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Diurnal Variations in the Analgesic Effectiveness of Morphine in Mice[1,2]

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BORNSCHEIN, R. L., R. S. CROCKETT AND R. P. SMITH. *Diurnal variations in the analgesic effectiveness of morphine in mice.* PHARMAC. BIOCHEM. BEHAV. 6(6) 621–626, 1977. – Response to thermal stimulation and the analgesic effectiveness of morphine during various phases of the diurnal cycle were assessed by the hotplate method. Saline treated controls exhibited shortest reaction times during the last quarter of the light-phase and first quarter of the dark phase. Longest reaction times were recorded during the last quarter of the dark phase. Doses of 4, 8, 16, and 32 mg/kg of morphine was administered IP at the peak and trough of the pain sensitivity rhythm. The ED_{50} (95% C.L.) during the last quarter of the light phase was found to be 14.60 (10.6–20.0) mg/kg while during the last quarter of the dark phase the ED_{50} was found to be 5.85 (4.5–7.7) mg/kg. In a second experiment, independent groups of ten mice each were injected SC with 8 mg/kg of morphine at three hr intervals over a 48 hr test session. Peak analgesic activity was obtained in the group injected during the last quarter of the dark phase while minimal analgesic effectiveness was obtained during the third quarter of the light phase. Central administration of morphine via the intraventricular route yielded the same relationship, i.e., maximal analgesic effectiveness during the last quarter of the dark phase.

Circadian Analgesia Morphine Hotplate Pain Central administration

MORRIS AND LUTSCH [8,9] have reported finding a circadian variation in the magnitude of morphine analgesia as assessed by a modification of the tail-pinch method of Hafner. In addition to demonstrating the presence of a rhythm, they successfully brought about a phase shift in the rhythm by reversing the light-dark cycle [7], thereby demonstrating that the light cycle is the entraining agent. We have previously reported [2] a diurnal variation in the latency and quality of response to thermal stimulation on a hot plate. This raised the possibility that the morphine analgesia rhythm reported by Morris and Lutsch was the result of an underlying pain rhythm.

The present series of experiments were conducted in order to examine the nature of the interaction, if any, between a diurnal pain reactivity rhythm and a morphine analgesia rhythm, and to quantify the diurnal variation in morphine analgesia by determining the ED_{50} at various points wtihin the 24-hr light-dark cycle.

GENERAL METHOD

Housing

The mice were housed in a specially designed environmental chamber which provided sound attenuation, temperature control and a rigidly maintained lighting schedule. The chamber was maintained at 22° ± 1°C by an air conditioning system which was entirely independent of the building air conditioning system. The chamber was maintained on a 12:12 light-dark cycle with the lights-ON from 0600 to 1800 hr. Illumination during the lights-ON phase was provided by eight, 40 W fluorescent light bulbs. During the lights-OFF phase, low level illumination was provided by three 60 W, red incandescent light bulbs.

Entrainment Protocol

CF-1 male mice were procured from Carworth Farms. Mice were six weeks old at the time of arrival in the laboratory. At this time they were housed five to a cage in translucent polypropylene cages (13 cm × 18 cm × 28 cm) obtained from Carworth. Bedding material was Litter Green supplied by McFadden Co. Food (Purina Lab Chow) and water, which were available ad lib, were replenished once a week during the lights-ON phase. Bedding material was also changed once a week at this time. Mice were maintained under this entrainment protocol for four weeks prior to the initiation of testing at seventy days of age.

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Drugs

Morphine sulfate was purchased from Merck, Sharpe and Dohme. Doses are expressed as mg/kg of salt. The concentration of drug was adjusted such that all injection volumes were 0.01 ml/g mouse. Each mouse was individually weighed and injected with the appropriate volume of drug. Control injections consisted of 0.9% saline.

Procedure for Central Administration

Mice were lightly anesthetized using a mixture of 50% O_2 and 50% CO_2 gas. Induction time was approximately twenty seconds; recovery time, approximately thirty seconds.

Anesthesia was maintained for sixty seconds during which an 8 to 10 mm long incision was made in the scalp on the longitudinal center line between the ears. A 26 g diameter burr hole was then placed in the skull 1.5 mm caudal to Lambda on the longitudinal center line. The mouse skull, being quite thin and transparent, permitted visualization of the posterior colliculi and cerebellum. Therefore, slight adjustments were made in the location of the burr hole to precisely locate the hole at the juncture between the right and left posterior colliculi and the cerebellum.

