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Diazepam Modulates the Period of Locomotor Rhythm in Mice (*Mus booduga*) and Attenuates Light-Induced Phase Advances

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SUBRAMANIAN, P. AND R. SUBBARAJ. *Diazepam modulates the period of locomotor rhythm in mice (*Mus booduga*) and attenuates light-induced phase advances.* PHARMACOL BIOCHEM BEHAV **54**(2) 393-398, 1996.—Experiments were carried out on the continuous action of diazepam (benzodiazepine) offered through drinking water in 2% ethanol on ψ and τ of the activity rhythms under LD (12 : 12) and DD conditions. Under entraining conditions diazepam failed to evoke striking changes in ψ. On the other hand, under free-running conditions period-lengthening and period-shortening effects were observed. Further experiments conducted on the continuous effect of diazepam on light pulse evoked phase shifts revealed that phase advances were attenuated significantly in diazepam-treated animals at CT 20 and 24. These results were discussed with regard to the action of diazepam on the light sensitivity of the circadian pacemaker.

Circadian Diazepam Locomotor activity γ-Aminobutyric acid

IN recent years, progress has been made in the identification and functional characterization of neural pathways by which photoreceptors located in the retina (10) communicate with the suprachiasmatic nucleus (SCN), a major site of circadian rhythm generation (8). 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. (25); lateral geniculate nucleus: Hendrickson et al. (6); SCN: Card and Moore (2)]. Agents that are known to affect GABA neurotransmission have been known to affect circadian rhythmicity (15,17,23). For instance, diazepam, which can potentiate GABA activity through benzodiazepine receptors (11), was known to i) evoke phase shifts (7,20) and ii) to facilitate faster resynchronization of circadian rhythms to shifted light cycles (3).

In hindsight, the observation that a single injection of a benzodiazepine can induce a phase shift under constant conditions could indicate the treatment with benzodiazepines may also be capable of altering entrainment pattern (change in phase angle differences, etc.) of animals synchronized to an LD cycle. Additionally, the phase-shifting effects of diazepam raise the possibility that continuous administration of diazepam might modify the τ of the activity rhythm. To test these predictions, experiments were conducted on the effect of chronic administration of diazepam on locomotor rhythms under constant darkness and under LD (12 : 12) conditions in *M. booduga*.

Intraperitoneal injection of β-methyl carboline (benzodiazepine reverse agonist) with triazolam was found to sharply attenuate the phase advances (16) by light pulses in hamsters. β-Methyl carboline when given alone had no effect on the phase of the rhythm. The light pulse blocking effect of baclofen (agonist of $GABA_B$ receptor), bicuculline (antagonist of GABA), and diazepam were studied in detail by Ralph and Menaker (13–15) in the locomotor rhythms of hamsters.

These studies suggest that the neurotransmitter GABA may be involved in the mediation of some, but not all, light input to the mammalian circadian system (15). In the present study, the attenuation of light pulse evoked phase advances by diazepam is described on the Indian field mouse *Mus booduga*.

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METHOD

Animals and Housing

Adult male mice were captured from the fields surrounding the University campus. They were kept in a light-tight, temperature-controlled experimental cubicle maintained at 31 ± 1°C. Food comprised of millets, maize, grains, and water were available ad lib. Dim red light of 610–700 nm was used while feeding the animals (18) and the hours of routine care were varied. A magnet-activated switch was used to record wheel revolutions. The revolutions were picked up by channels of an A620 X Esterline Angus Event Recorder. Actograms were constructed and double plotted in a manner that is now routine in chronobiological research (4).

