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Effects of Parenterally Administered Triazolam on Sleep in Rats With Lesions of the Preoptic Area

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MENDELSON, W. B. *Effects of parenterally administered triazolam on sleep in rats with lesions of the preoptic area.* PHARMACOL BIOCHEM BEHAV **61**(1) 81–86, 1998.—In previous work we have reported that microinjections of triazolam or pentobarbital into the medial preoptic area of the anterior hypothalamus produce a hypnotic effect. This finding raised the possibility that the sleep-enhancing actions after systemic administration of these compounds might be mediated by hypnogenic mechanisms in the preoptic area. The current study examined whether sleep enhancement by triazolam requires the anatomic integrity of the preoptic area. Nine rats with histologically confirmed lesions of the preoptic area induced by ibotenic acid (2.5 μg/μl in 0.4 μl), and 10 rats that had undergone a sham lesion procedure, had 2-h sleep studies that confirmed that by day 5 measures of total sleep time and sleep latency had returned to preintervention values. Rats were then given triazolam 0.8 mg/kg or vehicle intraperitoneally in counterbalanced order, on days 7 and 9 postlesion, in an environment with an ambient temperature of 25°C. Following injections at 1000 h, in conditions in which lights were on from 0800–2000 h, 2-h sleep studies were performed. In the lesioned rats, triazolam significantly decreased sleep latency and increased total sleep time, primarily by increasing NREM sleep, whereas injections of vehicle did not. In summary, parenterally administered triazolam was found to have hypnotic effects in rats who were 1 week post-preoptic area lesion. These data are interpreted in light of previous evidence of redundancy of sleep-regulating mechanisms in the nervous system. © 1998 Elsevier Science Inc.

Triazolam Benzodiazepines Medial preoptic area Hypothalamus

SINCE the studies of Von Economo (40) following the epidemic of encephalitis lethargica, there has been growing recognition that the hypothalamus is involved in regulation of sleep and waking. This view was strengthened by the lesion studies of Hess (14) and Nauta (28), which indicated that the anterior hypothalamus might be involved in sleep maintenance, whereas a more caudal area might regulate wakefulness. In a basal forebrain area including the medial preoptic area (MPA), stimulation enhanced (34) and lesions suppressed (21) sleep in cats. Lesions in the medial preoptic area of rats acutely decreased sleep, although in a manner dependent on ambient temperature (36).

The involvement of the MPA in sleep regulation raises the possibility that it might be a site of action in producing the sleep-enhancing effects of clinically used hypnotic medications. To explore this possibility, we injected triazolam (24) and pentobarbital (26) into the MPA and found that sleep was enhanced, whereas injections into nearby structures including the horizontal limb of the diagonal band of Broca had no effect (23). Other groups have found similar results after microinjection of ethanol (39) and adenosine agonists (38). These findings suggest that the preoptic area may mediate the hypnotic action of a wide range of compounds. To explore the possibility that the integrity of the preoptic area is necessary to produce the sleep-enhancing effects of systemically administered hypnotics, we gave triazolam intraperitoneally to preoptic area-lesioned rats.

METHOD

As a representative of the benzodiazepine hypnotics we chose the 1,4 triazolobenzodiazepine triazolam, which, for many years, was the most widely prescribed hypnotic in the U.S. It binds with high affinity to the central BZ recognition

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site (3). Like ^{3}H diazepam, it binds with lower affinity at 37° compared to 0–40°C (8). Although earlier indirect data had questioned whether it exhibited a GABA shift, observations using ^{3}H triazolam confirmed that, like other BZ agonists, its affinity for the BZ receptor was enhanced by GABA agonists at 0 and 37°C (8). Direct binding experiments in several brain areas showed pseudo-Hill slopes of unity and similar K_i values, indicating lack of receptor heterogeneity (8). Studies of inhibition of [^{3}H] flumazenil binding to recombinant receptors indicates high-affinity binding (K_i 0.6–1.3 nM) to a wide range including $\alpha_{1,3,5}\beta_2\gamma_2$ (13). The lipophilicity of triazolam based on HPLC retention index is in the midrange (0.643) of a variety of benzodiazepines, as is its brain:unbound serum concentration (19.52) after systemic administration (3).

