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Daily Rhythms in Spontaneous and Diazepam-Induced Anxiolysis in Syrian Hamsters

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YANNIELLI, P. C., B. I. KANTEREWICZ AND D. P. CARDINALI. *Daily rhythms in spontaneous and diazepam-induced anxiolysis in Syrian hamsters.* PHARMACOL BIOCHEM BEHAV **54**(4) 651–656, 1996. – The diurnal variations in spontaneous and diazepam-induced anxiolysis and exploratory behavior were examined in Syrian hamsters in a plus-maze paradigm. The administration of diazepam or flunitrazepam augmented the percentage of time spent in the open arms, the percentage of entries to the open arms, and the number of crosses to both arms, whereas ethyl-β-carboline injection decreased them. These three behavioral parameters showed significant daily variations, with the maxima being found at night (2400–0400 h). Flumazenil (5 mg/kg) injected at 0400 h decreased significantly the percentage of time spent in open arms and of entries to the open arms, without affecting significantly the total number of entries to both arms. Day-night differences in anxiety-related behavior persisted in hamsters kept under constant darkness for 3 days. Diazepam (0.5 mg/kg) increased the time spent in the open arms at 1600 and 2000 h only, and augmented the percent of entries to the open arms at 2000 h only. The total number of entries to both arms was augmented significantly by diazepam at all time intervals tested, except for 0400 h. The results indicate that Syrian hamsters exhibited significant diurnal changes in anxiolysis-related behavior in the plus-maze paradigm.

Diurnal rhythms Diazepam Anxiolysis Exploratory behavior Time-dependent effects

DAILY rhythms in biochemical and physiologic processes are widespread phenomena. In general, the rhythmicity is endogenous, resulting from the oscillatory activity of the suprachiasmatic nuclei (SCN) of the hypothalamus (26,28,34). Almost every neuron in SCN contains γ-aminobutyric acid (GABA), and the amino acid is considered a major transmitter for the circadian apparatus (29).

In a previous study we reported the existence of circadian modifications in GABA turnover rate of CNS regions of the Syrian hamster, including the preoptic-medial basal hypothalamic area (25). Under long days (i.e., under a daily 14 L : 10 D photoperiod), daily rhythm in the turnover of GABA in cerebral cortex, basal hypothalamus, cerebellum, and pineal gland exhibited clear phase relationships, with maximal values at night.

Because brain GABAergic function has been linked to anxiety-related behavior in a number of experimental and clinical studies (3,23,33,35), we considered it worthwhile to study whether spontaneous and drug-induced anxiolysis varied diurnally in Syrian hamsters as assessed in a plus-maze paradigm. Specifically, we sought to answer the following questions: a) Does the Syrian hamster exhibit typical anxiolytic and proexploratory behavior in the plus-maze? b) Does this behavior show a diurnal rhythmicity compatible with the circadian rhythms in brain GABA turnover previously reported? c) Do anxiolytic and proexploratory activities of diazepam in Syrian hamsters display a circadian rhythmicity?

METHOD

Animals

Male Syrian hamsters (*Mesocricetus auratus*, 100–150 g) were raised in our colony under a 14 L : 10 D daily photoperiod (lights on at 0600 h) and had free access to Purina chow

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(BioServ, Frenchtown, NJ) and water. An additional group of animals was isolated from the colony and maintained for 3 days in constant darkness before the tests.

Drugs

Diazepam, flunitrazepam, and flumazenil (kindly provided by Productos Roche S.A., Argentina), and ethyl-β-carboline (purchased from Sigma Chemical Co., St. Louis, MO) were dissolved in 50% dimethyl sulfoxide (DMSO), and 100 μl of the solution was injected IP. For all experiments, controls received 50% DMSO. In an initial experiment comparing DMSO-injected with noninjected animals, no differences in plus-maze behavior were detected between both groups (time spent in open arms: DMSO-injected: 11.9 ± 3.4%, untreated: 14.7 ± 3.6%; entries to open arms: DMSO-injected: 31 ± 6.8%, untreated: 49 ± 5.3%; total entries to both arms: DMSO-injected: 5.3 ± 1.3, untreated: 5.7 ± 2.3, mean ± SEM, n = 7-8/group, Mann-Whitney test: $p > 0.1$).