Morphine was introduced into the fourth ventricle by way of a 27 g needle inserted through the burr hole to a depth of 3 mm. Injection volume was 1 μl. The dose was 0.25 μg of morphine sulfate per mouse. The ventricular injection procedure has been previously described [1].

Apparatus and Testing Procedure

The apparatus employed for assessing reactivity to a thermal stimulus was a hot plate upon which the mice were placed [2]. The testing regimen adopted was similar to that used by Weller *et al.* [12]. When the animal came in contact with the surface, a timer was activated and then stopped when a response was made. A response was defined as (1) jumping, (2) paw licking, (3) kicking, or (4) paw withdrawal. Previous experience with this apparatus [2] indicated that essentially all responses could be classified into one of these groups. Mice not responding within thirty seconds were removed from the surface of the hot plate and assigned the maximum possible score of thirty sec.

Quantitative Procedures

Response latencies were expressed as a Percent of Maximum Possible Inhibition (%MPI) according to the method of Dewey *et al.* [3]. Mice not responding within thirty sec were removed from the apparatus and considered to be 100% analgesic. The % MPI was calculated for each animal as follows:

$$\% \text{ MPI} = \frac{\text{RT (Experimental animal)} - \text{mean RT (Control group)}}{30 \text{ sec} \quad \text{mean RT (Control group)}}$$

Control RT's were obtained from saline injected mice which were run concurrently with the morphine treated mice, i.e., at the same time of day and for the same number of trials. This was necessary since we have previously shown [2] that repeated testing and time of day interact to produce significant effects on the latency of mice to respond to thermal stimulation. A composite measure of both magnitude and duration of analgesia was also obtained. This was accomplished by integrating the area under the % MPI-time course curve. Mice 100% analgesic for the entire 120 min test session could obtain the maximum possible score of 120 MPI-min.

Although the above procedures have been widely used to operationally define the analgesic state they do not provide any indication of the relationship between an experimental animals' score and the distribution of scores seen in the control population. It would be more informative to define the analgesic state not only with respect to the mean of the control population but also with respect to its variance. For this reason analgesia scores for experimental animals were standardized with respect to the control group according to the following formula:

$$\text{Standardized Analgesia Score} = \frac{\text{RT (experimental subject)} - \text{mean RT (control group)}}{\text{one standard deviation (control group)}}$$

This formula expresses an individual animals' reactin time (RT) in terms of the number of standard deviation units its score is increased above the mean RT of the control group for any given time of day or trial, regardless of the mode of response. For the purposes of computing ED_{50} values, the method of Litchfield and Wilcoxon [6] was used. A state of analgesia was operatioally defined as an increase in RT of at least three standard deviation units above the control mean. This latter procedure was used for data derived from the first experiment.

METHOD OF PROCEDURE

Experiment 1

Morphine analgesia dose-response studies were conducted at 1500 hr and 0300 hr, i.e., at the peak and trough of the previously measured thermal reactivity rhythm [2]. During each of the two time periods, five groups of 24 mice each were injected intraperitoneally with saline vehicle or one of the following doses of morphine sulfate: 4, 8, 16 or 32 mg/kg. Each group was tested six times at thirty min intervals within a three hr period. Morphine or saline was injected immediately after the second trial. A different dose-group was run on each of four consecutive days (or nights) according to the following sequence: 8, 4, 16 and 32 mg/kg.

Experiment 2

Diurnal variations in the analgesic effectiveness of a single dose of morphine were assessed in different groups of mice subjected to analgesia testing at different times of the day. Sixteen groups of ten mice each were tested. A separate group of mice was tested every three hr over a 48 hr period. During a given three hr test session, each mouse was tested six times at thirty min intervals. Immediately following the second hot plate test, the mice were injected subcutaneously with a 8 mg/kg of morphine sulfate. The route of administration was changed from that used in the first experiment in order to determine whether the effects observed following intraperitoneal administration were route specific.

