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Midazolam, a Short-Acting Benzodiazepine, Resets the Circadian Clock of the Hamster

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WEE, B. E. F. AND F. W. TUREK. *Midazolam, a short-acting benzodiazepine, resets the circadian clock of the hamster.* PHARMACOL BIOCHEM BEHAV **32**(4) 901–906, 1989.—Treatment with the short-acting benzodiazepine, triazolam, has been found to induce changes in both behavioral and endocrine circadian rhythms in hamsters. The objective of this study was to determine if these effects of triazolam could be generalized to other short-acting benzodiazepines. Therefore, the effects of midazolam on the biological clock of the hamster were examined in detail. A phase-response curve and a dose-response curve were measured to determine the effects of a single intraperitoneal injection of midazolam on the circadian clock of hamsters free-running in constant light. Midazolam injections produced maximal phase advances at circadian time (CT) 6 and 9 and maximal phase delays at CT 15 and 21. Doses of 2.5 mg or larger produced phase shifts that were significantly different from those produced by the vehicle controls. In addition, the phase-shifting effects of midazolam were completely blocked by administration of the benzodiazepine receptor antagonist, RO 15-1788, indicating that the phase-shifting actions of midazolam are mediated via benzodiazepine receptors. These results indicate that the previously reported effects of triazolam on the circadian clock can be generalized to other short-acting benzodiazepines.

Benzodiazepine Circadian clock RO 15-1788 Midazolam Phase shift Benzodiazepine receptor antagonist Triazolam Phase-response curve Locomotor activity

THE benzodiazepines are a class of drugs which are widely used for the treatment of anxiety, insomnia, anxious depression, convulsions, and psychosomatic disorders (9). Treatment with benzodiazepines has been found to induce changes in both behavioral and endocrine circadian rhythms in hamsters (15, 27, 35, 37). In view of the fact that a number of mood and sleep disorders have been associated with abnormal circadian rhythmicity in humans [for review, see (13, 20, 38, 44)], the finding that benzodiazepines alter circadian rhythms in hamsters raises the possibility that at least some of the therapeutic effects of benzodiazepines may be due to alterations in the circadian system. In addition, drugs which can induce changes in circadian rhythmicity may be useful for the treatment of conditions associated with disrupted circadian synchronization such as with rotating work shifts and rapid travel across time zones.

To date, most of the published work on the effects of benzodiazepines on the circadian clock has focused on the short-acting benzodiazepine, 8-chloro-6-(o-chlorophenyl)-1-methyl-4H-s-triazolo[4,3-a][1,4]benzodiazepine, triazolam. A single injection of triazolam induces permanent phase shifts in the circadian rhythm of locomotor activity in golden hamsters free-running under constant conditions (35) and in the circadian rhythm of pituitary LH release [(37), also see (34)]. Both the magnitude and direction of these phase shifts are dependent upon the time when the drug is administered relative to the onset of locomotor activity. Triazolam's action at a specific benzodiazepine receptor is indicated by the observation that a benzodiazepine receptor antagonist, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxylate, RO 15-1788, completely blocked the phase-shifting effects of triazolam on the hamster circadian clock (42). Triazolam also has been found to facilitate the resynchronization of circadian rhythms following an 8-hour shift in the light-dark cycle in hamsters (41) and following an 8-hour shift in the sleep-wake cycle in humans (39).

While treatment with triazolam has major effects on the circadian system, the generalization of these effects to other short-acting benzodiazepines has received little attention. The benzodiazepine, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4benzodiazepin-2-one, diazepam, which has a much longer half-life than triazolam in humans (20–80 hours for diazepam versus 2–6 hours for triazolam) (7), has been reported to induce phase shifts in the circadian rhythm of locomotor activity of golden hamsters (15,29) and to block light-induced phase advances in the activity rhythm (27). However, no further results of the effects of

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diazepam on the circadian clock have been reported at this time. The present series of experiments were carried out to examine in detail the effects of another short-acting benzodiazepine, 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine, midazolam, on the circadian clock of the golden hamster.

METHOD

Animals and Housing

Adult male golden hamsters (*Mesocricetus auratus*) (Lak: LVG), purchased from Lakeview Hamster Colony (Newfield, NJ) were initially group housed under a 14 L:10 D light-dark cycle before being transferred to conditions of constant light (LL) (light intensity at the level of each cage = 1–60 lux). Upon transfer to LL, each animal was housed individually in a cage equipped with a running wheel to allow for the continuous recording of the circadian rhythm of locomotor activity. The animal was allowed to free-run under constant conditions for at least two weeks prior to drug treatment in order to establish a clear free-running rhythm before treatment. Food and water were available ad lib. The average body weight of these hamsters was approximately 150 grams at the time of drug treatment.