Experimental Procedure

An elevated plus-maze was built as described by Pellow and co-workers (31). The apparatus, made of wood, was composed of two open arms, 50 × 10 cm, and two closed arms, 50 × 10 × 40 cm; each pair of arms was opposed. The maze was suspended 50 cm above the floor. The rationale for the procedure employed relied on the observation made in rats that animals tend to enter and stay mainly in the closed arms, so that any increase in the time spent on the open arms or, additionally, in the number of entries to the open arms, was considered indices of an anxiolytic effect (14,31). Validation of the procedure for Syrian hamsters is described in Results.

Measurement of diurnal variation in anxiolytic behavior of hamsters was made at 0800, 1200, 1600, 2000, 2400, or 0400 h, under the same lighting conditions that the hamsters had in the breeding room (i.e., bright light or dim red light for animals studied at daytime or nocturnal intervals, respectively). Animals were handled, injected, and placed in individual home cages. Groups of seven to nine animals receiving diazepam (0.5 mg/kg) or vehicle (50% DMSO) were tested in the plus-maze 20 min after drug administration. The effect of flumazenil on nocturnal anxiolytic behavior in hamsters was assessed at 0400 h. As for diazepam, groups of seven to nine animals receiving flumazenil (5 mg/kg) or vehicle were tested 10 min after administration.

Each experiment was repeated two or three times. A different group of animals was used for each measurement at each time point. A total number of 230 hamsters were used for the study.

Animal behavior in the plus-maze was recorded in 5-min trials. The number of entries to the open or closed arms of the maze, as well as the time spent in each arm, were recorded. Percentage time on the open arms, and the number of crosses to the open arms, were taken as an index of anxiolysis; the total number of entries to open and closed arms was used as an index of the exploratory behavior of the animals.

Statistical Analyses

Results were statistically analyzed by nonparametric procedures, as data did not fulfill the assumption of normality. Either a Mann-Whitney test (for differences between control and treatment) or Kruskall-Wallis test followed by Dunn's multicomparison test (for differences between time points) was employed.

TABLE 1

SPECIFIC BEHAVIORS SHOWN BY SYRIAN HAMSTERS IN OPEN OR CLOSED ARMS OF PLUS-MAZE

	Open Arms	Closed Arms
Rearing	2.5 ± 1.9	18.0 ± 2.2
Grooming	1.2 ± 0.5	5.5 ± 3.1
Freezing	Present	Absent

Shown are means ± SEM (n = 7-9 animals/group) of number of behavioral events in Syrian hamsters confined for 20 min to an open or closed arm of plus-maze. Differences between open and closed arms are significant ($p < 0.001$, Mann-Whitney test).

TABLE 2

EFFECT OF BZP AND ETHYL-β-CARBOLINE ON BEHAVIOR OF SYRIAN HAMSTERS IN A PLUS-MAZE

	% Time in Open Arms	% Entries to Open Arms	No. Entries to Both Arms
Experiment 1			
Vehicle	10.8 ± 10.9	29.3 ± 12.9	10.1 ± 7.9
Diazepam (2 mg/kg; 20 min earlier)	44.1 ± 17.0*	53.3 ± 4.6*	29.6 ± 7.2*
Experiment 2			
Vehicle	18.4 ± 7.6	44.5 ± 22.3	7.7 ± 4.9
Flunitrazepam (0.5 mg/kg; 20 min earlier)	31.4 ± 11.7*	49.9 ± 13.9	29.7 ± 5.8*
Experiment 3			
Vehicle	23.1 ± 3.7	46.9 ± 3.7	18.9 ± 6.2
Ethyl-β-carboline (2 mg/kg; 10 min earlier)	9.2 ± 7.3*	31.4 ± 15.8*	7.1 ± 3.0*
Experiment 4			
Vehicle	9.6 ± 2.4	16.6 ± 6.1	11.6 ± 4.1
Ethyl-β-carboline (2 mg/kg; 5 min earlier)	4.4 ± 5.7	2.0 ± 4.5*	4.0 ± 3.5*

Each drug or the vehicle (50% DMSO) was injected IP between 1400 and 1800 h, and the behavior was tested in a plus-maze as described in Method. Shown are the means ± SEM (n = 7-9 animals/group).

*Significant differences ($p < 0.05$) compared to the respective vehicle-injected control (Mann-Whitney test).

