IL-1 include sleep alterations, fever, anorexia, and activation of the hypothalamus-pituitary-adrenal axis, in which 5-HT plays a role, suggests that this cytokine may exert some of its effects by interacting with the serotonergic system (2). Serotonin is involved in sleep control and thermoregulation (1, 21). As far as sleep is concerned, inasmuch as there are data suggesting that serotonin could act as both a sleep-enhancing and a sleep-suppressing agent, a unifying hypothesis has been proposed. In accordance with this hypothesis (1, 2), the 5-HT released during wakefulness (W) might induce the synthesis and/or release of hypnogenic factor(s) that would be subsequently responsible for sleep.

The aims of this study were to determine whether MDP- and IL-1-induced sleep changes are mediated through the serotonergic system. To this purpose sleep responses to centrally administered MDP and IL-1 were evaluated in rats pretreated with para-chlorophenylalanine (PCPA), which induces insomnia and depletes brain 5-HT by inhibiting tryptophan hydroxylase, the rate-limiting enzyme, in the formation of 5-HT from tryptophan (10).

MATERIALS AND METHODS

Animals and surgery. The experiments were performed on male albino rats (CD, Charles River; Calco, Italy; 250–300 g). Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with international "Guide for the Care and Use of Laboratory Animals" [DH EW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health DDR/NIH, Bethesda, MD 20892]; EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; and national (D.L. n.116, G.U. suppl. 40, Feb. 18, 1992) laws and policies. The animals were anesthetized (pentobarbital sodium 40 mg/kg + chloral hydrate 180 mg/kg ip), injected with a broad-spectrum antibiotic (penicillin G benzathine), positioned in a stereotaxic apparatus, and surgically prepared for chronic polygraphic recordings. Electroencephalographic (EEG) and electromyographic (EMG) electrodes, as well as a 30,000 Ω (at 25°C) calibrated thermistor (Omega Engineering, Stamford, CT), were implanted. EEG electrodes were unilaterally placed over the frontal [anterioposterior (AP) = 2 mm, lateral (L) = 2.5 mm from bregma] and parietal (AP = −5 mm, L = 3.5 mm from bregma) cortices. A third screw was placed over the cerebellum to ground the animal. Teflon-coated silver wires were inserted into the neck muscles to record electromyograms. To record cortical temperature (Tcort), the thermistor was implanted between the skull and the dura mater over the parietal cortex. A polyethylene cannula was stereotaxically implanted into the lateral ventricle (AP = −1 mm and L = 1 mm from bregma) for intracerebroventricular injections. An integrated circuit socket was attached to the skull with dental acrylic, and insulated leads were routed from this plug to the electrodes and the thermistor. The rats were allowed 1 wk to recover before they were connected to a flexible tether and slip ring and accustomed to the soundproof recording cham-
The animals, individually housed under a 12:12-h light-dark cycle (lights on at 3:00 AM when test compounds were administered at dark onset; lights on at 10:00 AM when test compounds were administered at light onset) at 21 ± 1°C, were allowed at least 48 h adaptation before testing began. Food and water were available ad libitum.

Recording apparatus. Signals from the EEG and EMG electrodes, as well as from the thermistor, were fed into a Grass model 7 polygraph (Quincy, MA) in the adjacent room. These signals were subjected to analog-to-digital conversion with 12-bit precision at a sampling rate of 128 Hz (NB-M10-16; National Instrument, Austin, TX). The digitized EEG waveform, the $T_{\text{cor}}$ samples, and integrated values for electromyography were stored as binary computer files until subsequent analysis.

Substances. PCPA was purchased from Sigma (St. Louis, MO), dissolved in pyrogen-free distilled water, and adjusted to pH 6.0 with 10 N NaOH. Rats were given PCPA 300 mg/kg. This dose was administered in a volume of 5 ml/kg ip. MDP was purchased from Sigma and dissolved in pyrogen-free saline (PFS). Rats were given MDP 150 pmol (74 ng). This dose was administered in a volume of 1 µl icv. IL-1 (human recombinant IL-1β produced in Escherichia coli) was purchased from R & D System (Minneapolis, MN). Lyophilized IL-1 was dissolved in PFS containing 0.1% bovine serum albumin, aliquoted, and frozen until used. Rats were given IL-1 1.25 ng. This dose was administered in a volume of 1 µl icv. A heat-inactivated preparation of IL-1 (90°C, 60 min) was injected into the animals as an additional control for potential endotoxin contamination.

Experimental design. Sixty-seven rats were used. To verify the patency and free drainage of the intracerebroventricular cannula, the rats were tested for the angiotensin-induced drinking response (4); only the animals in which a drinking response was elicited were used. Five animals were discarded because they failed to respond to the angiotensin test.