Drug Administration and Measurement of Locomotor Activity

All injections were administered intraperitoneally in a volume of 0.1 cc/animal. The vehicle was dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). Most animals received more than one injection, and consecutive injections were separated by at least two weeks. Some animals were used in more than one experiment and/or contributed more than one data point to the same experiment.

The phase-shifting effects of the vehicle or drug injections on the circadian rhythm of wheel-running activity were assessed by visual inspection of the chart record of each animal. Using the daily onsets of wheel-running activity for the 7–10 days preceding an injection as phase reference points, a time of activity onset for the succeeding day was projected (time 1). In addition, the daily onsets of activity for the 7–10 days after the injection (i.e., once a new steady state was achieved) were used as phase reference points, and a time of activity onset for the cycle following the injection was projected back (time 2). Time 2 was subtracted from time 1, yielding the magnitude of the phase shift of activity induced by the injection. Thus, a positive value indicates an advance and a negative value indicates a delay in the onset of the activity rhythm. This method of analyzing phase shifts has been validated by regression analysis (3). Phase shifts were measured to the nearest five minutes.

Experiments

Experiment 1: Effects of a single injection of midazolam at different circadian times on the biological clock. Hamsters were injected with a single injection of midazolam (2.5 mg) or vehicle (0.1 cc) at circadian times 0, 3, 6, 9, 12, 15, 18, and 21, with circadian time (CT) 12 being defined as the onset of locomotor activity. Each group consisted of 7–9 animals.

Experiment 2: Dose-response curve for the phase-shifting effects of a single injection of midazolam. Hamsters were injected with either 0.1 cc of vehicle or one of the following doses of midazolam: 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, or 10.0 mg. All injections were timed to occur 6 hours before the onset of activity (CT 6), a time when maximal phase advances were produced in Experiment 1. Each group consisted of 7–10 animals.

Experiment 3: Use of the benzodiazepine receptor antagonist, RO 15-1788, to block the phase-advancing effects of midazolam. A dose of 5.0 mg RO 15-1788 was used, because this dose effectively blocked the phase-shifting effects of triazolam (42). All animals received two injections, the first occurring 15 minutes prior to the second injection which was given at CT 6. The following four treatment groups were used: 1) vehicle + vehicle, 2) vehicle + midazolam (2.5 mg), 3) RO 15-1788 + midazolam (2.5 mg), 4) RO 15-1788 + vehicle. Each group consisted of 8–9 animals.

Statistics

In Experiments 1–3, one-way and/or two-way analysis of variance (ANOVA) followed by Dunnett's test were used to analyze the data. Data for the dose-response curve (Fig. 3) were approximated to the best-fit curve generated by the program ALLFIT (4), and EC_{50} and ED_{50} values were determined.

Because multiple injections were given to some of the animals, the possibility exists that phase shift was affected by the number of injections the animal received. Therefore, for each analysis of variance, an analysis of covariance was done, in which the number of the injection (first, second, . . . fifth) was a covariate. In none of the three experiments was the covariate significant.

RESULTS

Experiment 1

A single injection of midazolam produced clear and reliable phase shifts in the circadian rhythm of locomotor activity that were dependent upon the circadian time of administration (Fig. 1). Phase-response curves were generated for animals injected with either vehicle or midazolam at different circadian times (Fig. 2). The magnitude of phase shifts produced by injections of vehicle alone did not differ for different phases of the circadian cycle [one-way ANOVA, $F(7,52) = 1.02$, $p = 0.43$]. Further, the mean of all vehicle injections was not significantly different from zero, $F(1,52) = 0.00$, $p = 0.96$. Analysis of control and experimental data together in a two-way ANOVA revealed significant effects of circadian time, $F(7,108) = 13.77$, $p < 0.001$, and interaction of midazolam treatment with circadian time, $F(7,108) = 13.30$, $p < 0.001$. Post hoc tests comparing phase shifts produced by midazolam to the average phase shift of all vehicle injections revealed that significant phase advances were obtained by injections of midazolam at CT 6 and 9 (Dunnett's, $p < 0.001$), and significant phase delays were produced by injections of midazolam at CT 15 and 21 ($p < 0.05$).