As an agent for making lesions we used ibotenic acid (IBO), an excitotoxic analogue of glutamate that destroys neuronal cell bodies without injuring fibers of passage or structures distant from the site of injection (6,30). Preliminary studies indicated that the doses described here would produce discrete lesions of the MPA with essentially 100% survival.

The study was performed on 19 225–250 g male Sprague–Dawley albino rats (approximately 60 days old) purchased from the Harlan Co. Each rat was anesthetized with 75 mg/kg ketamine and 7 mg/kg xylazine intramuscularly (IM). The rat was then mounted in a Kopf stereotaxic apparatus with a mouth bar set at −5.0 mm. After a scalp incision was made and the skull exposed, a hole was drilled at a stereotaxically determined location. The dura was then gently disrupted at the hole and a 24-gauge stainless steel guide cannula was lowered to 1.0 mm above the brain site of interest. For the MPA, the stereotaxic coordinates of the tip of the guide cannulae (bilaterally placed), derived originally from Paxinos and Watson (29) and assessed from previous work and pilot studies, are as follows: A-P: −0.4 mm, M-L: +0.5 mm, D-V: −7.1 mm (from the skull surface, with the mouth bar at −5.0 mm).

During the same surgical procedure, four 0-80 stainless steel screws were implanted through the skull to serve as dural EEG electrodes. These screws were connected to an Amphenol socket by short lengths of 0.010-inch Teflon-coated stainless steel wire. The stripped ends of two other lengths of this wire were implanted in the neck musculature to act as electromyographic (EMG) electrodes and these, too, were connected to the Amphenol socket. Before releasing the rat from the stereotaxic apparatus, the entire assembly of cannulae, electrodes, and Amphenol socket was cemented in place with dental acrylic. The edges of the wound were then treated with an ointment containing bacitracin, polymyxin, and neomycin. Finally, the guide cannulae were occluded with 31-gauge stainless steel stylets of matching length, and a protective plug was placed in the Amphenol socket.

After the surgical implantation of cannulae and electrodes for electroencephalogram and electomyogram, the rats were housed individually in smooth-walled plastic cages for at least 1 week prior to lesioning. At 1000 h M on the morning prior to lesioning or sham-lesioning, a 2-h sleep recording was performed, which served as baseline data. In nine rats we then administered 0.2 μl of ibotenic acid (2.5 μg/μl in phosphate-buffered saline, pH 7.4) into each of the two cannulae, over a period of 3 min, leaving the syringe in place for 5 min subsequently. An example of a lesion is seen in Figs. 1. A comparable procedure was carried out in 10 rats, with injection of the vehicle for ibotenic acid, for purposes of having a sham lesion control group (Fig. 2).

Following lesioning, the rats were housed individually. For the first 12 hours after lesioning they were kept in an ambient temperature of 26°C, following which temperature was returned to 25°C. Studies were performed for purposes of tracking the effects of lesions on sleep, on days 1, 3, 5, and 8 post-lesion or post-sham lesion. The recordings to determine effects of injections of triazolam or vehicle were performed (with treatment condition in random sequence) on days 7 and 9 post-lesion or post-sham lesion. The night before an experiment the rats were housed in the chambers in which they were subsequently tested. Lights were on from 0800 h until 2000 h during housing throughout the study, and relative humidity was controlled at 55%. All recordings began at 1000 h. As previous studies had indicated that the effects of triazolam microinjection on sleep were largely confined to the first 2 h [e.g., (22)], recordings were done for that duration.

Triazolam was dissolved in a 1:1 mixture of Emulphor polyoxyethylated vegetable oil and ethanol. Just prior to the

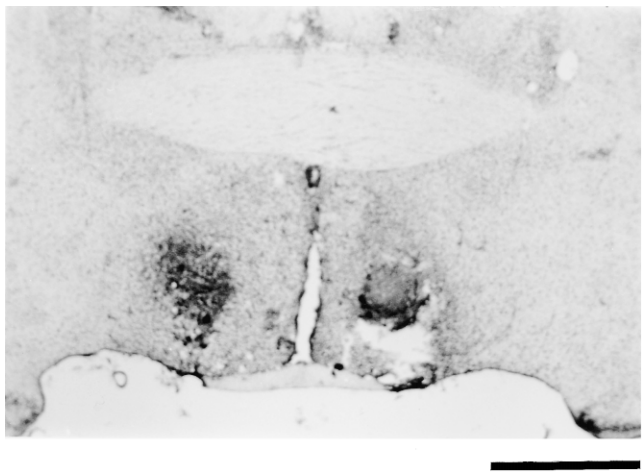

FIG. 1. Example of an ibotenic acid lesion of the preoptic area, 9 days post-lesion. Slide was stained using cresyl violet, and horizontal calibration line = 1 mm.