Experiment 3

Diurnal changes in the magnitude and time course of

morphine-induced analgesia were examined following the injection of 0.25 μg of morphine into the fourth ventricle. Mice were injected with either saline or morphine at either 0300 or 0900 hr. These times were chosen to correspond with the crest and trough of the analgesia rhythm obtained after peripheral administration of morphine. Thermal response latencies were determined 10 min and one min prior to injection, as well as 5, 10, 20, 40, 60, 80 and 100 min postinjection. Mice remained on the hot plate until two responses were made, the latency for each response was recorded. Mice failing to make two responses within 60 sec were removed and assigned a score of 60 sec. Both the initial response latency and the time elapsed between the first and second responses were recorded. This procedural change was introduced because of behavioral effects noted during pilot studies.

RESULTS

Experiment 1

Data derived from the dose-response study were subjected to an analysis of variance. The analysis was performed on the latency data within these factors: two time periods (P), five doses (D), six trials (T) per animal and 15 animals per period per dose. The period effect was found to be significant $F(1,230) = 20.24$, $p<0.01$. The mice exhibited significantly greater analgesia at 0300 hr. As might be expected, the dose effect was also significant, $F(4,230) = 61.01$, $p<0.01$. A significant trial effect, $F(5,1150) = 145.85$, $p<0.01$, reflected the time course of action of morphine analgesia. The significant P × D interaction, $F(4,230) = 3.59$, $p<0.01$, and T × D interaction, $F(20,1150) = 26.76$, $p<0.01$, appear in Fig. 1. The magnitude of analgesia and the duration of the analgesic effect are significantly greater for all doses when administered at 0300 hr.

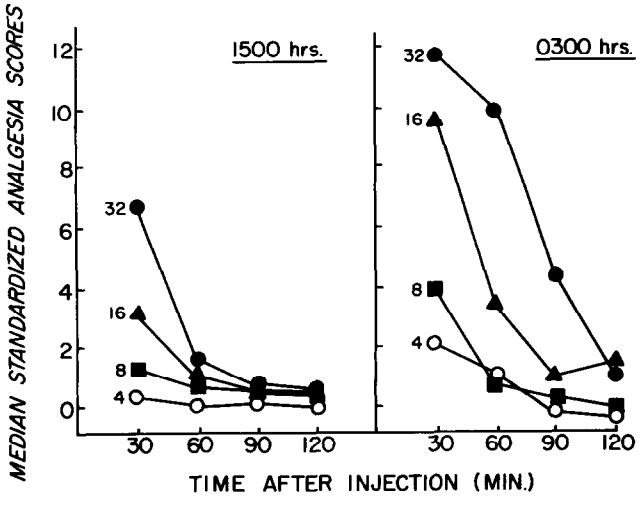

FIG. 1. Time course of analgesia as a function of dose (4, 8, 16, 32 mg/kg) and time of day after intraperitoneal injection of morphine to independent groups of 15 mice per dose.

The ED_{50} and 95% confidence limits computed from latency scores at thirty min postinjection were found to be 5.85 mg/kg (4.47–7.66) at 0300 hr and 14.60 mg/kg (10.66–20.00) at 1500 hr. The slopes of the two dose-response curves were found to be not significantly different as shown in Fig. 2.

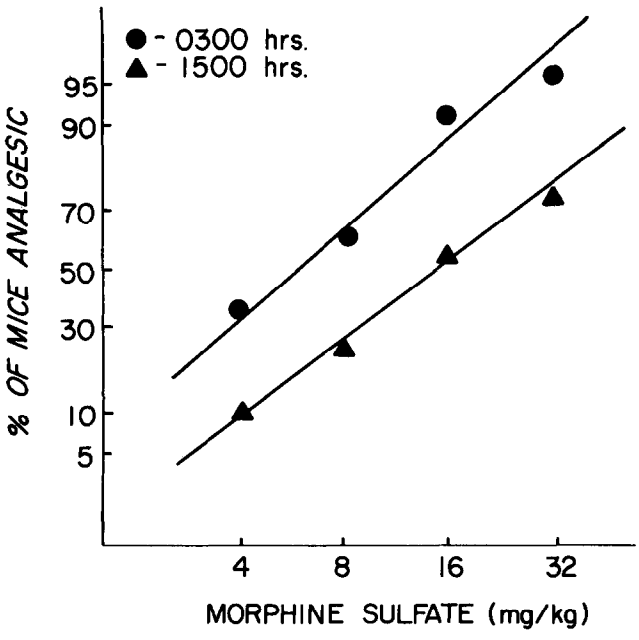

FIG. 2. Morphine analgesia dose-response curves at peak and trough of thermal reactivity rhythm. Groups of 15 mice per data point received intraperitoneal injections of morphine sulfate.

