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Circadian Fluctuations in Pain Responsiveness and Brain Met-Enkephalin-Like Immunoreactivity in the Rat

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KURUMAJI, A , M TAKASHIMA, K OHI AND K TAKAHASHI *Circadian fluctuations in pain responsiveness and brain Met-enkephalin-like immunoreactivity in the rat* PHARMACOL BIOCHEM BEHAV **29**(3) 595–599, 1988 —The 24-hour patterns of pain responsiveness and brain Met-enkephalin-like immunoreactivity (MLI) were determined in male Wistar rats housed under a 12-hour light and dark cycle (lights on from 0700 hr to 1900 hr) A circadian rhythm was observed in latencies to hot plate test (55°C), showing the peak level near the onset of the dark phase (2000 hr) Pretreatment with naloxone (5 mg/kg, subcutaneously) decreased the highest latency (2000 hr), but did not change the lowest latency (1100 hr) In the mesolimbic area and the striatum, MLI had a negative correlation with the circadian fluctuation in pain sensitivity MLI at 2000 hr was reduced significantly compared to that at 1100 hr in the basal ganglia, the frontal cortex and the substantia nigra These results suggest that the circadian variation in hot plate latencies follows a circadian change in the activity of the endogenous opioid peptides system, and that Met-enkephalin may participate in the enhancement of the opioid system in the brain

Hot plate test Met-enkephalin Circadian rhythm Striatum Mesolimbic area

IT has been demonstrated that the responsiveness of mice to nociceptive stimuli, measured by the hot plate test, shows a circadian rhythm, and that treatment with naloxone decreases the hot plate latency during the late hours in a day when the latencies are naturally high [4] A rise in whole mouse brain total opioid levels, determined by mouse vas deferens bioassay, was observed in mice sacrificed in late afternoon compared to early morning [13] A receptor binding study revealed that the amount of specifically bound (^{3}H) naloxone, an antagonist of opioid peptide, shows a circadian variation in the light-dark cycle, with a peak near the onset of the dark phase [11] Accordingly, it assumes that the circadian rhythm in pain sensitivity is mediated by endogenous opioid peptides However, it is not clear which opioid peptide participates in the circadian variation in pain sensitivity

Met-enkephalin, one of the endogenous opioid peptides, is believed to play a role as a neurotransmitter or neuromodulator in the central nervous system. This was demonstrated by its presence and unique distribution in the brain [7,8], its release from nerve terminals following depolarization [6], and its role in the modification of pain perception [2] Moreover , Xuan *et al* [14] reported that the injection of d-Ala-2-Leu-enkephalin, an analogue of enkephalin, into the nucleus accumbens of the rabbit produces a significant antinociception

In the present study, we examined 24-hour patterns of latencies to hot plate test and brain Met-enkephalin-like immunoreactivity (MLI) in the rat in order to clarify the role of Met-enkephalin in the circadian rhythm in hot plate latencies

METHOD

Animals

Male Wistar rats (220–300 g) were purchased from Sankyo Labo Service Corporation (Japan) and housed 4 per cage under controlled conditions (24±1°C temperature and 50% humidity) with a 12 12 light-dark cycle (lights on at 0700 hr) and free access to food and water They were used in the following experiments after acclimatisation to the environment for two weeks

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Hot Plate Test

To study a 24-hour pattern of pain sensitivity, eight animals were tested for hot plate latencies at 3-hour intervals according to the method of Frederickson *et al* [4], following five days of handling and succeeding two days of adaption to the apparatus A metal plate (10×23 cm) was electrically heated and thermostatically controlled at a temperature of 55°C A transparent acrylic resin box (40 cm high and open at the top) served to confine the rat to a defined area of the hot plate The time in seconds from contact with the plate until a paw lick or an escape jump was recorded as a hot plate latency In each sampling session, one latency was taken from one animal During the dark period, a dim red light was turned on during the test

Naloxone hydrochloride (Sigma) dissolved in physiological saline was given subcutaneously to rats at a dose of 5 mg/kg, 15 minutes prior to the test Control animals were treated with an equal volume of the vehicle