Sixty-two rats were subdivided into the following groups. Group 1 (PCPA) (n = 6) received pyrogen-free distilled water intraperitoneally twice on consecutive days at dark onset (as shown in Fig. 1, top) and PFS intracerebroventricularly 5 min, 12 h, and 24 h after the second PCPA administration. One week later the animals were given PCPA 300 mg/kg ip and PFS intracerebroventricularly according to the same protocol. Polygraphic recordings began immediately after the first vehicle or test substance administration and continued for 72 h. Groups 2a and 2b (MDP) were injected intracerebroventricularly at either dark (group 2a, n = 6) or light (group 2b, n = 5) onset with both vehicle (PFS) and MDP 150 pmol, so each animal served as its own control. Experiments were randomly scheduled with an interval of at least 1 wk between injections. Group 3a and 3b (IL-1) were injected intracerebroventricularly at either dark (group 3a, n = 6) or light (group 3b, n = 5) onset with both vehicle (PFS) and 1.25 ng IL-1, according to the same protocol used for group 2. Group 4 (n = 6) received PFS and a heat-inactivated preparation of IL-1 (90°C, 60 min) intracerebroventricularly, according to the same protocol used for group 2. Group 5a, 5b, and 5c (PCPA + MDP) received PCPA 300 mg/kg ip, according to the same protocol used for group 1. The animals were also given MDP 150 pmol icv 5 min after the second PCPA administration (group 5a, n = 5), 12 h after the second PCPA administration (group 5b, n = 6), or 24 h after the second PCPA administration (group 5c, n = 6). Group 6a, 6b, and 6c (PCPA + IL-1) received PCPA 300 mg/kg ip according to the same protocol used for group 1. Animals were also given IL-1 2.5 ng icv 5 min after the second PCPA administration (group 6a, n = 6), 12 h after the second PCPA administration (group 6b, n = 5), or 24 h after the second PCPA administration (group 6c, n = 6). In groups 2-6, polygraphic recordings began immediately after the injection and continued for 12 h. All the intracerebroventricular injections were performed over a 1-min period.

Data analysis. The postacquisition determination of vigilance state was made by visual scoring using custom software (Mark R. Opp, University of Texas Medical Branch, Galveston, TX) written in LabView (National Instruments, Austin, TX). Twelve-second epochs of the electroencephalogram, $T_{\text{cor}}$,...
and integrated electromyogram were displayed on a high-resolution computer monitor. The polygraphic signals were simultaneously displayed on the monitor to facilitate a visual determination of the behavioral state. The animal behavior was classified as either W, SWS, or desynchronized sleep (DS) on the basis of criteria published elsewhere (23).

Statistical analyses. In groups 1, 2, 3, and 4 statistical differences between control and experimental values were assessed by means of Student's t-test for paired data. When differences across time were evaluated, two-way analysis of variance (ANOVA) with repeated-measures design was performed for the duration of each vigilance state throughout 12-h time blocks for each group of rats. Two factors were taken into account in this analysis: treatment (vehicle or test substance) and time (hours). If a statistically significant treatment effect was detected, individual 1-h time blocks were analyzed by paired-samples t-tests to determine the specific time when the effect occurred. To evaluate the interaction between treatments A (PCPA) and B (either MDP or IL-1), control values from intraperitoneal (treatment A) and intracerebroventricular (treatment B) injections were pooled together and two-way ANOVA (between-subjects design) was performed. The main effect consisted of treatment A and treatment B.

RESULTS

PCPA. PCPA effects were dependent on the dark-light cycle. PCPA induced an increase in W and a decrease in SWS during light phases and a decrease in DS during both dark and light phases (Fig. 1). DS reduction was significant starting from the first 12-h time block after the first PCPA treatment (first day; Fig. 1). W enhancement and SWS reduction were already statistically significant during the light phase on the first day, but reached their highest level during the light phase on the second day (Fig. 1). PCPA induced a significant drop in $T_{cort}$; after the first administration a maximal decrease of $0.84 \pm 0.20°C$ (from preinjection values) was observed in the first hour postinjection. $T_{cort}$ returned to control values starting from hour 4. After the second administration, $T_{cort}$ remained significantly reduced by $0.69 \pm 0.09°C$ only in the first postinjection hour.