RESULTS

Because the plus-maze procedure to examine anxiolysis in the Syrian hamster had not been validated before, such a validation was performed following criteria described in rats by Pellow et al. (2,30). All measurements related to validation of the model were done between 1400 and 1800 h. Behavioral parameters in Syrian hamsters confined for 20 min to an open or closed arm of plus-maze are summarized in Table 1. Hamsters exhibited behavior closely similar to that of rats (e.g., grooming, rearing, and freezing). Frequency of grooming and rearing was significantly higher in hamsters confined to the closed arm, whereas freezing (an anxiety-related behavior) was only seen in hamsters staying in an open arm.

As shown in Table 2, diazepam (2 mg/kg) significantly augmented the percentage of time that hamsters spent in the open arms, as well as the percentage of entries to the open arms (as related to the total number of entries). Flunitrazepam (0.5 mg/kg) significantly augmented the percentage of time spent in open arms. Both diazepam and flunitrazepam augmented the total number of entries to the plus-maze arms. Conversely, the anxiogenic compound ethyl-β-carboline (2 mg/kg) decreased the percentage of time spent in open arms and entries to the open arms, and the total number of entries to both arms; the effect was found either 5 or 10 min after ethyl-β-carboline injection.

Figure 1 depicts the diurnal variation in anxiety-related behavior in Syrian hamsters. The percentage of time spent in open arms and entries to the open arms, and the total number of entries to both arms showed significant daily variations (Kruskal–Wallis test, KW value corrected for ties = 35.02, 19.6, and 24.74, respectively, $p < 0.001$). Maxima for the three parameters were found at 2400–0400 h. Daily changes of behavior in untreated and vehicle-injected hamsters were essentially similar (results not shown). As shown in Fig. 2, injection of 5 mg/kg of flumazenil at 0400 h resulted in a significant decrease of percentage of time spent in open arms and of entries to the open arms ($p < 0.005$, Mann–Whitney test) without affecting significantly the total number of entries to both arms.

Table 3 summarizes the results of an experiment designed to examine day–night differences in anxiety-related behavior in hamsters kept for 3 days in a dark environment. Day–night differences in the percentage of time spent in open arms and

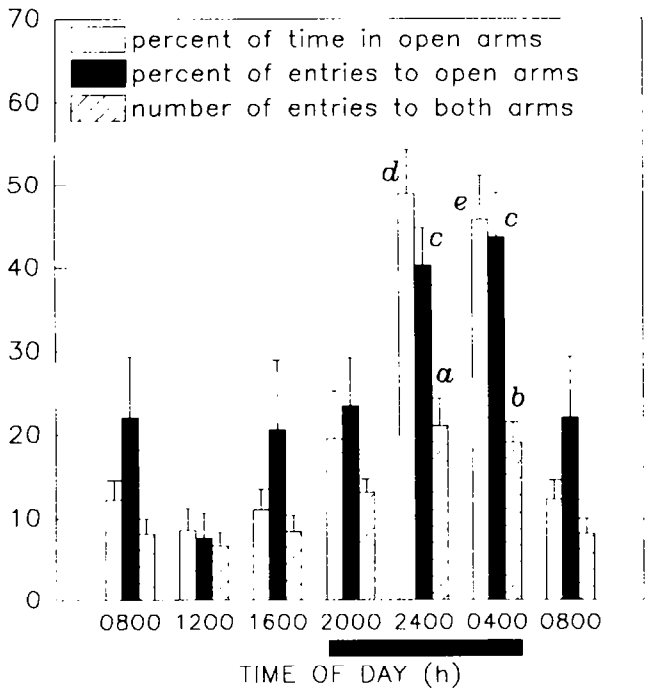

FIG. 1. Diurnal variation in anxiety-related behavior in Syrian hamsters, as assessed in a plus-maze. Shown are the means ± SEM (n = 7–9 animals/group) of behavioral parameters, tested as described in Method. Kruskal–Wallis test followed by Dunn's test showed the following significant differences as a function of time: $^{a}p < 0.01$ vs. 1200 h, $p < 0.05$ vs. 0800 and 1600 h; $^{b}p < 0.05$ vs. 1200 h; $^{c}p < 0.01$ vs. 1200 h; $^{d}p < 0.001$ vs. 1200 h, $p < 0.01$ vs. 0800 and 1600 h; $^{e}p < 0.001$ vs. 1200 h, $p < 0.01$ vs. 1600 h, $p < 0.05$ vs. 0800 h.