Measurement of MLI

Following one week of handling, animals were decapitated at 3-hour intervals over a 24-hour period In the dark phase, a dim red light was turned on during the procedure The brains were quickly removed in the cold, and stored at −80°C until biochemical analysis The following areas of the brain were dissected out or punched out from serial frozen coronal slices (600 μm) using a microknife or stainless steel tube (1 5 or 2 0 mm i d) as previously described [8] The following co-ordinates (atlas of Paxinos and Watson [12]) were used (+ anterior or − posterior to bregma mm) frontal cortex +4 2 ~ +2 4, mesolimbic area +2 7 ~ +0 9, striatum +1 7 ~ −0 7, thalamus −1 8 ~ −3 2, amygdaloid nuclei and piriform cortex −2 3 ~ −4 1, substantia nigra −5 3 ~ −4 7

The radioimmunoassay procedure and antibody specificity for Met-enkephalin were described in the previous paper [8] Brain tissues were heated 90°C in 1 N acetic acid containing 20 mM hydrochloride for 15 minutes, sonicated and centrifuged 8,800×g at 4°C for 30 minutes The supernatant fluid was then neutralised with sodium hydroxide and radioimmunoassayed for Met-enkephalin Anti-Met-enkephalin antiserum was produced in rabbits which were immunised with Met-enkephalin conjugated to bovine thyroglobulin using 1-ethyl-3-dimethylaminopropyl carbodiimide The antiserum so obtained showed no cross-reaction with Leu-enkephalin, α-, β-, γ-endorphin, β-lipotropin or α-neo-endorphin Standard or brain extract was incubated with antiserum diluted 2,000-fold and tritiated Met-enkephalin (20,000 cpm, 39 3 Ci/mmol, New England Nuclear) in 0 5 ml of 0 05 M phosphate buffer (pH 7 4) containing 0 25% bovine serum albumin and 0 5% 2-mercaptoethanol The incubation was carried out at 4°C for 2 days The tritiated Met-enkephalin bound to the antibody was separated from the free tritiated Met-enkephalin by adding 0 5 ml of 1% charcoal slurry containing 0 5% dextran, and the radioactivity of an aliquot of the supernatant fluid was measured in a liquid scintillation spectrometer Under our conditions, the minimum detection limit was 0 03 pmoles of Met-enkephalin The serial dilution of the extract in each brain region yielded a displacement parallel with that of authentic standards MLI in the pooled extract from the striatum, analysed by high performance liquid chromatography, coeluted with authentic Met-enkephalin

Protein was assayed according to the method of Lowry *et al* [9]

