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Light-Induced Phase Shifts and Fos Expression in the Hamster Circadian System: The Effects of Anesthetics

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Abstract In the present study, we examined the effect of administration of anesthetics on light-induced phase shifts of the circadian system. This information is of critical importance, because many studies of light input to the mammalian suprachiasmatic nucleus (SCN) have been performed on anesthetized animals. We found that light-induced phase shifts were blocked by all drugs used at anesthetic doses. We then determined the effect of two of these agents on light induction of Fos-like immunoreactivity in the SCN. We found that the administration of sodium pentobarbital prevented light induction of Fos expression in the SCN, whereas the administration of urethane did not. These results raise cautions about the use of anesthetized animals to answer questions about the photic regulation of neuronal activity in the SCN.

Key words anesthetics, circadian, Fos, photic, suprachiasmatic nucleus

The suprachiasmatic nucleus (SCN) of the hypothalamus is the site of circadian oscillators that act as pacemakers in the mammalian circadian system. In order to function adaptively, the SCN oscillators must be synchronized to the external environment. Light, acting through daily phase advances and delays of the oscillation, is the primary environmental signal utilized for this synchronization. Photic information received by the retina is transferred directly to the SCN via the retinohypothalamic tract (RHT), and less directly via a pathway that includes the intergeniculate leafleft of the lateral geniculate nucleus (Moore, 1973; Card and Moore, 1982; Pickard, 1982). Cells in the SCN have been shown to respond electrically to photic stimulation of the retina (Groos and Mason, 1978, 1980; Sawaki, 1979; Inouye, 1984; Meijer et al., 1986, 1989; Miller et al., 1987). Most of these studies have involved electrophysiological recordings from animals anesthetized with one of a number of different agents.

Although general anesthetics appear to share no common chemical structure, most tend to reduce excitatory postsynaptic potentials while increasing or maintaining inhibitory responses (e.g., Gage and Hamill, 1981; Franks and Lieb, 1982). A major problem, therefore, with any physiological investigation using anesthetized preparations is to control for the potentially confounding effects of the anesthetic itself; this may be particularly true for light input to the circadian system. The dissociative anesthetics ketamine and phencyclidine have been shown to prevent light-induced phase shifts of the hamster circadian system,

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presumably through their actions as antagonists of *N*-methyl-D-aspartate (NMDA) receptors (Colwell et al., 1990). In addition, the anesthetic sodium pentobarbital has been shown to affect *N*-acetyltransferase activity in the pineal (Zatz and Brownstein, 1979), which is regulated by both light and the circadian system, and to cause phase shifts of the circadian rhythm of wheel-running activity in mice (Ebihara et al., 1988). Given the pronounced impact of anesthesia on circadian rhythmicity, it is important to examine more closely the effects of these agents on light input to the mammalian circadian system.

In the present study, we investigated the effect of several anesthetics on light-induced phase shifts of the circadian rhythm of locomotor activity in the golden hamster. In addition, since recent work suggests that Fos induction may be a useful cellular marker of photic input to the SCN, we examined the effect of two commonly used anesthetics on light induction of Fos-like immunoreactivity (Fos-LI) in the hamster SCN. The results have bearing on the interpretation of physiological studies of the SCN in which animals are anesthetized, as well as the relationship of Fos induction to phase shifting.

MATERIALS AND METHODS

Male golden hamsters (*Mesocricetus auratus*, LVG-outbred), obtained from Charles River, Lakeview (Newfield, NJ) at 10 weeks of age, were housed individually, and their wheel-running activity was recorded. The animals were exposed to a light–dark cycle (LD 14:10) for 2 weeks, at which time they were placed in constant darkness (DD).

Hamsters remained in DD and were subjected to one of four treatments: (1) injection of vehicle alone; (2) injection of anesthetic; (3) injection of anesthetic plus light; or (4) injection of vehicle plus light. The treatments were delivered either 1.5 hr after the onset of activity at circadian time (CT) 13.5, when light would normally induce a phase delay, or 6.0 hr after the onset of activity at CT 18, when light would normally cause a phase advance (onset of activity is defined as CT 12 for nocturnal animals). Animals used for immunocytochemistry were killed 60 min after treatment. Otherwise, following each treatment the animals were allowed to run undisturbed in DD for at least 10 days, to enable us to determine the effects of treatment on the phase of the free-running rhythm.