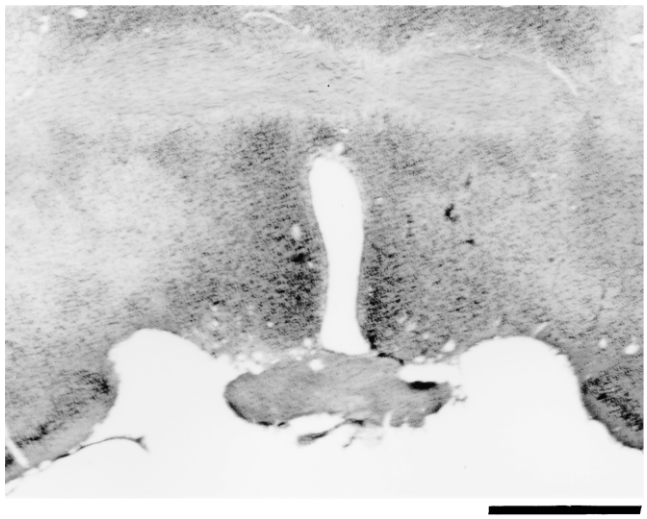

FIG. 2. Example of the preoptic area in an animal who has received a sham lesion, 9 days after the procedure. Staining and calibration line are the same as in Fig. 1.

injection, this solution was diluted tenfold with artificial cerebrospinal fluid (0.5 mM NaH_2PO_4, 0.25 mM Na_2HPO_4, 0.4 mM $MgCl_2$, 0.65 mM $CaCl_2$, 3 mM KCl, 128 mM NaCl, 25 mM $NaHCO_3$, and 250 mg/l bovine serum albumin, pH = 7.4). Animals received 0.8 mg/kg triazolam or vehicle intraperitoneally at 1000 h on the morning of the recordings. This dose was determined by previous studies of sedation [e.g., (10)] and pilot studies that indicated that it reduced sleep latency in naive rats. The elimination half-life of intraperitoneally administered triazolam in the rat has been found to be 2.53 h (17).

Following the injection of drug, each rat was placed back in its testing chamber, and a cable to a commutator (to allow free movement) and then on to a Grass Model 78 polygraph was attached to the Amphenol connector on the rat's headset. Three traces representing bifrontal EEG, fronto-occipital EEG (which was richer in theta band output), and EMG were recorded for 2 h for each rat. The polygraph paper speed was 10 mm/s, and the vertical deflection of the pen was calibrated so that 1.0 cm signified an electrical potential of 50 μV. After each recording, an investigator who was blind to the experimental condition scored the record, assigning a sleep or waking stage to each 30-s epoch (18).

After a rat completed the study, an injection of 400 mg/kg of sodium pentobarbital was given intraperitoneally (IP) and the animal was perfused transcardially with 0.9% NaCl followed by 4% formalin in 1.25% NaCl. The rat was then decapitated and the brain removed and stored in the formalin solution. Coronal brain sections (48 μm) were cut on a freezing microtome, mounted on slides, and stained with cresyl violet. The tip of the injection cannula track was then localized by light microscopy.

Changes in sleep parameters in the lesioned and sham-lesioned rats were examined by the use of two-way analyses of variance (ANOVAs), in which the independent variables were the intervention (lesion vs. sham lesion), the day (the number of days since the intervention), and the interaction between the two. The effect of triazolam was similarly assessed by two-way ANOVAs, in which the independent variables were the intervention (lesion vs. sham lesion), drug treatment (triazolam vs. vehicle) and the interaction between the two. When the ANOVAs showed significant effects, post hoc testing was performed using a Least Significant Difference test.