Experiment 2

Phase shift was significantly affected by dose of midazolam [one-way ANOVA, $F(10,73) = 13.25$, $p < 0.001$]. Phase shifts produced by injections of 2.5 mg or larger were significantly greater than phase shifts produced by vehicle injections at CT 6 (Dunnett's, $p < 0.001$, for 2.5 mg, 7.5 mg, and 10.0; $p < 0.01$ for 5.0 mg) (Fig. 3). In contrast, phase shifts produced by doses of less than 2.5 mg of midazolam were not significantly different from vehicle-induced phase shifts. The phase advances produced by doses of 2.5 mg or larger averaged 60–90 minutes. Using the program ALLFIT, the data were statistically fit to the curve shown in Fig. 3A, and the EC_{50} was determined to be 1.51 ± 0.380 mg.

To examine the proportion of animals responding to midazolam at different doses, we chose a threshold value for phase shifts to classify animals as responders. The mean and standard deviation for phase shifts produced by vehicle injection (Experiment 1) was 0.167 ± 8.6 minutes ($n = 60$, pooled across all circadian times).

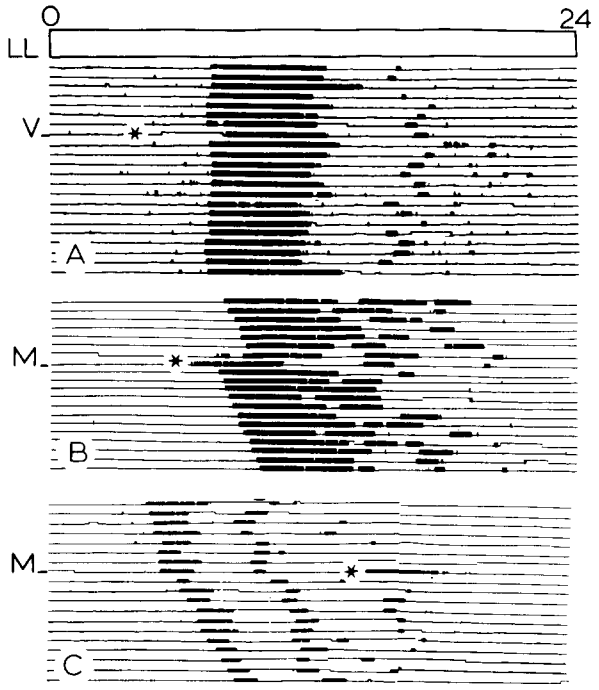

FIG. 1. Portions of wheel-running activity records from three hamsters free-running in constant light (LL). Each horizontal line depicts the activity pattern over a 24-hour period. Successive days are plotted from top to bottom. On the days indicated, each hamster was given an injection at the time designated by the star. Circadian time (CT) 12 is defined as the onset of locomotor activity. (A) An injection of vehicle (V) at CT 9 had no permanent effect on the phase of the activity rhythm. (B) An injection of midazolam (M) at CT 9 produced a phase advance of the activity rhythm. (C) An injection of midazolam at CT 21 produced a phase delay of the activity rhythm.

Twenty-five minutes is approximately three times the standard deviation of the vehicle-induced phase shifts, and this value was chosen as the threshold for classifying animals as responders to midazolam injections. Phase advances of at least 25 minutes were observed in 0/7, 2/8, 0/7, 0/7, 3/7, 4/10, 7/7, 6/7, 8/8, and 7/7 animals receiving injections of 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 mg of midazolam, respectively. The curve generated by ALLFIT is shown in Fig. 3B, and the ED_{50} was determined to be 0.847 ± 0.169 mg.

Experiment 3

The results from Experiments 1 and 2 demonstrated that maximal phase advances can be produced by injections of 2.5 mg of midazolam at CT 6. This information was used to determine if the benzodiazepine receptor antagonist, RO 15-1788, could block the phase advances produced by midazolam. Two-way ANOVA showed a significant interaction effect between midazolam and RO 15-1788, $F(1,30) = 21.98$, $p<0.001$. Post hoc comparisons between the vehicle-vehicle control groups and the other three groups showed that midazolam produced significant phase advances (Dunnett's test, $p<0.001$), but the administration of RO 15-1788 did not produce phase shifts that were significantly larger than those produced by vehicle alone (Figs. 4, 5). Further, comparisons between the vehicle-midazolam control and the RO 15-1788-midazolam groups showed that 5 mg of RO 15-1788 administered 15 minutes prior to injection of midazolam completely blocked the midazolam-induced phase advances.