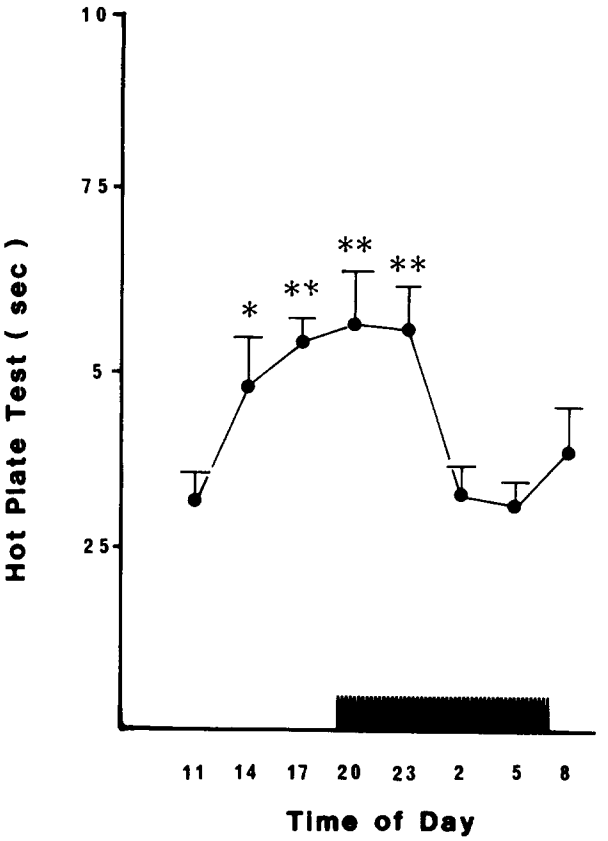

FIG 1 A circadian rhythm in pain sensitivity in the rat The hot plate test was used to evaluate the pain responsiveness The results were expressed as mean with S E M of data obtained from eight animals Lights were off during the time period marked by the hatched bars *$p<0$ 05, **$p<0$ 01 as compared to 1100 hr of day

Statistical Analysis

Results were expressed as mean with S E.M Data from the hot plate test were analysed by one-way ANOVA and followed by multiple comparison test (Duncan's method) MLI data were analysed using two-way ANOVA (region × time of day) and two-tailed Student's *t*-test Correlation coefficients of linear regression were calculated according to the method of least squares

RESULTS

Hot Plate Test

Hot plate latencies were found to vary significantly over the 24-hour sampling period, $F(7,56)=4$ 26, $p<0$ 001 The latencies were low during the early part of the light phase, began to rise 6 hours before the dark onset, and fell during the last 5 hours of the dark phase (Fig 1)

Effects of pretreatment with naloxone on the hot plate latencies are shown in Fig 2 The latency at 2000 hr was decreased by the administration of naloxone ($p<0.01$), while the effect of naloxone was not found at 1100 hr The naloxone-reduced latency at 2000 hr did not differ from the latency at 1100 hr in either the saline-treated rats or the naloxone-treated ones The diurnal difference between 2000

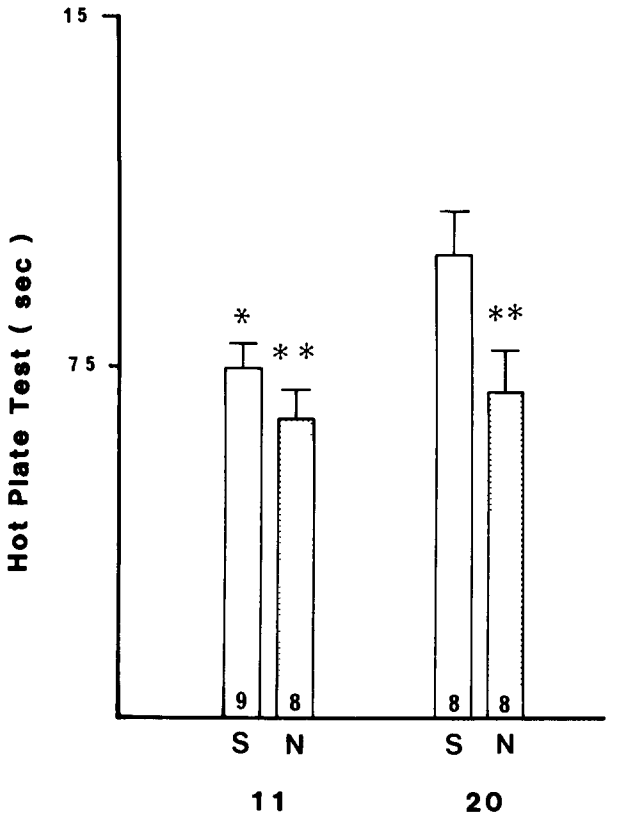

FIG 2 The effect of naloxone on pain sensitivity in the rat Naloxone (5 mg/kg, SC) was injected 15 minutes before the hot plate test Each column indicates the mean with S E M for the number of animals shown in the columns S saline, N naloxone, 11 1100 hr of day, 20 2000 hr of day *$p<0$ 05, **$p<0$ 01 as compared to the saline-treated rats at 2000 hr of day