Experiment 1

Two studies were conducted in Experiment 1. In the *first* series of experiments animals were subjected to 12 L : 12 D cycle. Light intensity during L phase was about 10 lx. Animals were allowed to drink tap water for the first 3 weeks of experiment. Then animals were administered with diazepam (0.25 mg/ml) in 2% ethanol solution (9) (n = 10). This dose was selected because it is effective (17) and below the LD_{50} value (LD_{50} for diazepam in *M. booduga* = 3 mg/ml with average drug uptake of 1–2 ml/day) (17). Control animals were administered with 2% ethanol for 3 weeks (n = 5). Diazepam and ethanol solutions were replenished daily. After removing drug or ethanol solutions animals were allowed to drink tap water for 2–3 weeks. ψ changes before, during, and after administration of drug or ethanol were calculated and compared.

In the *second* series of experiments animals were allowed to free run in DD for 2–3 weeks. After that, the water bottles were replaced with 0.25 mg/ml diazepam solution (in 2% ethanol). Diazepam treatment continued for the consecutive days of the experiment (n = 31). Control animals were ingested with 2% ethanol solution (n = 7). Diazepam/ethanol treatments were continued for about 2–3 weeks. Final steady-state values of τ obtained while mice were ingesting diazepam/ethanol solutions were compared to the τ values prior to administrations. After cessation of diazepam/ethanol treatments, animals were allowed to drink to tap water for 2–3 weeks. Period modulations, if any, were noted.

Experiment 2

Freshly caught animals were allowed to free run in DD for 10 days. Then they were given 0.1 mg/ml of diazepam continuously throughout the experiment (in 2% ethanol). This lower dose was selected because animals were kept under this drug administration for a longer time (i.e., 3–4 months). Animals (n = 15) received light pulses (cool light source, schott Mainz KL 150 E, Germany) were administered at 12 CT points (n = 4 for each CT point) with a minimum interval of 10 days between the pulses (CTO = subjective "sunrise"; CT 12 = "subjective sunset" of a given cycle). Animals (n = 15) subjected to DD received light pulses repeatedly with a minimum interval of 10 days.

Control animals (n = 15) were allowed to free run in DD for 10 days. They were administered with 2% ethanol solution throughout the experiment. Fifteen-minute light pulses were administered at 12 CT points (n = 4 for each CT point) similar to diazepam-treated animals. Phase shifts and period changes were calculated by drawing regression fitting lines as described by Daan and Pittendrigh (4).

RESULTS

Experiment 1

During the course of the experiment the daily drug uptake ranged between 1 and 2 ml (hence the drug uptake ranged between 0.25 and 0.5 mg/day).

Under LD conditions diazepam failed to evoke striking changes in ψ (Fig. 1B). Ethanol (2%) administered control animals also failed to evoke changes in ψ (Fig. 1A). On the other hand, under free-running conditions (DD) diazepam showed period-shortening and period-lengthening effects (Fig. 3A and B). Of the 31 animals tested, 17 animals showed shortening of τ and 14 animals showed lengthening. Ethanol (2%) administration did not evoke any period-modifying effect in the free-running rhythm (Fig. 2).