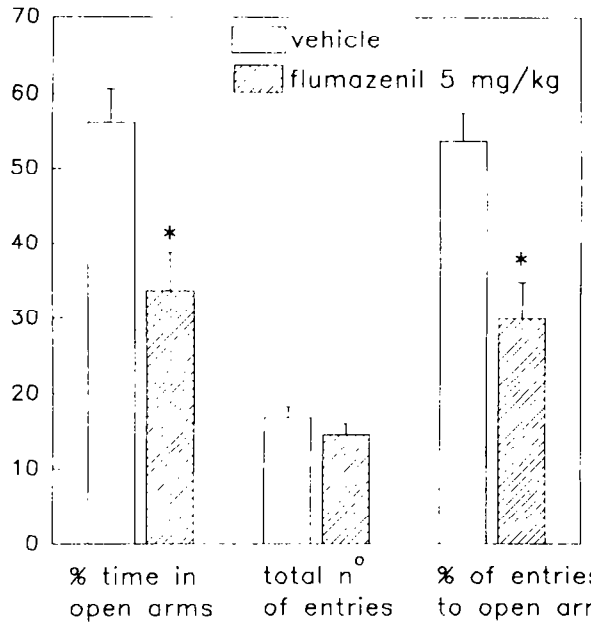

FIG. 2. Effect of flumazenil (5 mg/kg) on anxiety-related behavior in Syrian hamsters, as assessed in a plus-maze at 0400 h. Shown are the means ± SEM (n = 7–9 animals/group) of behavioral parameters, tested as described in Method. Flumazenil or vehicle was injected IP, and animals were tested 10 min later. *Significant differences compared to vehicle ($p < 0.005$, Mann–Whitney test).

TABLE 3

DAY–NIGHT DIFFERENCES IN BEHAVIOR OF SYRIAN HAMSTERS IN A PLUS-MAZE AFTER 3 DAYS OF CONSTANT DARKNESS EXPOSURE

	% Time in Open Arms	% Entries to Open Arms	No. Entries to Both Arms
1600 h	17.4 ± 5.7	14.4 ± 5.5	9.4 ± 1.7
0400 h	57.4 ± 4.8*	51.4 ± 3.4†	21.4 ± 1.5‡

Shown are the means ± SEM (n = 7–9 animals/group) of behavioral parameters tested as described in Method. Significant differences: *$p < 0.01$; †$p < 0.05$; ‡$p < 0.001$ compared to the 1600-h group (Mann–Whitney test).

of entries to open arms, or in the total number of arm entries persisted in animals under constant darkness.

Figure 3 depicts the diurnal variation in the anxiolytic activity of diazepam (0.5 mg/kg) in hamsters as assessed in the plus-maze. Diazepam increased significantly the time spent in the open arms at 1600 and 2000 h (Fig. 3A), whereas it augmented the percent of entries to the open arms at 2000 h only (Fig. 3B). The total number of entries to both arms increased significantly after diazepam at all time intervals tested, except for 0400 h (Fig. 3C).

DISCUSSION

The results indicate that the plus-maze is useful to assess anxiolytic and proexploratory activity in Syrian hamsters. A diurnal rhythmicity in anxiolysis, as revealed by the aug-