Experiment 2

The response latencies of all morphine treated mice were converted to % MPI scores as described previously. The distribution of % MPI scores for those groups of mice injected during the lights-OFF period was negatively skewed, i.e., a large proportion of the mice were 100% analgesic. Therefore, the data appearing in Fig. 3 and Fig. 4 are reported as median values rather than means. Figure 3 depicts the time course of analgesic action obtained at four equally spaced intervals during the 24 hr light-dark cycle. Both the magnitude and duration of action of analgesic activity were greatest during the lights-OFF periods, i.e, 2100 hr and 0300 hr. The ceiling effect which was encountered at thirty min postinjection should also be noted. Because of the thirty sec cut-off employed and the high degree of analgesia obtained with the 8 mg/kg SC injection, the apparent magnitude of the diurnal influence is attenuated. In spite of this, significant differences were found between 0300 and 0900 hr (Median test, Chi-square = 10, $df = 1, p<0.01$).

A composite measure of magnitude and duration of action was obtained by integrating the area under each of the 16 median time-effect curves obtained by injecting ten mice during each of 16 periods in this 48 hr study. Data collected at 2400 hr during the second 24 hr interval have been omitted. These data were considered invalid due to a change of experimenters necessitated by the fatigue of the original experimenters. The rhythm exhibits a peak at 0300 hr, i.e., during the last quarter of the animals activity phase, and a trough at 0900 to 1200 hr, i.e., during the middle of the animals inactive phase.

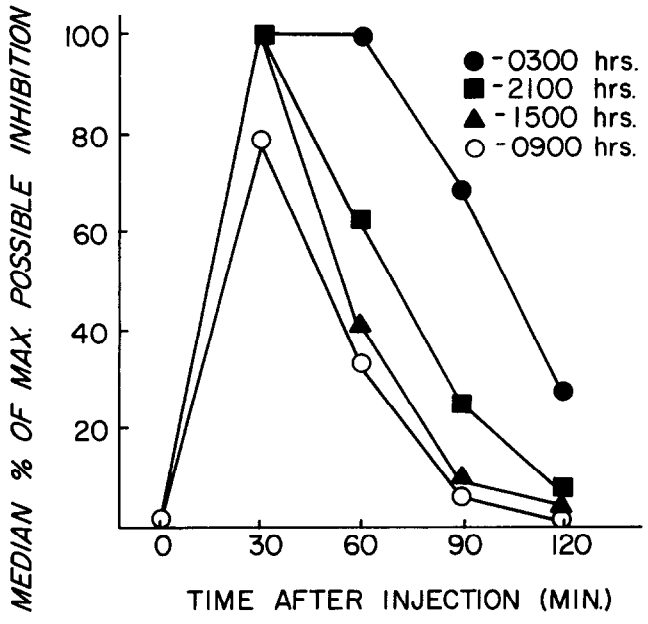

FIG. 3. Time course of morphine analgesia as a function of time of day. Groups of 20 mice each were injected SC with 8 mg/kg of morphine.

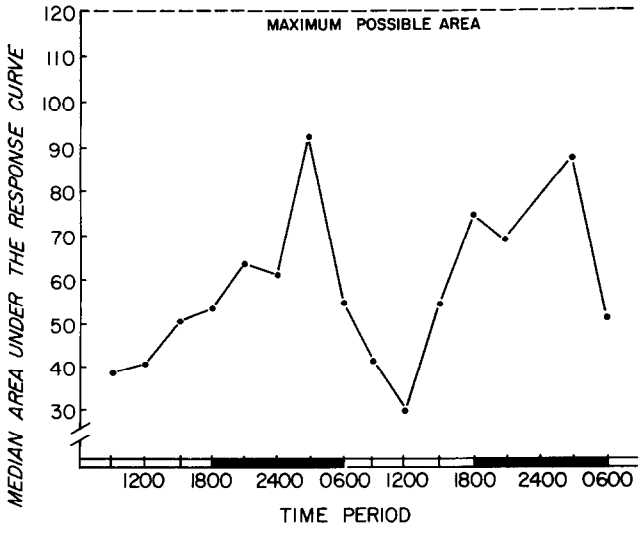

FIG. 4. Diurnal variation in the analgesic effect of 8 mg/kg of morphine sulfate (SC). Each point represents the median score for a group of 10 mice.