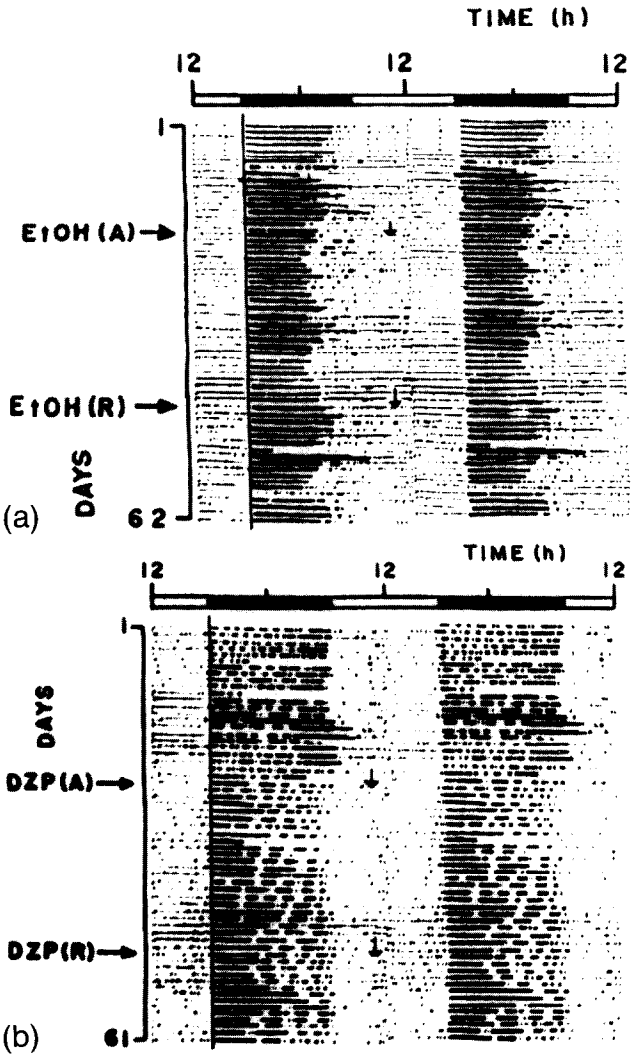

FIG. 1. Representative activity records (double plotted) of *M. booduga* in LD. EtOH(A) (A) and DZP(A) (B) indicate the day of diazepam/ethanol administration; EtOH(R) and DZP(R) indicate the day of diazepam/ethanol removal. Arrows inside of actogram denote the times of commencement of EtOH/diazepam administration and removal. Striking differences in ψ were not seen.

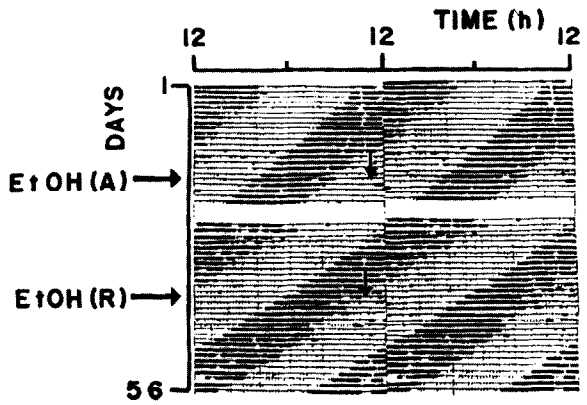

FIG. 2. Representative actogram showing the absence of period change during the course of ethanol treatment on the locomotor activity rhythm of *M. booduga* in DD. The gap (3 days) indictes missing data.

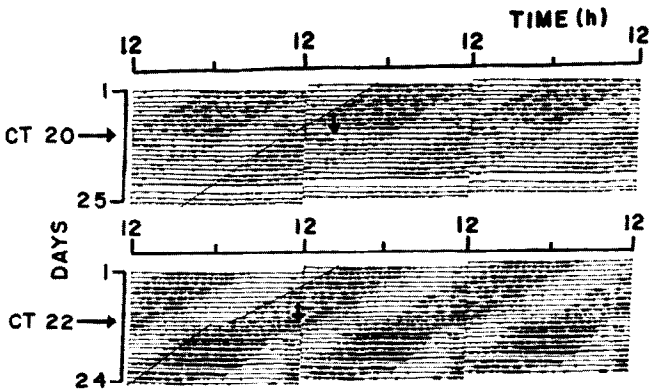

FIG. 4. Representative portions of the actograms (triple plotted) showing $\Delta\phi$s evoked by 15′ light pulses on control (2% ethanol-treated) animals at CT 20 and 22. The gap in the upper diagram denotes loss of data.