The light stimulus used to induce phase shifts and Fos-LI was a 15-min pulse of monochromatic light (515 nm) at an intensity of 1.0×10^{-1} $\mu W/cm^2$. The stimulus parameters (duration, irradiance, and wavelength) were chosen to produce submaximal phase shifts (Takahashi et al., 1984). The irradiance was approximately doubled for the "bright-light" treatments, in order to produce saturating phase shifts. Stimulus intensity (irradiance) was measured before each trial with a radiometer (United Detector Technologies, Hawthorne, CA). Following the light pulse, the hamsters were returned to DD. All handling and treatment of animals were carried out in complete darkness with the aid of an infrared viewer (FJW Industries, Elgin, IL).

With the exception of halothane, all anesthetics were administered by intraperitoneal injection at least 15 min prior to the light treatment, to ensure adequate anesthesia. Halothane was administered by inhalation 3 min prior to the light treatment and was maintained throughout the pulse. The following drugs and doses were used: althesin (a steroid anesthetic), 0.05 ml/kg; chloral hydrate, 500 mg/kg; α-chloralose, 80 mg/kg; sodium pentobarbital, 40

mg/kg; urethane (subanesthetic), 1250 mg/kg; urethane (anesthetic), 2000 mg/kg. In addition, two drug combinations were tested: α-chloralose, 40 mg/kg plus urethane, 1000 mg/kg; chloral hydrate, 200 mg/kg plus sodium pentobarbital, 20 mg/kg. Animals were considered adequately anesthetized when a toe pinch failed to elicit a behavioral response. For each anesthetic, with the exception of urethane and halothane, the doses used produced anesthesia for 30–60 min in test animals. At anesthetic doses, urethane produced anesthesia for many hours, and some animals never recovered clear wheel-running rhythms and had to be excluded from the analysis (6 of 14 animals). Because of the potential effects of anesthetics on sympathetic control of pupillary dilation, an experiment was conducted in which both experimental and control groups were treated with atropine delivered topically to each eye, so that pupils would remain dilated during the light treatment. All drugs were purchased from Sigma Chemicals (St. Louis, MO), with the exception of althesin, which was provided by Glaxo (Research Triangle Park, NC).

Phase shifts in the activity rhythm were determined by measuring the phase difference between eye-fitted lines connecting the onset of activity for a period of 7 days before and 10 days after an experimental manipulation. In order to estimate the steady-state phase shifts produced, 4 days of data after treatments that caused phase advances were excluded from the analysis. In other respects, the method for calculating phase shifts was the same as has been reported elsewhere (Takahashi et al., 1984).

For perfusion, animals were removed from their light-tight boxes and anesthetized with a lethal dose of halothane by inhalation in the dark. Then, under dim red light, the hamsters were perfused and standard immunohistochemical procedures were followed. The antisera used in this study was an anti-Fos (4-17) rabbit polyclonal antiserum (Oncogene Science, Uniondale, NY; dilution 1:250). The sites of the antibody–antigen binding were visualized with an avidin–biotin–peroxidase procedure (Elite ABC kit, Vector Labs, Burlingame, CA). Methods of anlaysis of immunostaining have been previously described (Colwell and Foster, 1992).

The phase-shifting effects of a treatment were considered to be significant when the 95% confidence interval of the group mean did not overlap zero. Differences between treatment groups were evaluated using a Kruskal–Wallis one-way analysis of variance, followed by a Mann–Whitney U test where appropriate. Values were considered significantly different if $p < 0.05$. In the text, values are shown as means ± *SEM*; phase advances are shown as positive values, while phase delays are shown as negative values.

RESULTS

The intraperitoneal administration of the anesthetic sodium pentobarbital (40 mg/kg) significantly inhibited both light-induced phase advances and delays of the circadian rhythm of wheel-running activity (Fig. 1). Control injections of sodium pentobarbital or vehicle alone, administered in the absence of a light treatment during the subjective night, had no significant effect on the phase of the free-running rhythm. Examples of locomotor activity records from experimental and control animals treated with sodium pentobarbital and light are shown in Figures 2A–2C. Phase shifts produced by bright-light treatments at CT 18 were also significantly reduced by sodium pentobarbital anesthesia (15 ± 7 min vs. 126 ± 6 min for vehicle controls; $n = 6$ per group; $p < 0.001$). These results were not altered by the concomitant