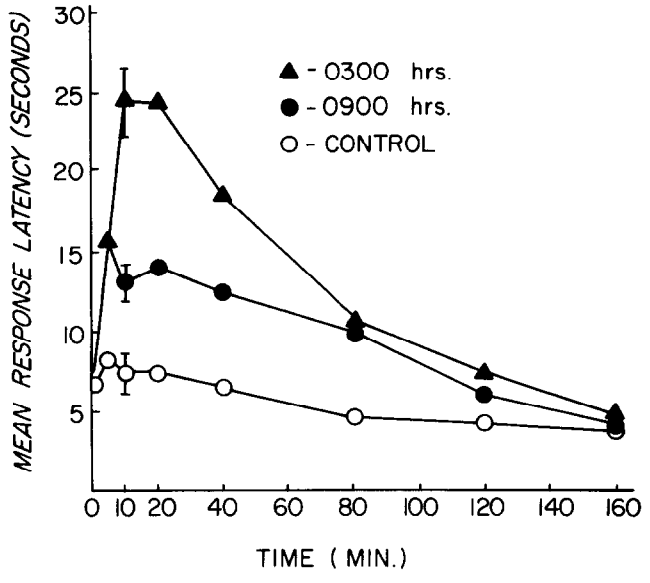

FIG. 5. Diurnal variation in analgesic activity (latency to make two consecutive responses) following fourth ventricular administration of 0.25 μg of morphine. Control groups were combined into a single group of 18 mice. Morphine groups each contained 15 mice.

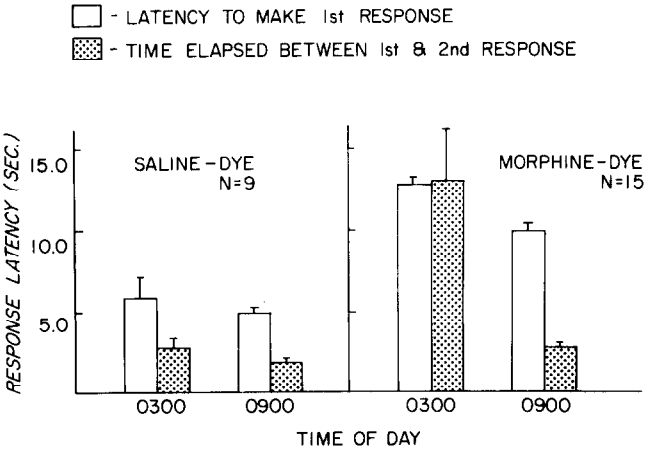

FIG. 6. Response latencies 10 min after IV ventricle injection of either saline or morphine (0.25 μg/mouse). Data is reported as mean ± SEM for each of the four groups.

Experiment 3

The effects of intraventricular morphine injections are shown in Fig. 5. The rate of onset of analgesia was the same at 0300 and 0900 hr. The major difference seen in these two groups was the significantly greater magnitude of analgesia as reflected in longer response latencies at 0300 hr. The differences between the two groups disappeared by eighty min postinjection. Figure 6 depicts the latency to make the first response and the time elapsed between the first and second response for control mice (N = 9 per time period) and morphine treated mice (N = 15 per time period) ten min after injection at 0300 and 0900 hr. First and second response latencies for saline treated mice were significantly lower at 0900 hr than at 0300 hr in agreement with the previously reported diurnal change in pain thresholds. The first response latency for morphine treated mice was significantly longer at 0300 hr than at 0900. This finding fully supports the relationship obtained following SC administration of morphine, i.e., maximal analgesia at 0300 and minimal analgesia at 0900 hr. Even more dramatic was the latency required to make a second response to continuing thermal stimulation. At 0300 hr, the morphine treated mice appeared indifferent to the thermal stimulus after making the first response and remained relatively immobile for another 10 to 15 sec before making

a second response. At 0900 hr, the first response was followed by an increased activity level similar to that seen in saline treated mice and then within two seconds by the second response.

DISCUSSION

Experiments 1, 2 and 3 demonstrate that a significant diurnal variation in the effectiveness of morphine is present in the mouse-hot plate test. The morphine analgesia rhythm was found to parallel the pain rhythm (cf. [2]).