Experiment 2

The drug uptake ranged between 0.1 and 0.2 mg/day. Representative recordings of phase shifts induced by 15-min light pulses on diazepam-treated animals are shown in Figure 5. Phase advances were found to be attenuated in diazepam-treated animals at late subjective night (CT 20, 22, and 24; Fig. 5). Distinctly, in two animals at CT 20 and 24, phase advances were blocked completely (Fig. 5). Phase shifts evoked in 2% ethanol-administered animals are shown in Fig. 4. Diazepam and ethanol PRCs were characterized by phase

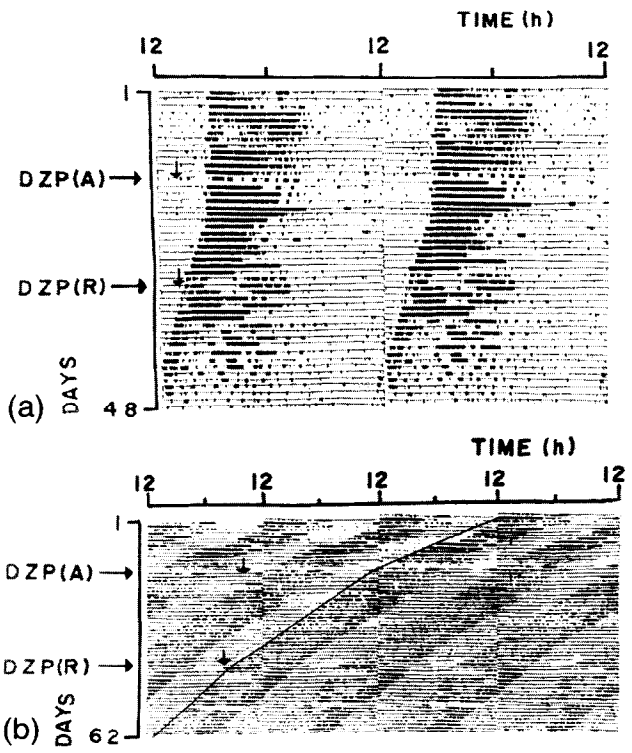

FIG. 3. Representative actogram (A) showing the period-shortening effect of diazepam on free-running rhythms in DD. Note minor modulation of τ after cessation of drug treatment. Representative activity record (quatriplotted) (B) showing the period-lengthening effect of diazepam in *M. booduga*.

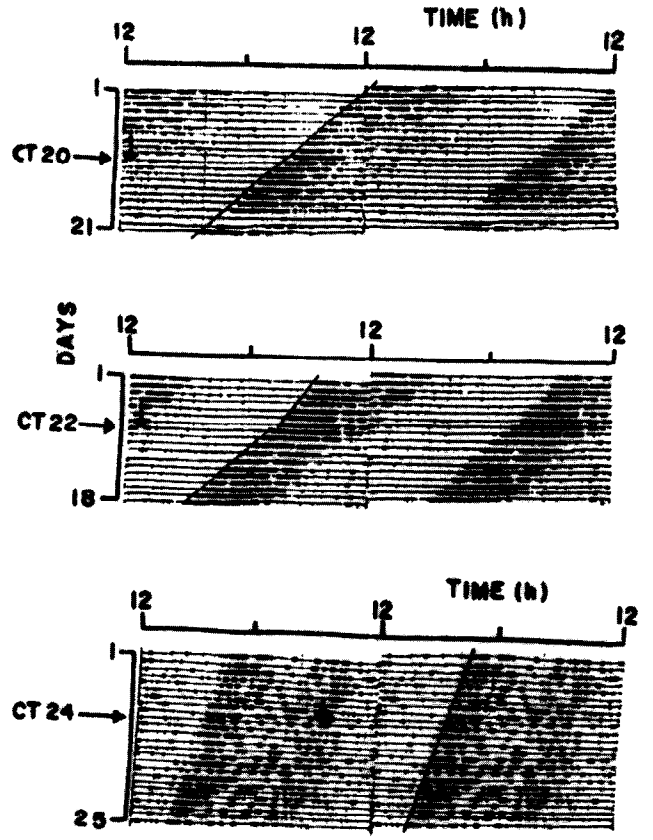

FIG. 5. Representative portions of actograms showing the effect of light pulses (on drug-treated animals) at CT 20, 22, and 24. In the above cases, 15′ light pulses at CT 20 and 24 induced the phase shifts. Light pulse perturbation at CT 22 produced a small advance phase shift (see text).

delays during early subjective night and phase advances during late subjective night (Fig. 6A). However, in the diazepam PRC small phase advances were observed at late subjective day.