RESULTS

Table 1 presents the data on prelesion sleep, and postintervention sleep in rats with real and sham lesions. In the sham lesion group there were no significant changes in sleep. The ANOVA indicated a significant effect of the lesion, in comparison to the sham lesion group, on sleep latency, total sleep time, and NREM sleep time. Following the active lesion, there was a significant but transient increase in sleep latency compared to baseline values. Similarly, total sleep decreased initially compared to the baseline, as a consequence of decreased NREM sleep. Both returned to prelesion values by the fifth day.

As seen in Table 2, the ANOVA on the data from the drug treatment days (days 7 and 9) revealed a significant effect of the lesion, compared to the sham lesion group, on sleep latency and NREM sleep, such that sleep latency was higher, and NREM sleep was lower, in the active lesion group. An examination of drug effects revealed that triazolam significantly increased total sleep time and NREM sleep, and decreased sleep latency regardless of lesion status. A significant interaction term indicated that the drug effect on sleep latency was more potent in the active lesion group. There were no significant changes in the amount of REM sleep.

CONCLUSIONS

In considering the implications of these data, it may be worthwhile to consider two aspects of the conditions under which the recordings were performed: 1) the circadian timing

TABLE 1

EFFECT OF IBOTENIC ACID LESION AND SHAM LESION OF THE PREOPTIC AREA ON SLEEP

						ANOVA Results		
Lesion* Day Intervariable	Baseline†	Day 1	Day 3	Day 5	Day 8	Lesion*	Day	Interaction
Lesion								
TST	55.7 ± 2.4	46.6 ± 3.0*	47.9 ± 2.7*	51.8 ± 2.9	55.4 ± 2.5	0.001	0.03	0.01
SL	22.5 ± 3.3	43.2 ± 4.4*	32.3 ± 1.2*	26.5 ± 1.1	18.1 ± 1.3	0.001	0.001	0.001
IWT	43.4 ± 3.3	37.8 ± 3.7	46.4 ± 3.7	46.8 ± 3.0	49.6 ± 2.8	NS	NS	NS
NREM	*53.2 ± 2.5*	*41.2 ± 3.0**	*44.4 ± 3.2**	*48.8 ± 3.1*	*52.4 ± 2.4*	0.001	0.001	0.01
REM	*2.6 ± 0.5*	*5.4 ± 0.8**	*3.5 ± 1.0*	*3.0 ± 0.7*	*3.0 ± 0.7*	*NS*	*0.05*	*NS*
RL	*84.2 ± 7.1*	*56.4 ± 6.1*	*54.2 ± 9.4*	*55.2 ± 4.1*	*62.0 ± 8.0*	*NS*	*0.06*	*0.01*
Sham Lesion								
TST	60.7 ± 1.2	60.6 ± 0.9	61.2 ± 1.2	61.8 ± 1.3	60.2 ± 0.9			
SL	16.2 ± 1.7	14.3 ± 1.4	13.8 ± 1.8	14.6 ± 0.9	14.4 ± 1.3			
IWT	44.0 ± 2.0	46.0 ± 1.7	46.0 ± 1.1	44.6 ± 1.8	44.1 ± 1.6			
NREM	57.6 ± 1.0	57.2 ± 0.8	58.0 ± 1.1	59.0 ± 1.2	57.4 ± 0.9			
REM	3.0 ± 0.5	3.5 ± 0.7	3.2 ± 0.4	2.8 ± 0.5	2.8 ± 0.5			
RL	69.8 ± 4.5	70.8 ± 6.2	78.0 ± 6.8	71.8 ± 3.3	73.3 ± 3.6			

Abbreviations: TST = total sleep time; SL = sleep latency; IWT = intermittent waking time (waking time after initial sleep onset), NREM = total NREM sleep; REM = total REM sleep; RL = REM latency.

*Definition of terms used in ANOVA: "Lesion": main treatment effect (effect of lesion vs sham lesion); "Day": effect of day of recording (Baseline through day 8) regardless of lesion status; "Interaction": interaction of lesion status and day of recording. All values represent mean ± SEM minutes.

† Prior to lesioning or sham lesioning.