However, the magnitude of the analgesia rhythm was so great that a rhythm was still demonstrable following statistical correction for the diurnally changing base line in pain reactivity. Morphine, IP, was found to be 2.7 times more effective at 0300 hr than at 1500 hr. There was no detectable diurnal variation in the onset of analgesia thereby ruling out the possibility that the diurnal rhythm reported by Morris and Lutsch was a reflection of a diurnal change in time course of action. Thus it would appear that the work of Morris and Lutsch [8,9] truly reflects a diurnal change in the analgesic effectiveness of morphine even though their rhythm would be somewhat attenuated following correction for the changing base line in pain reactivity. The present study also demonstrates that the analgesia rhythm is neither sex nor task specific since this study used mice of the opposite sex and an entirely different analgesiometry task than that used by Morris and Lutsch. Although they conducted no dose-response studies, their reported rhythms are similar to that shown in Fig. 4. In the present study, morphine was more effective in increasing the response latency to thermal stimulation during the latter half of the dark phase of the lighting cycle irrespective of the route of administration.

A number of factors could account for the observed diurnal changes in the analgesic activity of morphine including diurnal changes in (1) drug metabolism and other factors influencing bioavailability (2) pituitary-adrenal rhythms, and (3) central neurohumoral rhythms.

Several factors argue against peripheral metabolic factors being responsible. First, morphine was most effective during the dark period for all routes of administration, i.e., IP, SC and intraventricular. Circadian variations in liver metabolism also argue against peripheral factors. Data reported by Radzialowski and Bousquet [10] indicate that enzymes known to be responsible for N-demethylation and glucuronide conjugation of morphine exhibit maximal in vitro activity during the middle of the dark cycle. Thus one would predict morphine to be metabolized most rapidly during the dark. In the presence of relatively lower levels of morphine in the blood one would be led to predict reduced magnitude and duration of analgesia following the administration of morphine at night. The data shown in Figs. 2, 3, and 4 clearly indicate that this was not the case. Assuming that morphine metabolism is greater at night, the elevated analgesic activity of morphine at night must be accounted for by factors other than diurnal variations in peripheral metabolism. Data derived from intraventricular injections of morphine (see Figs. 5 and 6) also support the contention that central factors not peripheral metabolic factors, are responsible for the observed analgesia rhythm. As seen following peripheral administration, morphine's analgesic effectiveness after ventricular administration was greater at 0300 hr than at 0900 hr.

Pituitary-adrenal reactivity may also be involved in the morphine-analgesia rhythm. The responsiveness of the rodent pituitary-adrenal system to environmental stress undergoes diurnal changes and is highest during the last half of the dark phase [4]. Furthermore, sensory acuity in humans is reportedly decreased in the presence of elevated circulating steroid levels [11]. Therefore, the peripheral or central actions of corticosterone may be augmenting in some manner the effectiveness of morphine. However it is not presently possible to go beyond this speculation since no circadian examination of these systems has been made in the presence of morphine. Likewise, although central neuro-transmitters with which morphine is thought to interact, e.g., serotonin and dopamine, exhibit cyclic variations in concentration and release [11], no examination of the rhythms has been made in the presence of morphine.

The significance of the increased latency to make a second response at 0300 hr following fourth ventricular administration of morphine is unclear at this time. Whether this effect is related to the fourth ventricular administration of morphine or the time at which the morphine was administered remains to be determined. The clinical literature contains many subjective reports which imply two different mechanisms of morphine action. The first pertains to a reduction in the level of pain while the second pertains to a tranquilizing action wherein the patient reports a sensory awareness of the pain but is no longer emotionally aroused by the pain. In the present study, the absence of agitated behavior of any kind following the initial response may indicate a diurnal variation in the ability of morphine to suppress the emotional response to pain as well as block the initial sensory component.

Conclusion

Significant diurnal variations in morphine analgesia have been demonstrated with the mouse-hot plate test thus confirming and extending the work of Morris and Lutsch [8,9]. Central administration of morphine as well as current information regarding diurnal changes in liver enzyme activity suggest that changes in the responsiveness of the CNS to morphine may underlie the observed analgesia rhythms. The demonstration of a three-fold difference in the efficacy of morphine as a function of time of day has important implications for both the therapeutic use of morphine and experimental investigations of mechanisms of morphine action, development of morphine congeners and basic studies of CNS processes.

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