MDP. MDP effects on sleep-wake activity and $T_{cort}$ were also dependent on the dark-light cycle. MDP, when given at dark onset, induced a decrease in W and an increase in SWS during the first 6 h after administration (Fig. 2). DS reduction was observed when MDP was given at light onset (Fig. 2). MDP always induced a febrile response, but $T_{cort}$ increase was higher when MDP was given at light onset than when it was administered at dark onset. During the light phase MDP induced the maximal increase in $T_{cort}$ in the third hour, when it rose from $36.04 \pm 0.12°C$ in control conditions to $36.74 \pm 0.24°C$ after treatment; during the dark phase the maximal increase in $T_{cort}$ was still observed in the third hour, but $T_{cort}$ rose from $36.80 \pm 0.04°C$ in control conditions to $37.25 \pm 0.03°C$ after treatment.

PCPA + MDP. In PCPA-pretreated rats, MDP did not induce any significant change (in comparison to the PCPA condition) in W and SWS (Fig. 2) and in $T_{cort}$ (data not shown), regardless of the phase of the dark-light cycle when MDP was given, whereas DS was further reduced during the light phase (Fig. 2). Although data in Fig. 2 refer to the first dark and light phases after the second PCPA administration, the same pattern of changes in sleep-wake activity and $T_{cort}$ was observed when MDP was given 24-h later (data not shown).

IL-1. When administered at light onset, IL-1 induced a decrease in W and DS and a biphasic increase in SWS (Fig. 3, left). In particular, SWS was enhanced in the first postinjection hour and in hours 3-6. When IL-1 was given at light onset, no consistent pattern of changes was observed in W and SWS (Fig. 3, right). W and SWS were modified only at certain times, and, as far as SWS is concerned, changes were observed in the direction of both an increase and a decrease. DS was consistently inhibited. A febrile response was observed when IL-1 was given at both dark or light onset, but, similar to what happens with MDP, the effect was dependent on the phase of the dark-light cycle (Fig. 3). Not only was the maximal $T_{cort}$ increase higher during the light phase, but the time course of the increase was also different, showing a biphasic pattern that was not present during the dark phase (Fig. 3). No changes were observed in any of the variables considered when heat-inactivated IL-1 (2.5 ng, 90°C, 60 min) was administered (data not shown).
PCPA + IL-1. In PCPA-pretreated rats, sleep-wake activity was modified by IL-1, but with a different pattern (Fig. 4). In particular, the first phase of SWS increase was completely suppressed and SWS enhancement was monophasic during the dark phase (Fig. 4, right and left). During the light phase, SWS was monophasically increased by IL-1 in these PCPA-pretreated animals (Fig. 4). The febrile response to IL-1 was not modified by PCPA pretreatment (Fig. 4, top), even during the third day, when the PCPA-pretreated group was given PFS intracerebroventricularly.

DISCUSSION

The results of the present study suggest that 1) MDP effects on sleep-wake activity and \( T_{\text{cort}} \) depend on the light-dark cycle and are both mediated by the serotonergic system; 2) IL-1 effects on sleep-wake activity and \( T_{\text{cort}} \) depend also on the light-dark cycle, although they are mediated through different mechanisms; 3) the mechanisms mediating the first and the second phases of IL-1-induced SWS excess are different: 5-HT could be involved in the first phase, but not in the second one; and 4) the serotonergic system does not appear to be involved in IL-1-induced fever.

Sleep-wake activity. The finding that MDP does not induce any changes in sleep-wake activity when given to PCPA-pretreated rats suggests that 5-HT is essential for MDP to exert its effects on sleep-wake activity. MDP interactions with the serotonergic system, which plays an important role in sleep regulation (1), take place at different levels. 5-HT competes for MDP specific binding sites, and MDP modifies 5-HT release and turnover, acting both as an antagonist of 5-HT actions mediated through 5-HT\(_2\) receptors and as a 5-HT uptake blocker (17, 24, 28).

5-HT may also be involved in mediating MDP effects on sleep in a different way. It has been shown that MDP-induced SWS excess can be mediated via endogenous, brain-derived IL-1 and TNF (8, 26, 27). Because the endogenous production of IL-1 can be stimulated by 5-HT (25), data showing that MDP increases serotonergic turnover (17) suggest that 5-HT may be involved in mediating MDP-induced stimulation of IL-1 production and/or release. Opposing evidence that extracellular 5-HT levels in the anterior hypothalamus/preoptic area do not increase after MDP administration (28) puts forward a different hypothesis, i.e., 5-HT could exert a permissive role on MDP-induced IL-1 activation. According to this hypothesis, physiological levels of 5-HT may be necessary for MDP to induce IL-1 activation. When these levels are reduced (below a certain level) MDP could not induce its effects on IL-1.

