—	-0.58 $\pm$ 0.14	—
Muscimol (8 mg/kg)	-0.07 $\pm$ 0.03	n.s.	-0.52 $\pm$ 0.11	n.s.
THIP (10 mg/kg)	+0.12 $\pm$ 0.03	n.s.	-0.78 $\pm$ 0.10	n.s.
CT 18				
Vehicle (0.2 ml)	-0.03 $\pm$ 0.03	—	+0.95 $\pm$ 0.08	—
Muscimol (8 mg/kg)	-0.08 $\pm$ 0.08	n.s.	+0.91 $\pm$ 0.10	n.s.
THIP (10 mg/kg)	+0.23 $\pm$ 0.08	<0.05	+1.09 $\pm$ 0.19	n.s.
Diazepam (5 mg/kg)	-0.12 $\pm$ 0.05	<0.05	+0.54 $\pm$ 0.16	<0.01
Clonazepam (1 mg/kg)	—	—	+0.68 $\pm$ 0.08	<0.05
Flunitrazepam (2 mg/kg)	—	—	+0.67 $\pm$ 0.14	<0.05
Triazolam (5 mg/kg)	-0.29 $\pm$ 0.04	<0.01 ^c	+0.24 $\pm$ 0.12	<0.01 ^d
Midazolam (15 mg/kg)	-0.37 $\pm$ 0.05	<0.01 ^c	+0.58 $\pm$ 0.18	<0.01 ^d

Drugs were administered via i.p. injection either without light at CT 13.5 or CT 18 (drug only) or prior to a phase shifting light pulse at CT 13.5 or CT 18 (drug + light). Significance was tested by ANOVA; $n = 5$ per group. +, hours of phase advance; —, hours of phase delay.

^a Compared with vehicle control group.

^b Compared with vehicle + light control groups.

^c Results are from a different experiment and are therefore compared with separate vehicle controls (-0.05 $\pm$ 0.08).

^d Results are from a different experiment and are therefore compared with separate vehicle + light controls (+1.35 $\pm$ 0.24).

Figure 5. Hypothetical model of the relationship between GABA neurons, circadian pacemaker cells, and retinal input to the circadian system. *P*, pacemaker cell. Numbers refer to the adjacent synapse. Retinal input reaches the pacemaker neuron via the photic entrainment pathway. Signals may be transmitted by excitatory amino acids (1). GABA reduces synaptic transmission by inhibiting the release of excitatory neurotransmitter (2) and/or by reducing the sensitivity of the pacemaker cell to excitatory input (3). The phase dependence of the drug effects suggests a phase-dependent modulation of GABA activity. GABA metabolism, release, uptake, or efficacy may be controlled by the clock (4). If we postulate a direct retinal input to the GABA neuron (5), then the pacemaker and the GABA cell occupy almost identical positions with respect to each other and to the photic entrainment pathway (see text).

1788 (Fig. 4). DAVA itself had no effect on this response to light when given without baclofen. Neither of the vehicle solutions had a significant effect on the response to the light pulse alone (Fig. 4) nor on the phase of the free-running rhythm (Table 3). Pupil diameter was not affected by either baclofen or DAVA. At CT 13.5, intracranial infusion of the DAVA vehicle resulted in highly variable responses to baclofen plus light (mean phase delay = -0.20 ± 0.25 hr; $n = 6$). Given the initially small size of the light-induced phase delay (-0.52 ± 0.10 hr), it was not practical to test the effect of DAVA on the baclofen blockade at this time point.

Discussion

The blockade of light-induced phase delays by bicuculline and the reduction of this effect both by the GABA_A agonist THIP and by diazepam suggest that these drugs exert their effects on the circadian system through interactions at GABA_A-BZ receptors. GABA binding at these receptors results in an increase in the conductance of the chloride channel associated with the complex. This action of GABA can be mimicked by the competitive GABA agonist THIP, blocked by the competitive antagonist bicuculline, or potentiated by diazepam via the BZ site. The reversal of the bicuculline blockade by THIP and diazepam (Fig. 1), therefore, may reflect their opposite effects on GABA activity and chloride conductance.