TABLE 2
EFFECTS OF INTRAPERITONEALLY ADMINISTERED TRIAZOLAM ON SLEEP VARIABLES ON DAYS 7 AND 9 POST-LESION OR POST-SHAM LESION

Variable	Vehicle	Triazolam	ANOVA		
			Lesion	Drug	Interaction†
Lesion					
Total Sleep	*44.1 ± 4.7*	*70.6 ± 6.6**	*NS*	*.001*	*NS*
Intermittent waking	*46.0 ± 5.7*	*43.3 ± 6.7*	*NS*	*NS*	*0.5*
Sleep latency	*29.3 ± 3.7*	*6.0 ± 1.2*	*0.001*	*0.001*	*0.001*
NREM sleep	*42.6 ± 4.6*	*67.8 ± 7.0**	*0.05*	*0.001*	*NS*
REM sleep	*1.6 ± .3*	*2.8 ± .7*	*NS*	*NS*	*NS*
REM latency	*83.6 ± 10.6*	*58.5 ± 8.0*	*NS*	*0.05*	*0.01*
Sham Lesion					
Total sleep	59.6 ± 0.9	81.6 ± 2.3*			
Intermittent waking	46.3 ± 1.6	30.2 ± 1.8*			
Sleep latency	14.8 ± 1.1	7.4 ± 0.8*			
NREM sleep	57.1 ± 0.9	80.0 ± 2.4*			
REM sleep	2.4 ± 0.5	1.6 ± 0.4			
REM latency	55.4±4.6	64.0 ± 4.2			

Abbreviations are the same as in Table 1.

* $p < 0.01$ compared to vehicle.

† Definition of terms used in ANOVA: "Lesion": main treatment effect (effect of lesion vs sham lesion); "Drug": effect of triazolam vs. vehicle regardless of lesion status; "Interaction": interaction of lesion status and drug treatment. All values represent mean ± SEM minutes.

of the studies, and 2) the postlesion recovery sleep of the animals. We chose to study the hypnotic effects of triazolam during the daytime with the lights on. Our interest has long focused on the mechanism of action when clinical hypnotics are given to humans during the part of the circadian cycle associated with sleep—the nighttime. When such studies are done in humans, the enhancement of sleep is relatively small. In a review of all hypnotic efficacy studies meeting basic design criteria, for instance, we found that hypnotics increased nocturnal sleep by a mean of only 35 min—an increase of approximately 8% (12). Similarly, one of the most cited studies of the efficacy of flurazepam, which was then the most widely prescribed hypnotic, found that it increased sleep by only 6–8% (16). Our basic science interests have been to elucidate the actions of a widely prescribed hypnotic (triazolam) when given to rats at the circadian time associated with sleep (the daytime). The advantage of this approach is that it keeps us closer to the clinical situation, and answers the question that most interests us clinically—how triazolam interacts with a brain "primed" for sleep. The disadvantage is that because of high baseline sleep levels, the amount by which it can be increased may be relatively small. However, even in the present daytime study, total sleep was increased by triazolam by 60%, a very potent effect even by the standards of human hypnotic studies. It is also comparable to that seen in a study of rats without lesions. Mendelson and Monti (25), reported an increase in total sleep time of 40% after administering the same dose of IP triazolam to animals with an intact MPA.

In designing this study, another goal was to administer triazolam to lesioned rats in which there was a substantial amount of baseline sleep. Because a very serious disruption of sleep under baseline conditions might potentially alter any hypnotic actions of the drug, we recorded the degree of sleep disruption induced by the lesion across time. It was found that, in keeping with previous studies from this laboratory, even the acute disturbances in sleep induced by the preoptic area lesions were modest (9), lending support to the view that larger decrements in sleep occur only when lesions are large, and ambient temperature is low [e.g., (36)]. For example, on day 1 postlesion, we found total sleep time to be reduced to 83.2% of typical sleep, compared to a reduction to 15.2% in animals with large lesions kept at a cooler ambient temperature (20°C) in the Szymusiak and Satinoff study (36). On day 5, total sleep and sleep latency had returned to prelesion baseline values. Thus, our triazolam injection data were gathered at a time at which sleep parameters had returned to essentially normal values. In passing, it should be mentioned that preliminary evidence indicates that IP triazolam also has significant hypnotic properties when given 1 day postlesion, when sleep is still disrupted. In a pilot study, we have recorded sleep in rats receiving triazolam or vehicle (4 each) 1 day after IBO lesions of the MPA, using the same doses and experimental design as the main study presented here. On day 1 postlesion, in the animals receiving vehicle IP, total sleep was reduced to 82.8% of baseline, a comparable reduction to that seen on day 1 in the main study. Under these conditions, IP triazolam significantly increased total sleep by 58%, an amount comparable to the 60% increase seen on days 7 and 9 postlesion in the main study presented here.