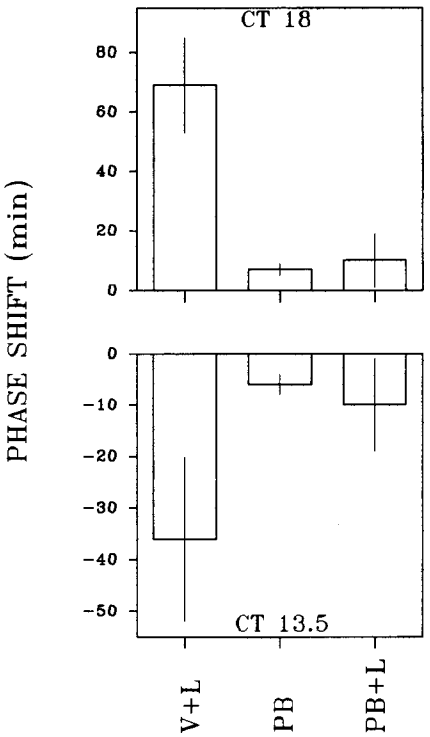

FIGURE 1. Sodium pentobarbital (40 mg/kg) can prevent light-induced phase advances and delays. Mean phase shift in the rhythm of locomotor activity of hamsters in DD that received a treatment of either vehicle plus light (V + L), sodium pentobarbital (PB), or sodium pentobarbital plus light (PB + L). Top: Light treatments (15 min) were delivered at CT 18. Bottom: Light treatments (15 min) were delivered at CT 13.5. The vehicle or drug treatments were always administered 30 min prior to the light treatments. $n = 6$ for all points; vertical bars represent *SEM*.

administration of the pupillary dilator atropine to the eyes of the treated hamsters (24 ± 10 min vs. 102 ± 9 min in atropine/vehicle controls; $n = 4$ per group; $p < 0.01$).

The intraperitoneal administration of the anesthetic urethane (2000 mg/kg) also significantly inhibited light-induced phase advances of the circadian rhythm of wheel-running activity (15.9 ± 6.3 min; $n = 8$). Control injections of urethane alone, administered in the absence of a light treatment, had no significant effect on the phase of the free-running rhythm (−7.5 ± 4.0 min; $n = 6$). A locomotor activity record from an experimental animal treated with urethane and light is shown in Figure 2D. Light-induced phase shifts were not significantly inhibited by a lower dose (1250 mg/kg) of urethane. However, these animals were not fully anesthetized and still responded to a toe pinch.

The administration of a number of other anesthetics also inhibited light-induced phase advances (Table 1). With one exception, none of these anesthetics were found to cause significant phase shifts by themselves (the group treated with α-chloralose plus urethane at CT 18 showed a significant phase delay). The effects of some anesthetics may have been additive. For example, neither urethane at 1250 mg/kg nor α-chloralose at 40 mg/kg prevent light-induced phase advances when given separately. However, when administered together, they significantly inhibited light-induced phase advances (Table 1). A similar result was obtained using sodium pentobarbital with chloral hydrate (Table 1).

The effect of the anesthetics urethane and sodium pentobarbital on light induction of Fos-LI in the SCN was also examined. The administration of sodium pentobarbital, at a dose (40 mg/kg) that prevented light-induced phase shifts, also inhibited light induction of

Fos-LI in the SCN. In contrast, photic induction of Fos-LI in animals anesthetized with urethane (2000 mg/kg) was unimpaired. There were no obvious differences in either the amount or distribution of Fos-LI in the SCN of animals treated with urethane plus light, compared with those treated with vehicle plus light. Neither drug by itself caused a significant induction of Fos expression in the SCN. Data are shown in Figures 3 and 4.