Similarly, the blockade of light-induced phase advances by diazepam and other BZs and the reversal of the effects of diazepam by antagonists of both GABA and diazepam (Fig. 2) suggest that these drugs, too, are exerting their effects through interactions at the GABA_A-BZ receptor complex. As one action of BZ at this site is to increase chloride conductance, effects of diazepam that require an increase in conductance should be blocked by agents which competitively reduce diazepam binding

Table 3. Lack of effects of baclofen and DAVA on the phase of free-running rhythms

Treatment	Phase shift (hr $\pm$ SEM)	<i>p</i> ^a
Baclofen vehicle (0.2 ml)	$+0.03 \pm 0.04$	—
Baclofen (15 mg/kg)	$+0.04 \pm 0.03$	n.s.
DAVA vehicle (0.2 μ l)	-0.09 ± 0.07	—
DAVA (10^{-3} M)	-0.12 ± 0.05	n.s.

Drugs were administered without light at CT 18. Significance was tested by ANOVA; $n = 5$ per group. +, hours of phase advance; —, hours of phase delay.

^a Compared with vehicle control group.

(e.g., RO15-1788) or which directly block the chloride channel (e.g., picrotoxin). Therefore, the ability of RO15-1788 and picrotoxin to reverse the diazepam blockade of light-induced phase advances might be explained by these 2 mechanisms, respectively. Bicuculline may reduce chloride conductance either directly by blocking the GABA_A binding site or indirectly by reducing the affinity of the BZ site for diazepam (Tallman et al., 1978). However, the relatively poor ability of bicuculline to reverse the effects of high doses of diazepam suggests that diazepam may exert its effects through more than one mechanism, perhaps a direct effect on the chloride channel or an entirely different process. Nonetheless, taken together, these results suggest that GABA plays an important role in modulating the responsiveness of the circadian system to light and that the most likely site of action is the GABA_A-BZ receptor chloride-ionophore complex.

Modification of chloride conductance appears to be the most likely effector mechanism for most of the results reported here. However, there are inconsistencies with this interpretation. If the bicuculline blockade of light-induced phase delays at CT 13.5 were due to decreased chloride conductance, then picrotoxin, which blocks chloride channels through a third binding site on the GABA_A-BZ receptor, and FG 7142, an inverse agonist at the BZ site, should have yielded similar results. This is clearly not the case (Table 1). The inability of these drugs to mimic the effects of bicuculline suggests that a reduction in chloride conductance may not be sufficient to block phase-delay responses to light. Furthermore, as high doses of picrotoxin also induced convulsions in these animals, a general (i.e., convulsive) effect of bicuculline is ruled out as a mechanism for the blockade. Hence, although GABA_A-BZ receptors appear to be the most likely site of action for bicuculline, the effector mechanism mediating the action of this drug is unclear.

The results obtained at CT 18 present a similar problem. If the effects of BZs were due to the potentiation of GABA activity, then GABA_A agonists would be expected to have similar effects, and they do not (Table 2). It seems unlikely that the inability of GABA_A agonists to block light-induced phase advances is due to insufficient dosage, as sufficient drug reaches the brain in all cases to induce sedation. Furthermore, the doses used in this experiment were sufficient to reduce the bicuculline blockade of phase delays (Fig. 1). Therefore, the lack of any consistent effect of these agonists (a significant effect of THIP was obtained in only one experiment, see Table 2) on either free-running rhythms or on the response to light suggests that the BZ blockade of light-induced phase advances may not be due to the potentiation of GABA activity; and, like the effects of bicuculline, the

effects of BZ, although mediated by GABA_A-BZ receptors, may not require changes in chloride conductance.