IL-1 could in turn induce SWS by activating the growth hormone (GH)-releasing hormone-GH system and nitric oxide (11). Because the first phase (but not the second one) of IL-1-induced SWS increase is suppressed in PCPA-pretreated animals, 5-HT could play a role in this first phase (5). The hypothesis that the mechanisms mediating the first and the second phase of IL-1-induced SWS excess may be different and that 5-HT may be involved only in the first phase is in agreement with recent observations showing that the first phase is specifically associated with an increase in serotonergic activity in the medial preoptic area (5).

MDP can restore SWS to normal (control) levels in PCPA-pretreated rats when it is given intravenously (18). The discrepancy with the results of the present study seems to suggest that there might be a difference between peripheral versus central MDP administrations, but other factors (such as the moment of the dark-light cycle when MDP is given) should also be taken into account.

Because MDP effects on sleep are mediated through both IL-1 and TNF (8, 26, 27) and they are completely blocked by PCPA pretreatment, it could be hypothesized that 5-HT might also be important in mediating
MDP induction of TNF, but direct or indirect evidence supporting this hypothesis is lacking (2) and further studies are needed to investigate the possible interactions between MDP, TNF, and the serotonergic system.

Although PCPA is not a specific 5-HT depletor, causing decreases also in brain levels of norepinephrine and dopamine, it has much less of a depleting effect on these monoamines (16, 20). Moreover, insomnia induced by PCPA is specifically related to serotonin depletion because it can be reverted by administering 5-hydroxytryptophan, the direct precursor of 5-HT (1).

Because SWS and DS are differently and specifically modulated by several experimental manipulations (including the ones mentioned in the present study), these different sleep phases may be regulated by different mechanisms.

Although PCPA effects on 5-HT synthesis are irrespective of the light-dark cycle (16), PCPA is unable to increase W during the dark phase (the active phase for rats). On the other hand, both MDP and IL-1 are ineffective in enhancing SWS during the light phase (the resting phase for rats). These findings support the hypothesis that the amount of W and of SWS during the dark and light phases, respectively, represents a physiological limit beyond which it may be very difficult to force sleep-wake activity. Data in the present paper are in agreement with previous works describing circadian variations in responses to MDP and IL-1 (15, 19, 22).

Thermoregulation. The observation in this study that MDP-induced fever is blocked in PCPA-pretreated rats suggests that MDP effects on Tcort are mediated by the serotonergic system, and it is consistent with data showing that fever induced by different bacterial pyrogens can be attenuated by prevention of 5-HT synthesis, lesions of the raphe nuclei, administration of the 5-HT receptor antagonist cyproheptadine, or whole-brain depletion of 5-HT (28). Because, on the other hand, IL-1-induced fever is unaffected by PCPA pretreatment, this suggests that the serotonergic system is not involved in mediating IL-1-induced changes in Tcort and supports the hypothesis (put forward above to explain sleep changes observed in this study) that 5-HT might be involved in the process linking MDP to the stimulation of cytokine(s) production, particularly IL-1. The hypothesis that 5-HT is not involved in IL-1-induced fever is supported by recent data showing that two
different doses of IL-1 (2.5 and 25 ng iv) induce an identical increase in Tcort, but an activation of the serotonergic system that is specific and different for each dose (5). However, 5-HT is involved in thermoregulation (21) and, in physiological conditions, the serotonergic activity in the medial preoptic area has been shown to be closely linked to thermoregulatory processes (7), suggesting that mechanisms regulating body temperature in physiological versus pathological conditions may be different.

Because in rats body temperature displays a clear circadian rhythm (by increasing during the dark phase and decreasing during the light phase), the observation that both MDP and IL-1 induce a higher febrile response when given at light onset than at dark onset suggests that the physiological temperature level may affect pyrogen-induced febrile responses. Circadian rhythms of different hormones involved in thermoregulation, such as corticotropin-releasing hormone or corticosteroids, can account for the findings in the present paper, which are in agreement with previous data (19). The present data also suggest that not only the extent but also the time-course of IL-1 febrile response (monophasic versus biphasic fever) may be modulated by circadian rhythms.

The observation that MDP induces an increase in SWS when given at dark onset, whereas the febrile response is higher when MDP is given at light onset, suggests that 1) MDP effects on sleep-wake activity and Tcort are specifically and differently modulated by the dark-light cycle, and 2) sleep and temperature responses can be dissociated (6, 14).

The authors thank Cristina Sollazzo for help in preparing the manuscript.

This work was partially supported by Sigma-Tau (Pomezia–Rome, Italy).

Address for reprint requests: L. Imeri, Istituto di Fisiologia Umana II, Università degli Studi, Via Mangiagalli, 32, I-20133 Milano, Italy.

Received 18 February 1997; accepted in final form 22 July 1997.

REFERENCES