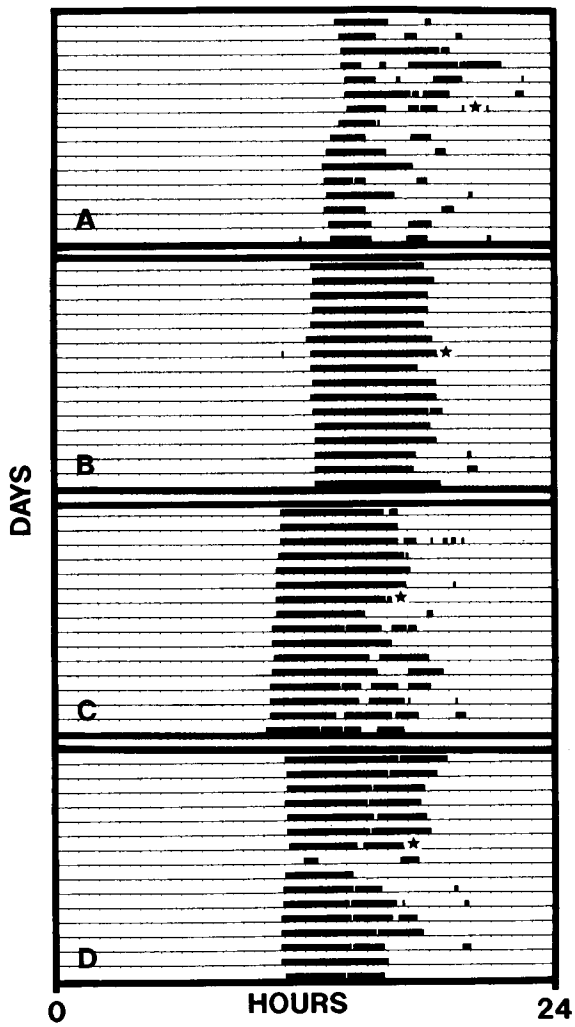

FIGURE 2. Locomotor activity records from experimental and control animals maintained in DD. Each horizontal line represents the activity record for a 24-hr day, and successive days are plotted from top to bottom. Stars represent the times of light and/or drug treatments. (A) Activity records illustrating the phase advance in a hamster that received a vehicle injection followed by a 15-min exposure to light at CT 18. (B) Lack of effect of an injection of sodium pentobarbital (40 mg/kg) at CT 17.5 on the phase of the circadian rhythm in locomotor activity. (C) Blockade of light-induced phase advances by an injection of sodium pentobarbital (40 mg/kg) 30 min prior to the light treatment at CT 18. (D) Blockade of light-induced phase advances by an injection of urethane (2000 mg/kg) 30 min prior to the light treatment at CT 18.

TABLE 1. The Effects of Various Anesthetics on Light-Induced Phase Advances of the Circadian Rhythm of Locomotor Activity in the Hamster

Treatment	Phase shift (min ± *SEM*)
Vehicle + light	69 ± 16
Althesin (0.05 ml/kg) + light	5 ± 2*
α-Chloralose (80 mg/kg) + light	−7 ± 9*
Chloral hydrate (500 mg/kg) + light	6 ± 7*
Halothane + light	11 ± 7*
Sodium pentobarbital (40 mg/kg) + light	10 ± 9*
Urethane (2000 mg/kg) + light	16 ± 6*
α-Chloralose (40 mg/kg) and urethane (1000 mg/kg) + light	19 ± 16*
Chloral hydrate (200 mg/kg) and sodium pentobarbital (20 mg/kg) + light	−5 ± 9*

Note. Light pulses were delivered 6 hr after the onset of activity (i.e., CT 18), when light would normally induce a phase advance. Positive values represent phase advances; negative values represent phase delays. There were six to eight animals per treatment group.

* Significantly different ($p < 0.05$) from vehicle + light controls.

DISCUSSION

The results presented here indicate that anesthetics can severely disrupt light input to the mammalian circadian system. The administration of sodium pentobarbital was found to prevent light-induced phase advances and delays of the circadian rhythm of wheel-running activity. By itself, sodium pentobarbital did not cause phase shifts at the times tested. A number of different anesthetics were investigated, and all showed the property of preventing light-induced phase advances (possible effects on light-induced phase delays were not investigated for some anesthetics). In addition, sodium pentobarbital, but not urethane, was found to inhibit photic induction of Fos expression in the SCN.

These results suggest that anesthetics interfere with the normal transmission of photic information to the circadian system. Possible anesthetic-sensitive sites include the retina, the retinal projections to the SCN, and the SCN itself. Since phase shifts were inhibited even when saturating light treatments and atropine were used during stimulation, it seems unlikely that the effect can be attributed to a reduced amount of light reaching the retina. The observations that photic stimulation of the retina can evoke electrical responses in the SCN of anesthetized animals, and that urethane anesthetized animals still display light-induced Fos-LI in their SCN, suggest that at least under urethane anesthesia, photic information can reach SCN neurons. Whether this is a normal amount of transmission to all normally innervated cell types is unclear. It may be that anesthetics have the general property of depressing the electrical excitability of SCN neurons. Interestingly, Rusak and Groos (1982) found that phase shifts of the feeding rhythm in rats could still be elicited under anesthesia with direct electrical stimulation of the SCN. Unfortunately, it is not known whether the anesthetic used in this study (a mixture of fluanisone and fentanyl) would prevent light-induced phase shifts. However, these results do raise the possibility that anesthesia does not render the circadian oscillator incapable of phase shifting, but instead raises the level of excitation required to produce this behavioral response.