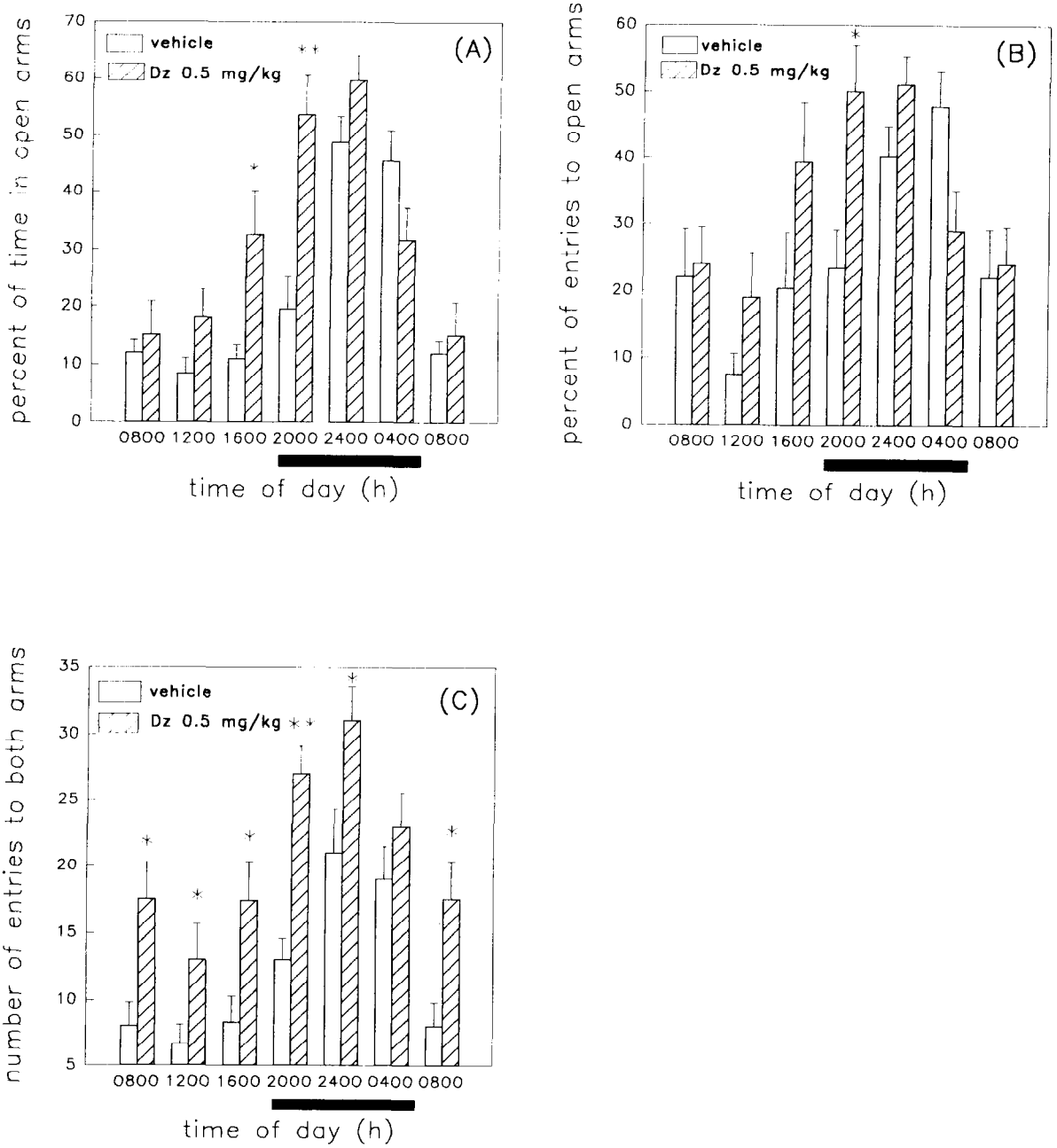

FIG. 3. Diurnal variation in the anxiolytic activity of diazepam (Dz) in Syrian hamsters as assessed in the plus-maze. Shown are the means ± SEM of behavioral parameters (n = 7–9 animals/group). Dz or the vehicle (50% DMSO) was injected IP at selected time intervals throughout a 24-h cycle, and the behavior was tested in the maze as described in Method, 20 min later. Significant differences: $*p < 0.05$; $**p < 0.01$, compared to vehicle (Mann–Whitney test).

mented percentage of time spent in open arms, or by the increase in the percentage of entries to the open arms in the plus-maze [both validated indicators of anxiolysis in rats (30)] was uncovered. The maxima for both parameters were found at night. Although these maxima coincided with the peak in locomotor and proexploratory behavior found in nocturnally active hamsters, it is interesting that in the rat, which is also a nocturnally active rodent, no diurnal change in anxiolytic-related behavior was apparent (21). Moreover, injection of the central benzodiazepine (BZP) antagonist flumazenil at 0400 h brought about a dissociation between anxiolytic and locomotor activities by significantly decreasing the former without modifying significantly the latter. In view of the link between anxiety and brain GABAergic function (3,23,33,35), and because we previously reported a diurnal rhythmicity in brain GABA turnover in Syrian hamsters with maxima at night (25), our results suggest a possible correlation between anxiolytic-related behavior and brain GABA turnover.