Closer visual inspection and statistical analyses revealed several differences between the PRCs. In the diazepam-treated animals advances were smaller at CT 20, 22, and 24 (Fig. 6A). At CT 20 and 24 phase advances were attenuated significantly (CT 20, $p < 0.005$; CT, $p < 0.05$; Student's t-test). At CT 22 the reduction of phase advance in diazepam PRC failed to achieve statistical significance ($p > 0.1$) when compared with

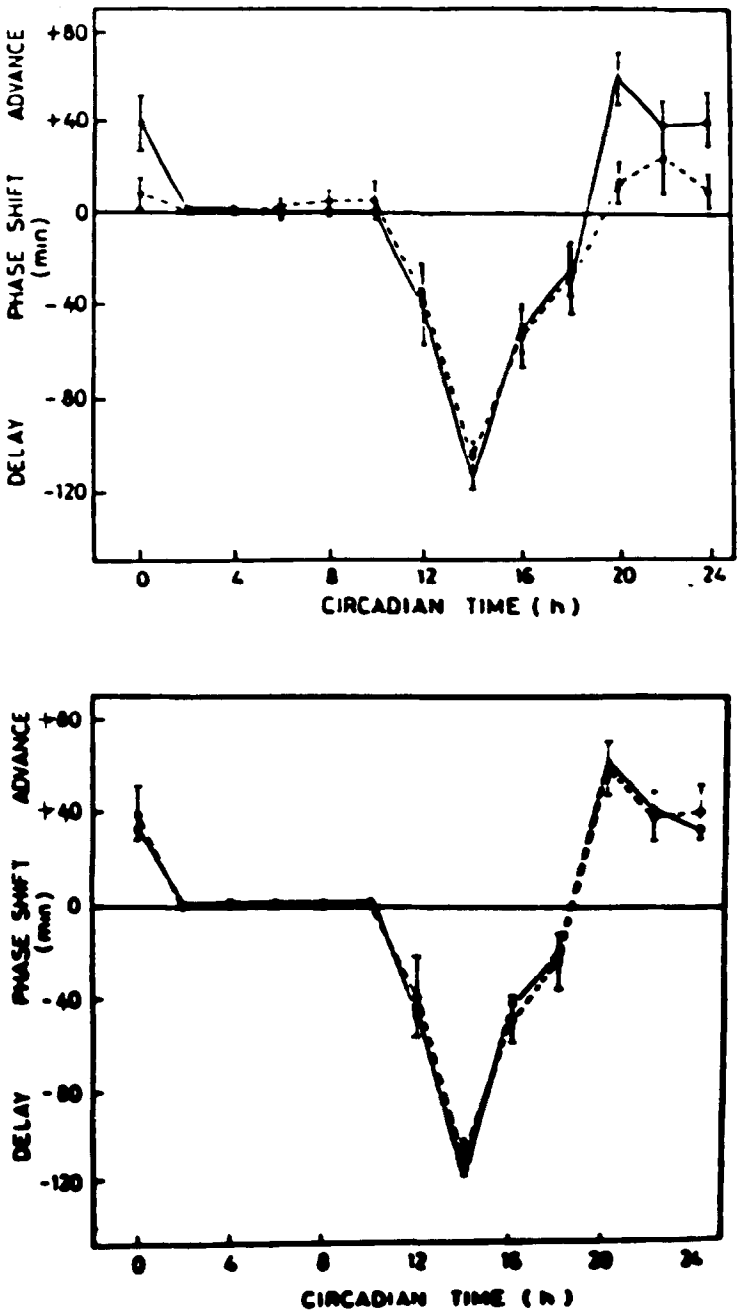

FIG. 6. Phase response curves to 15′ light pulses (1000 lx) delivered to (A) chronic diazepam (●-----●) or 2% EtOH-treated (●——●) mice. The phase-shifting response (mean ± SD) of wheel-running onset is plotted as a function of the circadian time of pulse administration. (B) Phase-shifting responses of 20% EtOH-treated (●-----●) and H_2O-treated (●——●) (Kumarasamy, unpublished) animals. The phase-shifting response (mean ± SD) was shown in "2% EtOH PRC." In "H_2O-treated PRC" each point represents the mean value of phase shifts (n = 4–5; Kumarasamy, unpublished).