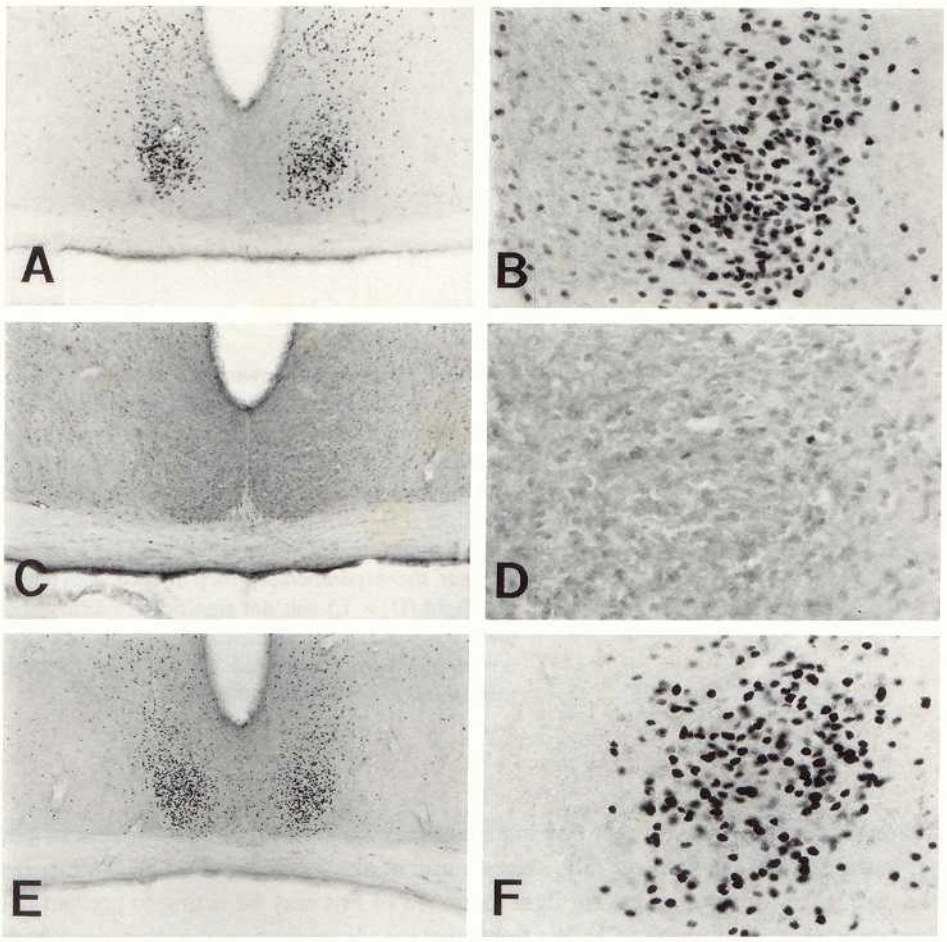

FIGURE 3. Sodium pentobarbital but not urethane inhibits photic induction of Fos-LI in the hamster SCN. Photomicrographs of coronal sections through the SCN region of hamsters stained for Fos-LI. Hamsters in DD were treated with either vehicle plus light (A, B), sodium pentobarbital plus light (C, D), or urethane plus light (E, F). Animals were killed 60 min after exposure to light at CT 18. Magnification: ×100 for A, C, E and ×400 for B, D, F. Reduced 27% for reproduction.

Data from the present study show that anesthetics prevent light-induced phase shifts. This finding suggests caution in the interpretation of results from electrophysiological studies of chronically anesthetized preparations. The obvious problem inherent in studies using anesthetized preparations is to determine whether spontaneous and/or evoked electrical activity is representative of what occurs normally. Anesthesia may result in a general depression of SCN electrical activity, or may reduce activity in only a subpopulation of cells. Because anesthetics prevent light-induced phase shifts, those SCN cells that respond electrically to light in anesthetized animals may not be involved in transferring light information to the circadian oscillator. These cells may not represent the total population of retinally driven SCN cells, and their responses may be abnormal.