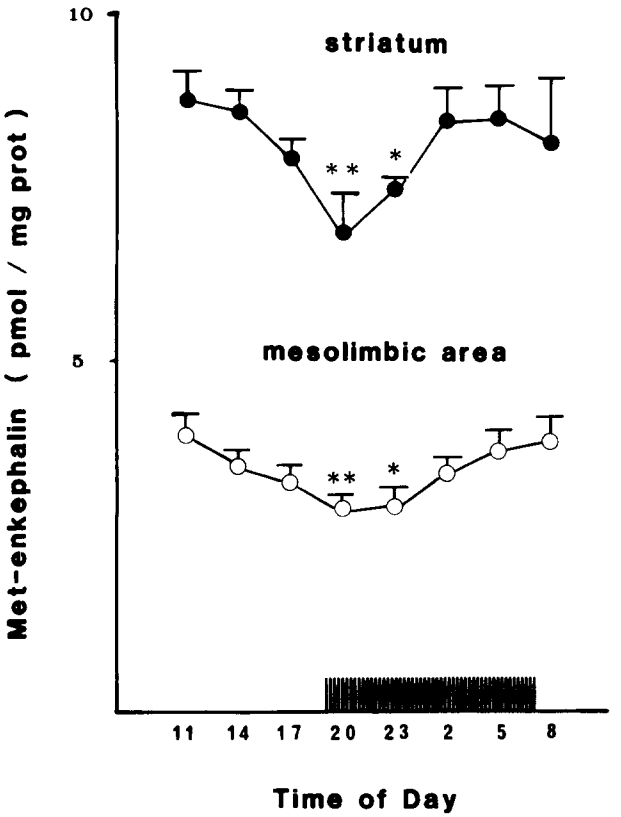

FIG 3 A circadian variation in Met-enkephalin-like immunoreactivity in the mesolimbic area and the striatum Results are expressed as mean with S E M of data obtained from 6 or 7 animals Lights were off during the time period marked by the hatched bars *$p<0$ 05, **$p<0$ 01 as compared to 1100 hr of day (two-tailed Student's t-test)

hr and 1100 hr in the saline-treated animals was statistically significant ($p<0$ 05)

MLI

Circadian variations in MLI are shown in Fig. 3. A two-way (region × time of day) analysis of variance (ANOVA) yielded a significant effect of region, $F(1,95)=474$ 52, $p<0$ 01, and effect of time, $F(7,95)=2$ 71, $p<0$ 05, but not of interaction between time and region, $F(7,95)=0.42$ During the light-dark cycle, MLI showed a circadian variation in the mesolimbic area as well as the striatum, with a nadir between 1700 hr and 2300 hr. The changes in MLI in both areas were found to be parallel ($r=0$ 822, $p<0$ 02)

The MLI content at 2000 hr was lower than that of MLI in 1100 hr in the frontal cortex and the substantia nigra, but not in the thalamus or the amygdaloid nuclei and piriform cortex (Table 1).

Correlation Coefficient

The hot plate latencies over the 24-hour period were found to have a negative correlation with MLI not only in the mesolimbic area ($r=-0$ 817, $p<0$ 02, Fig. 4) but also in the striatum ($r=-0.787$, $p<0.05$)

DISCUSSION

We demonstrated that the hot plate latencies over a 24-hour period show a circadian rhythm, and that treatment with naloxone is effective at the time of the highest latency, but not the lowest latency These results indicate that the elevation of latencies depends on the activity of the endogenous opioid peptides system As the rhythm of hot plate latencies under constant darkness was found to be the same as that under the light-dark cycle (data not shown), the latency to the hot plate test exhibited an endogenous rhythm Supporting our findings, Frederickson *et al.* reported that the responsiveness of mice to nociceptive stimuli, using the hot plate tests, shows a circadian rhythm, and that pretreatment with naloxone (3 mg/kg, SC) significantly reduced the hot plate latencies during the late hours (1530 hr to 0430 hr) when the latencies are naturally high [4] Other reports have also assumed that naloxone would be expected to have hyperalgesic effects only when the opioid system is activated [3,10]