If changes in chloride conductance are not required for the blocking actions of bicuculline and BZs, then the question arises as to what role, if any, GABA itself plays in the regulation of circadian responses to light. Perhaps GABA does play a role but via a quite different mechanism. One possibility is an effect on cyclic nucleotide metabolism. It has been reported recently that the BZ receptor may mediate an inhibition of cAMP activity (Fung and Fillenz, 1984; Gray et al., 1984). Cyclic nucleotide involvement has been demonstrated in other circadian systems (Eskin et al., 1982, 1984; Eskin and Takahashi, 1983). If the effects of diazepam on the circadian system involve a GABA_A-BZ-mediated inhibition of cAMP synthesis, then agents that reduce diazepam binding may reduce its behavioral effects. All 3 of the antagonists used in this study have been shown, albeit through different mechanisms, to reduce BZ binding (Tallman et al., 1978; Chweh et al., 1985). Thus, an alternative mechanism that could account for the effects of diazepam may be through an inhibition of cAMP synthesis. Bicuculline might also act via a similar mechanism. Although not identified by these data, a hypothetical effector mechanism for bicuculline could be GABA sensitive and chloride independent.

The ineffectiveness of the GABA agonists by themselves, in either producing phase shifts or altering light responses, may be due to many reasons. First, the agonists used are relatively short acting and even at high doses may not have their maximal effect at a time that is appropriate to affect responses to light. Second, the results indicate that the classic mechanism of action for these agonists (i.e., chloride conductance) may not be involved in the observed effects on light responses. GABA and its agonists may have different effects on mechanisms other than chloride conductance. A more likely explanation, however, is that since GABA appears to modulate photic responses rather than mediate photic input, GABA agonists would not be expected to mimic the effects of light and the additional effects of GABA agonists given together with light may be too small to detect. The latter 2 explanations are emphasized by the fact that bicuculline at very high doses neither completely blocks light-induced phase delays (see Ralph and Menaker, 1985) nor completely negates the diazepam blockade of phase advances (Fig. 2). In the circadian system, therefore, the efficacy of drugs that act at the GABA_A site may be relatively low.

A role for GABA in the regulation of circadian photic responses is supported, nonetheless, by the results obtained using GABA_B-specific drugs. Insofar as baclofen is an agonist and DAVA an antagonist at GABA_B sites, the blockade of circadian responses to light by baclofen and the reduction of some of these effects by DAVA suggest that the behavioral effects reflect the interaction of the 2 drugs at GABA_B receptors. Since neither bicuculline nor RO15-1788 altered this effect of baclofen at CT 18, and neither had an effect of its own, it is unlikely that baclofen produces its effect through GABA_A-BZ receptors.

Phase dependency of drug effects

An explanation for the phase dependency of many of the drug effects that we have described is suggested by the report that GABA uptake decreases at light offset (Barkai et al., 1985). This is likely to be a reflection of a decrease in GABA release. If such a change in GABA levels persists in constant conditions, then GABA activity could be high in the subjective day and early subjective night following the rhythm in electrical activity in

the SCN (Green and Gillette, 1982; Groos and Hendricks, 1982; Inouye and Kawamura, 1982). In this situation, GABA could have a maximal effect on light responses in the early subjective night.

The antagonistic effect of bicuculline would appear higher early in the subjective night due to competitive antagonism of GABA activity. Conversely, diazepam might be relatively ineffective at this time as a result of either a high level of GABA-stimulated cAMP synthesis, receptor down-regulation, and/or the presence of endogenous BZ ligands that may be released during periods of high GABA activity (Alho et al., 1985; Ferrero et al., 1986). Moreover, the classic actions of BZs are mediated by an increase in affinity of the GABA_A site for GABA, so the apparent effect of diazepam could be reduced even further when GABA activity is high. Late in the subjective night, when GABA activity is postulated to be low, an effect of bicuculline would be less apparent even though the drug could compete more effectively for receptor sites, and conversely, a GABA-independent effect of diazepam could be relatively enhanced.

Baclofen blocked light-induced phase shifts at both CT points tested; however, the doses required to reduce delays were higher than those required to reduce advances. This difference was small, but in the same direction as the differential effects of diazepam reported previously (Ralph and Menaker, 1986); both drugs have greater effects on phase advances than on phase delays. The small phase dependency of these effects may reflect interactions with modulatory GABA activity that is changing over time. However, there are differences between these effects: Whereas baclofen can block light-induced phase delays, diazepam has no effect at any dose tested; diazepam consistently induces small phase delays in the absence of light, whereas baclofen had no effect on the free-running rhythms. These differences may reflect differences in the location of GABA_A-BZ and GABA_B receptors in the light input pathway or, alternatively, differences in the coupling of the receptors to effector mechanisms. In spite of these differences, it is possible that the blocking effects of baclofen and diazepam involve the same intracellular events mediated by different receptors. This suggestion is strengthened by the fact that the 2 drugs share a number of pharmacological effects, including inhibition of cAMP synthesis, inhibition of voltage-dependent Ca²⁺ conductance (Gray et al., 1984; Taft and DeLorenzo, 1984), and reduction of neuronal excitability.