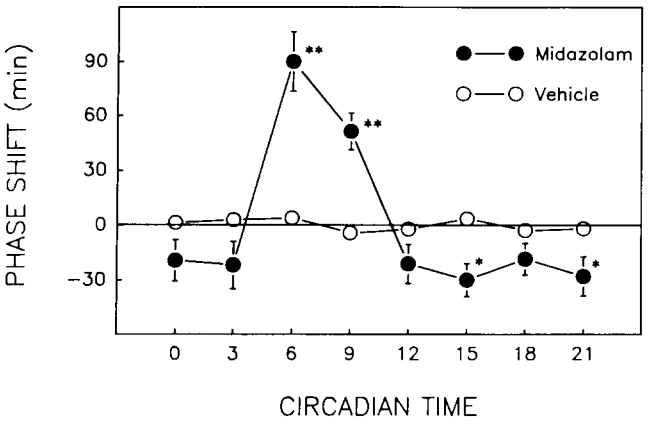

FIG. 2. Phase-response curves describing the phase-shifting effects of midazolam (●) or vehicle (○) on the circadian rhythm of locomotor activity in hamsters free-running in constant light. The mean (±S.E.M.) phase shift (in minutes) produced by an intraperitoneal injection of midazolam (2.5 mg) or vehicle (0.1 cc) is plotted versus the circadian time of administration. The onset of locomotor activity is designated as circadian time (CT) 12. A value below the solid line indicates a phase delay in the activity rhythm; a value above the line indicates a phase advance. Phase shifts to midazolam injections were compared (two-way ANOVA, followed by Dunnett's test) to the average of all phase shifts produced by intraperitoneal injections of the vehicle (*$p<0.05$, **$p<0.001$). The SEM bars for the vehicle injections are contained within the open symbols.

DISCUSSION

The results reported here establish that intraperitoneal injections of midazolam perturb the circadian clock of the golden hamster in a manner which is dependent upon the circadian time of administration. Doses of 2.5 mg or larger consistently produced maximal phase advances when administered at CT 6. Further, midazolam appears to be acting at a benzodiazepine receptor, because RO 15-1788, a highly selective benzodiazepine receptor antagonist (16), blocked the phase-advancing effects of midazolam.

The effects of midazolam on the circadian clock of the hamster are similar to those observed following treatment with triazolam. For example, the phase-response curves for intraperitoneal injections of midazolam and triazolam in hamsters free-running in LL differ only in the absolute amplitudes of the phase shifts, but the direction of the phase shifts at each circadian time is the same [compare with (35)]. The dose-response curve for midazolam (Fig. 3A) shows that doses of 2.5 mg or more are needed to produce a maximal phase advance at CT 6. In comparison, doses of 0.5 mg of triazolam produce maximal phase advances at CT 6 (36). However, the difference in the dose-response curves may be due to differences in protocol, because the midazolam dose-response curve was measured for hamsters free-running in constant light, whereas the dose-response curve for triazolam was measured for animals free-running in constant darkness. Finally, 5.0 mg of the benzodiazepine receptor antagonist, RO 15-1788, effectively blocked the phase advances produced by injections at CT 6 of either midazolam (Fig. 5) or triazolam (42).

The benzodiazepines are a class of drugs which are similar in structure, but vary in their physicochemical properties, routes and rates of metabolism and clearance, and patterns of distribution (7). Midazolam, an imidazobenzodiazepine whose salts are soluble and stable in aqueous solution (24), is structurally very similar to