A recent study of c-*fos* activity has indicated activation of ventrolateral preoptic (VLPO) neurons during sleep (31), raising the question of the relation of this area to the nearby site we have been studying. We have previously reported that microinjections of triazolam into the medial preoptic area enhance sleep, whereas injections into the lateral preoptic area had no effect (23). The latter injections were in the center of the lateral preoptic area, slightly dorsal to the VLPO. Thus, the issue of whether the VLPO is involved in the hypnotic ac-

tions of triazolam will need to be determined by studies of direct microinjection of triazolam into the area, or of the effects of parenterally administered triazolam in animals with lesions centered on the VLPO.

The relationship of the MPA to the lateral preoptic area (LPA) also needs to be considered. Microinjection of the reversible local anesthetic marcain into both areas in rats induces wakefulness, while injection at night induces sleep. The degree of responsiveness in the two areas differs, however, and has been interpreted to suggest that the MPA is more involved in maintaining tonic sleep, while the LPA may be more involved in maintaining tonic wakefulness (1,2).

The preoptic area contains cells sensitive to thermal, osmotic, and cardiovascular signals (15) as well as glucose and steroids (4). It contains neurons that become more active during non-REM and REM sleep (18). It appears to receive inputs from olfactory, visual, auditory, and tactile sources, and may coordinate these many inputs in reproductive and homeostatic systems (32). The projections seem consistent with this view, insofar as they are widely distributed throughout the forebrain and brainstem (33). A major output projects to the median and dorsal raphe nuclei, and possibly to the locus coeruleus (35). Stimulation of the MPA has long been thought to suppress neural activity in the midbrain reticular formation, or MRF (5), and a later stimulation study of the preoptic area revealed initial excitation followed by postexcitatory discharge suppression (37). Conversely, low-frequency stimulation of the caudal brainstem reticular formation (which induces EEG synchronization), and of the rostral brainstem reticular formation (which has little effect on the EEG) produces an excitatory action on preoptic neurons, while high frequency stimulation has the opposite effect (19,20). These observations suggest that the preoptic area plays a critical role in the regulation of sleep, involving interaction with reticular structures and the integration of sleep with other behaviors. It seemed, then, to be a candidate as an area that might mediate the sleep-promoting effects of benzodiazepine hypnotics. The presence of significant concentrations of some forms of the $GABA_A$-benzodiazepine receptor complex in the MPA also indicated that these compounds might bind there (11). Indeed, it was found that microinjections of triazolam into the MPA enhanced sleep (24). This begs the question as to whether the MPA mediates the hypnotic actions of parenterally administered benzodiazepines. We addressed this by determining whether there is an interaction between lesions of the MPA and the hypnotic properties of intraperitoneally administered triazolam. The hypnotic effects of intraperitoneally administered triazolam do not appear to be dependent on the anatomic integrity of the preoptic area once sleep has returned to baseline levels, which may suggest the likely redundancy of sleep-promoting structures sensitive to benzodiazepines in the CNS. To put this in context, the history of exploring the anatomy of sleep regulation is replete with examples of the redundancy of the mechanism, which seems to emphasize the importance of sleep to the organism. One of the best examples has been the study of lesions of the dorsal raphe nuclei (7,27): although anatomic or pharmacological lesions of the raphe acutely induce large reductions in sleep, within a short period of time the total amount of sleep returns to normal, as other systems assume control. It is possible that we are seeing a similar process—both for systems regulating baseline sleep as well as for mechanisms involved in the hypnotic response to benzodiazepines—in the MPA-lesioned rats.

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