Our results concerning the concentration of MLI in the discrete brain areas were in agreement with the report by Hong *et al* [7], indicating that the MLI content was high in the basal ganglia, whereas it was moderate in the thalamus

TABLE 1

DIURNAL DIFFERENCE BETWEEN 1100 HOUR AND 2000 HOUR IN BRAIN MET-ENKEPHALIN-LIKE IMMUNOREACTIVITY

	Met-Enkephalin-Like Immunoreactivity (pmol/mg prot)		
	1100 hr	2000 hr	
Frontal cortex	0 47 ± 0 04	0 34 ± 0 02	$p<0$ 02
Thalamus	1 15 ± 0 06	1 07 ± 0 07	N S
Amygdaloid Nuclei and Piriform Cortex	1 61 ± 0 18	1 51 ± 0 08	N S
Substantia nigra	0 81 ± 0 02	0 56 ± 0 05	$p<0$ 01

Results were expressed as mean with S E M of data obtained from 6 or 7 animals Statistical significance was determined by two-tailed Student's t-test

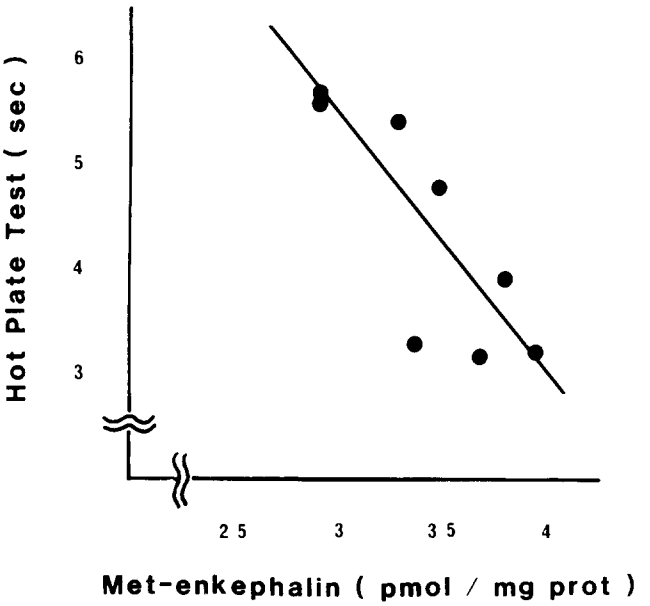

FIG 4 The correlation between hot plate latencies and Met-enkephalin-like immunoreactivity in the mesolimbic area over a 24-hour period A correlation coefficient of linear regression was calculated according to the method of least squares ($r=-0$ 817, $Y=-2$ 3X + 12 5, $p<0$ 02 N=8)

and the amygdala and low in the cortex and the substantia nigra

In the mesolimbic area and the striatum, MLI showed a circadian fluctuation with minimum content between 1700 hr and 2300 hr The concentration of MLI at 2000 hr was found to be significantly lower than at 1100 hr in the following areas of the brain the basal ganglia, the frontal cortex and the substantia nigra There may be two factors giving rise to the change in the brain MLI content One factor is the utilization of Met-enkephalin, another is synthesis and storage The circadian variation in MLI in the basal ganglia was found to be a mirror image of that of pain responsiveness, having a high correlation coefficient At the time of day when the brain MLI content was lowest, the hot plate latencies were at their highest levels and the activity of the opioid peptides system was enhanced as suggested by the results of naloxone administration In view of the rapid degradation of Met-enkephalin in synaptic clefts, it is likely that an increase in the release of the peptide resulted in the decrease in brain MLI content and activated the neuronal activity when the latencies were high This possibility is supported by Bayon *et al* who reported that in vivo spontaneous release of Leu-enkephalin in the globus pallidus in the rat increases between noon and evening by 100% [1]