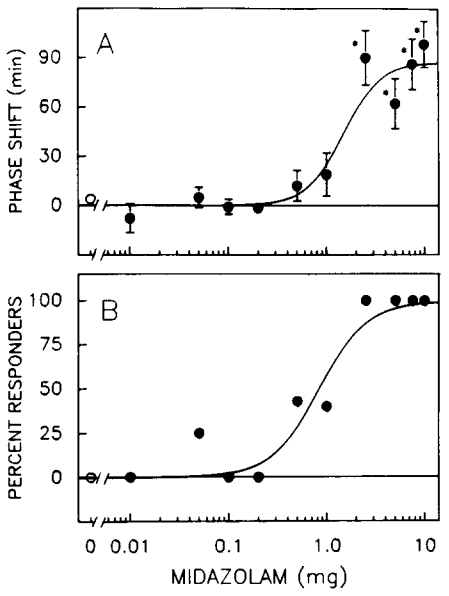

FIG. 3. Mean phase shifts and percent of animals responding to various doses of midazolam. (A) The mean (±S.E.M.) phase shift (in minutes) induced by an intraperitoneal injection of vehicle (○) or various doses of midazolam in hamsters free-running in constant light. All injections were given at CT 6, 6 hours prior to the onset of locomotor activity. *Significantly greater than phase shifts produced by vehicle injections at CT 6 ($p<0.01$, one-way ANOVA). The value of the mean phase shift in response to injections of 2.5 mg of midazolam was taken from CT 6 in Fig. 2. With no constraints used, the data were statistically fit to the curve generated by ALLFIT, and the EC_{50} was determined to be 1.51 ± 0.380 mg. (B) The percent of animals that responded with phase shifts of at least 25 minutes to intraperitoneal injections of midazolam, as described in text. Using the ALLFIT program, constraints were set at 0 and 100 as the minimum and maximum percent. The ALLFIT-generated curve indicates that the ED_{50} was 0.847 ± 0.169 mg.

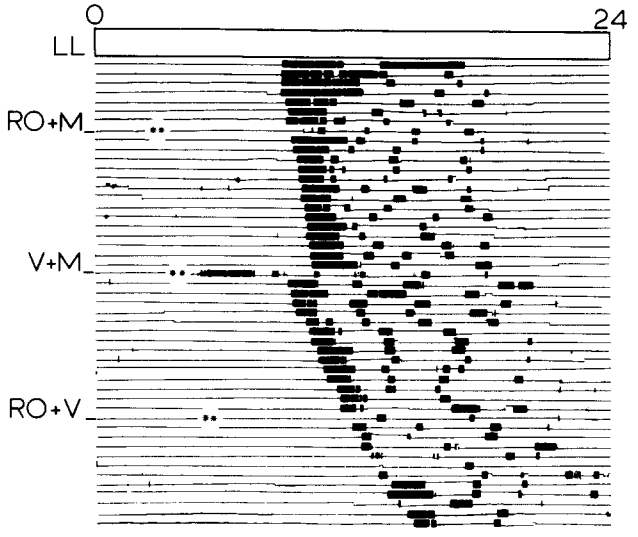

FIG. 4. Portion of a wheel-running activity record from a single hamster free-running in constant light (LL). On three occasions the animal was given two intraperitoneal injections indicated by the double asterisks. The injections were given fifteen minutes apart, with the second injection timed to occur at CT 6. RO = RO 15-1788 (5.0 mg), M = midazolam (2.5 mg), V = DMSO vehicle (0.1 cc). Injection of RO 15-1788 not only blocked the phase-advancing effects of midazolam, but it also blocked the burst of activity which followed the injection of midazolam (compare RO + M with V + M). Injection of RO 15-1788 plus vehicle did not alter the phase of the activity rhythm. See Fig. 1 legend for further details.

triazolam, differing only by the substitution of a fluorine in the place of a chlorine. Midazolam has greater lipid solubility, is more extensively plasma protein-bound, and has approximately the same or a slightly shorter elimination half-life than triazolam [see review by (7)]. All of these factors may have contributed to the differences observed between the dose-response curves for triazolam and midazolam. A lower potency of midazolam than triazolam with respect to the phase-shifting effects of the two drugs would be consistent with results from a variety of preclinical tests. Midazolam was about 1/10 as active as triazolam in motor performance tests (e.g., rotarod and chimney tests) in mice, approximately 1/50 as potent as triazolam for prevention of tonic seizures induced by 3-mercaptopropionic acid and pentetrazole in mice, and approximately 1/100 as potent as triazolam in its effects on the sleep-wakefulness cycle in rabbits (24). Despite differences in potency, midazolam produces all of the characteristic effects of the benzodiazepine class, including anticonvulsant, sleep-inducing, muscle relaxant, anticonflict, anxiolytic, and "sedative" effects, and midazolam also interacts with specific high-affinity binding sites for benzodiazepines [(24,25), also see review by (26)].