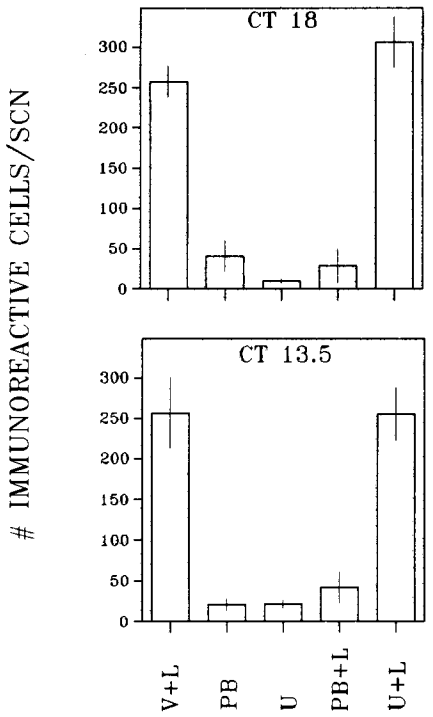

FIGURE 4. Histograms showing the number of Fos-positive cells in the SCN of the experimental and control groups (mean ± *SEM*). The light treatments were administered at either CT 18 (top) or CT 13.5 (bottom). The experimental group treated with sodium pentobarbital plus light (PB + L) was significantly different from the control group treated with vehicle plus light (V + L), whereas the experimental group treated with urethane plus light (U + L) was not significantly different from these controls. Drug treatments of pentobarbital (PB) or urethane (U) were delivered 30 min prior to the light treatments. n = 4–6 for all points.

Previous studies suggest that Fos induction may be a useful cellular marker of photic input to the SCN of the hamster (e.g., Kornhauser et al., 1990; Rusak et al., 1990). If this is the case, then agents that prevent light induction of Fos may be acting to prevent photic information from reaching the SCN. We found that the administration of sodium pentobarbital (but not urethane) inhibited photic induction of Fos-LI in the hamster SCN. Since sodium pentobarbital also prevents light-induced phase shifts, these results are consistent with the idea that this anesthetic acts to inhibit the transmission of photic information before it reaches the circadian oscillator within the SCN. The fact that an anesthetic dose of urethane does not prevent induction of Fos suggests that it is not the general state of anesthesia per se that interferes with Fos induction. These results also indicate that urethane may be the anesthetic of choice for physiological studies of the SCN in chronically anesthetized preparations.

It is now possible to compare aspects of the pharmacological regulation of light-induced phase shifts with those of Fos induction in the SCN. Some interesting parallels have been found: For example, NMDA receptor antagonists (e.g., Abe et al., 1991), the γ-aminobutyric $acid_B$ ($GABA_B$) receptor antagonist baclofen (C. S. Colwell and C. M. Kaufman, unpublished data), and sodium pentobarbital (present study) have all been found to inhibit both light-induced phase shifts and Fos induction in the SCN. However, our present results demonstrate that the anesthetic urethane can prevent light-induced phase shifts without any obvious effect on Fos induction. In addition, recent studies have demonstrated that it is possible to cause phase shifts of the circadian system without concomitant Fos induction (e.g., Mead et al., 1992; Colwell et al., 1993). Thus, it is clearly possible to dissociate Fos induction and phase shifting pharmacologically; Fos expression in the SCN is not sufficient for phase shifting.

These studies do not address the question of whether Fos expression is necessary for light-induced phase shifts.

Anesthetics are a structurally heterogeneous group of compounds, and although they have some common behavioral effects, they are likely to prevent light-induced phase shifts through a variety of cellular mechanisms. For example, the dissociative anesthetic ketamine acts to block the ion channel regulated by the NMDA receptor (e.g., Anis et al., 1983; Kemp et al., 1987), whereas sodium pentobarbital acts to enhance the chloride conductance regulated by the $GABA_A$ receptor (e.g., Barker and Ransom, 1978; Mathers and Barker, 1980; Nicoll and Wojtowicz, 1980; Schultz and MacDonald, 1981; Olsen, 1982). Both NMDA and GABA receptors appear to play a role in mediating light input to the circadian system (Ralph and Menaker, 1985, 1986, 1989; Colwell et al., 1990, 1991; Colwell and Menaker, 1992). The mechanism(s) underlying urethane's actions in the central nervous system are less clear (e.g., Pichon, 1979; Miller et al., 1986; McGivern and Scholfield, 1990). The possibility that urethane or any other agent tested blocks phase shifts through some nonspecific action cannot be eliminated, especially because urethane is toxic at the high dose used. Nevertheless, the results of the present study suggest that specific anesthetics with known mechanisms of action may be useful as tools for examining the pharmacology of the circadian system. In addition, the observation that anesthetics prevent light-induced phase shifts raises cautions about the use of anesthetized animals to answer questions about the photic regulation of circadian oscillators.

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