The injection of d-Ala-2-Met-enkephalin, an analogue of Met-enkephalin, into the caudate-putamen changed the motor function, e g , ipsiversive rotation behavior and bodily asymmetry [5] Xuan *et al* has recently reported that Met-enkephalin plays an important role in the antinociception in the nucleus accumbens of the rabbit, interacting with the serotonergic neuron [14] The physiological and behavioral responses to the hot plate test, such as nociception of heat stimuli and a paw lick or an escape jump, seem to be influenced by the enkephalinergic neuronal activity in the basal ganglia

Our findings seem to be contradicted by those of Wesche and Frederickson, who reported that an increase in whole brain total opioid levels, determined by mouse vas deferens bioassay, is observed in mice scarificed in late afternoon (when they are least responsive to pain) compared to early morning (when they are most responsive to pain) However, they could not find any elevation in the levels of Met- and Leu-enkephalin measured by radioimmunoassay [13] This discrepancy might be due to the different kind of species, or the method of sample preparation or the specificity of the antiserum for the Met-enkephalin assay We dissected out each rat brain area by the precise procedure described in the method section, whereas they used the whole mouse brain for the bioassay and the radioimmunoassays Concerning the specificity of antiserum for Met-enkephalin, we confirmed it with high performance liquid chromatography MLI in the pooled extract from the striatum coeluted with authentic Met-enkephalin

In conclusion, the present paper indicates that the change in the neuronal activity of brain Met-enkephalin may play an important role in the circadian rhythm in pain sensitivity of the rat It will be necessary to determine brain regions in which the peptide regulates the circadian variation in pain threshold

REFERENCES

1 Bayon, A and B Anton Diurnal rhythm of the in vivo release of enkephalin from the globus pallidus of the rat *Regul Pept* **15** 63–70, 1986

2 Belluzzi, J D , N Grant, V Garsky, D Sarantakis, C D Wise and L Stein Analgesia induced in vivo by central administration of enkephalin in rat *Nature* **260:** 625–626, 1976

3 El-Sobky, A , J O Dostrovsky and P D Wall Lack of effect of naloxone on pain perception in humans *Nature* **263·** 783–784, 1976

4 Frederickson, R C A , V Burgis and J D Edwards Hyperalgesia induced by naloxone follows diurnal rhythm in responsivitiy to painful stimuli *Science* **198:** 756–758, 1977

5 Geula, C and D Asodourian Asymmetric behavior induced by enkephalinergic agents in the basal ganglia *Pharmacol Biochem Behav* **23:** 207–213, 1985
6 Henderson, G , J Hughes and H W Kosterlitz In vivo release of Leu- and Met-enkephalin from the corpus striatum *Nature* **271:** 677–679, 1978
7 Hong, J S , H -Y Yang, W Fratta and E Costa Determination of methionine enkephalin in discrete regions of rat brain *Brain Res* **134:** 383–386, 1977
8 Kurumaji, A , M Takashima and H Shibuya Cold and immobilization stress-induced changes in pain responsiveness and brain Met-enkephalin-like immunoreactivity in the rat *Peptides* **8:** 355–359, 1987
9 Lowry, O H , N J Rosebrough, A L Farr and R J Randall Protein measurement with the folin phenol reagent *J Biol Chem* **193:** 265–275, 1951
10 Madden, J , IV, H Akil, R L Patrick and J D Barchas Stress-induced parallel changes in central opioid levels and pain responsiveness in the rat *Nature* **265:** 358–360, 1977
11 Naber, D , A Wirz-Justice and M S Kafka Circadian rhythm in rat brain opiate receptor *Neurosci Lett* **21:** 45–50, 1981
12 Paxinos, G and C Watson *The Rat Brain in Stereotaxic Coordinates* Sydney Academic Press, 1982
13 Wesche, D L and R C A Frederickson Diurnal differences in opioid peptide levels correlated with nociceptive sensitivity *Life Sci* **24.** 1861–1868, 1979
14 Xuan, Y T , Y S Shi, Z F Zhou and J S Han Studies on the mesolimbic loop of antinociception-II A serotonin-enkephalin interaction in the nucleus accumbens *Neuroscience* **19:** 403–409, 1986