RO 15-1788, a pure, specific benzodiazepine receptor blocker, very potently and selectively prevents or abolishes in a competitive manner all major actions of the benzodiazepines in the central nervous system [(9,16), also see reviews by (2,26)]. The selectivity of RO 15-1788 is very high; the blockade of receptors other than benzodiazepine receptors has not been observed up to subtoxic doses (10). Five mg of RO 15-1788 completely blocked the phase-advancing effects of midazolam in the present study and also effectively blocked the phase-advancing as well as the phase-delaying effects of triazolam (42). The antagonist properties of RO 15-1788 are dose-dependent, as doses of 0.1 or 1.0 mg were ineffective in blocking the phase-shifting effects of triazolam (42). Although RO 15-1788 alone did not have any effect on the circadian clock [Figs. 4, 5, this study; (42)], a variety of studies have provided evidence either for or against the idea that RO

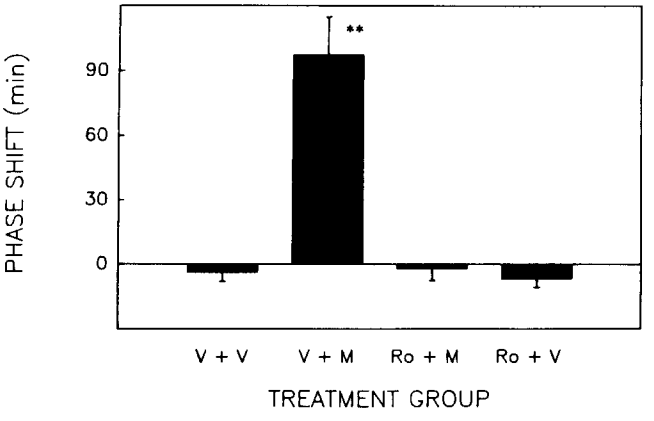

FIG. 5. Mean (±S.E.M.) phase shifts (in minutes) in the circadian rhythm of locomotor activity of hamsters free-running in constant light that received an intraperitoneal injection of either vehicle (V) or RO 15-1788 (RO) (5.0 mg) followed 15 minutes later by a second injection of either vehicle or midazolam (M) (2.5 mg) at CT 6. **Significantly different from RO + M ($p<0.001$, two-way ANOVA and Dunnett's test).

15-1788 has benzodiazepine agonist properties [(6, 9, 16, 17, 23, 43), also see (29), and review by (5)].

In the present study, RO 15-1788 blocked not only the phase-advancing effects of midazolam, but also the burst of activity following injection of the drug (see Fig. 4). Recent evidence has suggested that the phase-shifting effects of various chemicals are related to their ability to alter behavioral states, and the increased arousal following chemical treatment may be responsible for the phase shifts (21,22). Whether or not the phase shifts induced by midazolam are mediated by the increase in arousal following treatment is unclear at this time.

The benzodiazepines are believed to produce their pharmacological and clinical actions by combining with a benzodiazepine receptor which is structurally and functionally coupled to a gamma-aminobutyric acid (GABA) receptor (1,12). A generally accepted hypothesis is that the benzodiazepines act by enhancing GABAergic transmission, and this enhancement of GABA action involves benzodiazepine facilitation of chloride channel opening by GABA [see reviews by (8, 11, 12, 18)].

The effects of the benzodiazepines on the circadian clock also may be mediated via GABAergic transmission. The administration of the GABA-A agonist, muscimol, into the area of the suprachiasmatic nucleus (SCN) of the hypothalamus, induced phase shifts in the circadian rhythm of locomotor activity of blind, free-running hamsters that are similar in direction to the phase shifts induced by triazolam or midazolam (32). The SCN, a major pacemaker for the generation of many circadian rhythms in mammals (19,30), has been shown to contain a rich network of GABA fibers and cell bodies (40). In addition, both GABA and benzodiazepine receptor sites are present in the lateral geniculate nucleus (14,31), which sends afferents to the SCN (28,33). However, despite this evidence in support of GABAergic transmission as a mechanism for the benzodiazepine's phase-shifting effects, the site of action of benzodiazepines on the mammalian circadian clock remains unknown.

In conclusion, the results presented here indicate that the phase-shifting effects on the circadian rhythm of locomotor activity reported previously for triazolam (34,35) can be generalized to another short-acting benzodiazepine. In addition, the observation of antagonism by RO 15-1788 of both midazolam and triazolam-induced phase shifts supports the hypothesis that both of these benzodiazepines are exerting their effects on the circadian system via a GABA/benzodiazepine receptor. These results should provide the basis for further studies with midazolam and the circadian system in animals, as well as for studies involving attempts to manipulate the human circadian clock.

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