control PRC. The shape and amplitude of the ethanol light pulse PRC (control) was similar to the light pulse PRC obtained in H_2O-treated animals in *M. booduga* (Kumarasamy, unpublished) (Fig. 6B).

DISCUSSION

Diazepam offered through drinking water in mice had no effect on phase angle difference during entrainment. When the tenets of oscillation theory are applied to biological rhythms, the prime generalization is that the conditions that influence the τ of the organisms also influence the phase angle during entrainment. Such congruity of τ and ψ was categorically observed in birds (12,19) and in bats (23). Nevertheless, diazepam failed to exert such congruous action in entraining rhythms of *M. booduga*.

It seems possible that in the present experiment alternating steady-state conditions such as D/L (on) and L/D (off) transitions of entrainment exerted a strong effect that inhibited the modulation of phase angle by diazepam. However, the period-modulating effects of diazepam (period-shortening and period-lengthening effects) under constant conditions resembled the period-modulating effect of sodium valproate (a GABA elevating drug) (1).

The effects of diazepam on the activity rhythm are thought to be due to an action of the drug either on neurons that are on an input pathway to the circadian clock regulating this rhythm or on neurons that are part of the oscillator itself (14). Recent anatomical studies indicate that GABAergic neurons are quite prevalent within the SCN (24); the effect of benzodiazepines upon the rhythm of locomotor activity may be due to an action on benzodiazepine—GABA receptors located within the SCN region (22). However, because diazepam is reported to have a number of effects in the central nervous system (14), it will be necessary to characterize the effects of diazepam on circadian rhythms in more detail before firm conclusions regarding underlying mechanisms can be drawn.

Reports on the effect of ethanol on mammalian locomotor rhythms are very limited. Zucker et al. (26) reported that ingestion of 20% ethanol in hamsters, in some instances, lengthened the period of wheel-running rhythm. This effect of 20% ethanol on τ for a group of 12 hamsters tested was statistically significant in only 25% of individual hamsters (26). In our case, the lower concentration of ethanol used (2%) is unlikely to have directly affected the locomotor rhythms. Furthermore, no significant changes in shape and contour between ethanol PRC and "H_2O PRC" (Kumarasamy, unpublished) in *M. booduga* were detected (Fig. 6B). This indicates that ethanol has no additive/interactive action with diazepam to light pulses.

The attenuation of phase advances in the diazepam PRC (Experiment 2) confirmed the previous findings of Ralph and Menaker (14) where blocking of phase advances by diazepam was reported. Similar reduction in the amplitude of phase advances due to continuous administration of clorgyline was reported previously (5).

In hamsters, previous research on the actions of bicuculline and diazepam support the interpretation that different neurochemical mechanisms may participate in the transmission of light responsible for light-induced phase delays and advances (11,13,14). Unlike bicuculline and diazepam, which are blocking light pulse induced phase shifts completely, chronic administration of diazepam in the present study only attenuates the response to light pulses that phase advance the onset of wheel-running activity. This difference can be attributed by the different mode of administration of the drug (i.e., through drinking water) in the present study.

Of late, the light pulse blocking effect of bicuculline and diazepam is explained by their actions on retinal GABAergic inhibition of dopamine neurons and their relations to circadian rhythms (21). Attenuation of phase advances in the PRC in the present study can be interpreted by the action of diazepam on GABA not only in SCN but also in retina (21). It is anticipated that studies on the influence of psychoactive drugs on circadian responses to light perturbations may unravel the characteristics of light sensitivity of mammalian circadian pacemakers.

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