Some animal models of anxiety are based on the aversive properties of light to induce the anxiogenic state (5,10). Thus, we wished to know whether the diurnal rhythmicity in behavior found in hamsters persisted after keeping the animals in a constant dark environment. Inasmuch as the expected time-dependent difference in anxiolysis-related behavior was found after 3 days of exposure to a constantly dark environment, we concluded that daily changes in behavior depended on endogenous mechanisms and were not merely the result of the anxiogenic effect of bright light exposure.

In the context of circadian organization, the effects of several drugs on the brain, measured by their behavioral consequences, change during the day (13,18–22). Previous studies indicated that a number of pharmacologic agents (e.g., agonists and inverse agonists of GABA type A receptor) and non-pharmacologic procedures (e.g., handling) modify animal behavior in the plus-maze (1,6,7,17,31). Also, diurnal variations in the effect of some drugs have been reported (2).

As in rats (21), we found daily variations in the anxiolytic response to BZP in hamsters. Diazepam (0.5 mg/kg) increased significantly the percentage of time spent in the open arms as well as that of entries to the open arms when evaluated at 1600 and 2000 h or at 2000 h, respectively.

A possible criticism for interpreting the results in terms of diurnal changes of anxiety-related behavior is that the total number of arm entries were elevated during the dark phase; the animals therefore exhibited greater exploratory locomotion on both open and closed arms. Indeed, some authors have pointed out that measurements of anxiolysis could be overinterpreted when total number of entries became modified (24,30). However, some indications allowed us to dissociate between activity and anxiolysis profiles. For example, diazepam induced an increment in total activity at 0800, 1200, and 2400 h without affecting the percentage of time spent in open arms or the number of entries to open arms. Moreover, flumazenil (5 mg/kg), when administered at 0400 h, selectively diminished anxiolytic-related parameters without affecting total number of entries. This suggested that although an increase in locomotor activity is found after administering low doses of BZP (8,21), arm preference index in plus-maze, as well as the percentage of time spent in open arms in the absence of sedation, give valid measurements of anxiolysis even when the number of entries to both arms increases (24). Collectively, the data indicate that an increase in the total number of entries is not necessarily linked to an increase in anxiolytic parameters.

Because in previous studies we observed a maximal GABAergic activity during the dark phase of daily photoperiod in Syrian hamsters, as revealed by the increase in brain GABA turnover (25) or the increase in the affinity and biologic response of chloride ionophore to GABA in cortical membranes (B. I. Kanterewicz, P. C. Yannielli, D. P. Cardinali, unpublished results), the results suggest a correlation between an augmented anxiolytic-like state (endogenous anxiolysis due to an increased GABAergic tone) and the lack of anxiolytic activity of diazepam during the night. To what extent the changes in activity found were related to changes in diazepam pharmacokinetics deserves to be explored further.

Several studies have indicated that a number of endogenous substances influence the behavioral manifestations of anxiety in animals (4,9,11,27). For example, File and Pellow (15) and De Robertis et al. (12) postulated the existence of two BZP receptor-mediated systems in the brain, one activated by endogenous BZP-like compounds and the other by endogenous β-carboline-like compounds. In addition to the neurochemical data indicating an augmented GABAergic tone at night (25), a predominant endogenous BZP tone may also occur, as indicated by the observation that hamsters administered with the specific central type BZP antagonist flumazenil exhibited 40% inhibition of anxiolysis-related behavior in the plus-maze when injected at night. Another endogenous modulator of GABAergic activity could be melatonin, which displayed time-dependent anxiolytic activity in rats when examined in the plus-maze paradigm; this activity was maximal at night (21), coinciding with an augmented effect of melatonin on brain GABAergic neurons (32).

In summary, the questions posed earlier may now be answered. The Syrian hamster exhibited typical anxiolytic and proexploratory behavior in the plus-maze paradigm, with nocturnal maxima that correlated with the neurochemical changes of the brain GABAergic system reported previously (25). Day-night differences in behavior were still revealed after maintaining the animals in a constantly dark environment, suggesting the endogenous nature of the changes found. The anxiolytic activity of a small dose of diazepam also displayed a circadian rhythmicity, attaining maxima at the time when the GABAergic tone was minimal (i.e., late afternoon), whereas the proexploratory activity of diazepam was found at all examined times except for the 0400-h interval, when GABAergic activity attained its maximum. Collectively, the data indicate that the Syrian hamster may be an appropriate experimental model to examine the chronopharmacologic properties of BZP.

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