Relationship of GABA and the circadian system in mammals

The general conclusions derived from the data presented here form the basis of a model for the regulation of circadian responses to light by GABA. The photic entrainment pathway can be represented as a serial progression of events that occur after the initial reception of the photic signal. GABA may affect neural transmission or biochemical events at any or multiple points in the pathway. However, other results reported in the literature (Zatz and Brownstein, 1979; Smith and Turek, 1986) and preliminary results from localization studies (M. R. Ralph and M. Menaker, unpublished observations) suggest that the SCN, in which GABAergic neurons are concentrated, is the most likely brain site of action for most of these drugs. Unfortunately, the course of the photic entrainment pathway within the SCN is unknown, and the precise relationship of these intersecting pathways remains obscure.

Nonetheless, we can construct a simple, neural model for the relationship between GABA and circadian pacemaker neurons

(Fig. 5). In this arrangement, GABA may either: (1) regulate at a presynaptic site the release of neurotransmitters from terminals in the entrainment pathway, or (2) regulate directly the sensitivity of pacemaker cells to photic input. Both connections are equally possible, and no distinction is made between GABA_A-BZ and GABA_B receptors at these 2 sites.

One possible mechanism at the presynaptic location might involve a GABA_B-mediated, cAMP-dependent inhibition of voltage-dependent Ca²⁺ channels (Gray et al., 1984). Baclofen may block neurotransmitter release from the cells forming part of the neuronal pathway over which light information reaches the clock. Because excitatory amino acids have been implicated as candidate neurotransmitters in the retinohypothalamic projection (Shibata et al., 1986; Cahill and Menaker, 1987), this explanation is supported by the report that excitatory amino acid release in the SCN (Liou et al., 1984) and the amplitude of evoked field potentials in the SCN (Shibata et al., 1986) are reduced in the presence of baclofen.

Our results suggest that GABA regulation of the clock is phase dependent. Phase-dependent regulation by GABA implies clock control of GABA neurotransmission. Hence, one or many aspects of GABA activity must be regulated by the clock. Regulation could occur at any point in the metabolic pathways for GABA, at the point of GABA release (possibly via the regulation of neuronal excitability), or postsynaptically at the receptor or transduction levels. Many, though not all, of these possibilities require reciprocal connections between the pacemaker cells and GABAergic neurons (see Fig. 5).

An interesting feature that falls out of the model as constructed is that the pacemaker cell and the GABA neuron occupy very similar positions with respect to the photic input pathway. One only need postulate a direct retinal input to the GABA neuron for these positions to be essentially identical. The circadian pacemaker cells in the SCN have not been identified, and it is possible that the GABAergic neurons of the SCN contain circadian pacemakers. Indeed, it is entirely consistent with current data to propose that the clock in mammals is composed of a population of oscillating cells that are coupled to the environment by excitatory amino acids and to each other by GABA. If this model is correct and if GABA activity in the circadian system is rhythmic, as some of these data suggest, then the strength of coupling between pacemaker cells must also be rhythmic.

The data presented here support the hypothesis that circadian responses to light are regulated in a phase-dependent manner by GABA and that regulation occurs through both GABA_A-BZ and GABA_B receptors. GABAergic neurons have been found in the major brain nuclei known to be associated with the generation and control of circadian rhythms in mammals (retina: Wu et al., 1981; lateral geniculate nucleus: Hendrickson et al., 1983; SCN: Card and Moore, 1984; Van den Pol and Tsujimoto, 1985), but it is unclear what role GABA plays in each of these structures. Nonetheless, the phase-dependent nature of the effects reported here supports the suggestion made earlier (Ralph and Menaker, 1986) that in mammals differences may exist between the neural regulation of light-induced phase delays and advances.

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