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Rat brain opioid peptides-circadian rhythm is under control of melatonin

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Abstract

Several experiments have revealed an Endogenous Opioid System (EOS)-circadian rhythm. The brain-borne hormone, melatonin (MEL) has been shown to regulate the organism photoperiodic activity and may be implicated in the EOS-circadian rhythm. To explore this hypothesis, we studied the effect of functional pinealectomy on the EOS-circadian rhythm by measuring the immunoreactive content of Met-Enkephalin, Leu-Enkephalin and Synenkephalin in both hypothalamus and hippocampus of the rat brain, using standard radioimmunoassay procedures. Experimental animals exposed to white fluorescent light (WFL) for 15 days (<50 lux), displayed a disruption of the EOS-circadian rhythm, showing that absence of MEL induced a significant decrease of tissue content of enkephalin peptides at 01:00 h during the dark-phase of the 24-h circadian rhythm, when compared to control rats. Functional pinealectomized rats exposed to 4 or 6 h period of darkness (used to revert the effects induced by the absence of melatonin) significantly increased the tissue content of ME-IR and LE-IR, when compared to both controls and non-exposed WFL-treated rats. In addition, subcutaneous administration of exogenous melatonin (10, 100, 150, 300, 600 μg/kg), in WFL-treated animals produced significant dose-dependent increases of ME-IR in both brain regions tested. Finally, luzindole (melatonin receptor antagonist) administration, was not able to prevent the enkephalin tissue increase, induced with the MEL administration (150 μg/kg). This data suggest that MEL not only regulates the EOS-circadian rhythm, but also appears to modulate their synthesis in the rat brain from their respective neurons.

Keywords: Melatonin; Enkephalins; Functional pinealectomy; Circadian rhythm; Synthesis

1. Introduction

The physiology of the Endogenous Opioid System (EOS) is crucial for the homeostatic maintenance and survival of the organism. The EOS has been shown to be implicated in the regulation of a wide range of neuronal and extra neuronal functions produced by different subset of opioid peptides that once released, act either as neurotransmitters and/or neuromodulators at their cell targets (Andersson, 2000; Terenius, 2000). Mainly, the EOS has been shown to modulate the nociceptive transmission and pain perception (Machellska and

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Stein, 2000; Cabot et al., 2001; Cabot, 2001; Przewlocki and Przewlocka, 2001), stress responses (Saravia et al., 1998), cardiovascular activity (Dunbar and Lu, 2000; Rao et al., 2003) and immune-related responses (Maestroni and Conti, 1992).

Opioid peptides have been shown to be synthesized in both peripheral and CNS, from large propeptide protein precursors (Noda et al., 1982) and released to the extracellular *millieu*, under a circadian-rhythm control. Different studies have shown that opioid peptides reach a maximum tissue concentration (Przewlocki et al., 1983; Asai et al., 1988) at night-time (24:00–01:00 h), detecting high levels of released opioid peptides (Asai et al., 1998) and an increased expression of opioid receptors (Naber et al., 1981). Further studies showed that Synenkephalin (SYN), a bioactive non-opioid peptide encoded and released from the Proenkephalin A (amino acids 1–72), reaches its highest tissue concentration and maximum release responses during the dark-phase period (Asai et al., 1998). Thus, besides of the aforementioned observations, there is no clear evidence yet about the neurochemical and/or molecular mechanism that drives the circadian-rhythm control of the EOS in mammals.

In order to explore which endocrine substances could be involved in the circadian-rhythm control of EOS in the rat brain, we focused on the well known serotonin derivative, melatonin (MEL). MEL is an indoleamine (5-methoxy-*N*-acetyltriptamine) synthesized and secreted from the pineal gland at nighttime under a regular 24-h circadian rhythm (Reiter, 1993). Moreover, it has been shown to convey photoperiodic information and synchronize the biological activity during the 12-h light/dark cycle (Simonneaux and Ribelayga, 2003). Functional regulatory interaction between EOS and MEL could be postulated based on that pinealectomy abolishes the 12-h light/dark circadian rhythm of morphine analgesia (Golombek et al., 1991), and disrupts the diurnal rhythm content of Met-Enkephalin in the hypothalamus of the rat brain (Kumar et al., 1982). In addition, pinealectomy has been shown to disrupt GABA and general opioid peptides content in both rat and mouse CNS (Gomar et al., 1993), enhancing a naloxone reversed dose-dependent analgesic effect induced after administration of exogenous MEL (Kavaliers et al., 1983). Moreover, MEL has been shown to induce the expression of the Proopiomelanocortin (POMC) gene in several regions of the rat brain (Kotler et al., 1998; Yu et al., 2000) and in immune organs from rodent species (Wajs et al., 1995). Taken together, all these information suggest that MEL could be considered as a brain-borne hormone candidate, that not only regulates the circadian rhythm of the endogenous opioids, but also could be related with their synthesis in mammals.

The present study demonstrates a relationship between MEL and enkephalins after assessing the circadian variations on the tissue content of these opioid peptides in the rat brain in the presence and absence of endogenous MEL. Additionally, we studied the effect of the darkness stimulus, as well as melatonin and luzindole (MEL receptor antagonist) administration, in the rats subjected to functional pinealectomy.

2. Material and methods

2.1. Drugs and antiserum

Melatonin (*N*-acetyl-5-methoxytryptamine) and luzindole (*N*-acetyl-2-benzyltryptamine; *N*-[2-[2-(phenylmethyl)-1H-indol-3-yl]ethyl] acetamide, were purchased from Sigma Chemical Company (USA). Synenkephalin antiserum (SYN-IR to [Tyr^{63}](Syn 63-70) was generously donated by Osvaldo Vindrola (Neurochemistry Department, University of Puebla, Mexico).

2.2. Control animals

Male Wistar rats (230–260 g), were used in all experiments. Animals were fed with rodent pellet diet and water *ad libitum*. The rats were housed individually in animal cages, under a 12:12-h light-dark regime (lights on at 06:00 h, light off 18:00 h) at a room temperature of 23 ± 1 °C.

All experimental protocols were approved by the ethics committee of the National Institute of Psychiatry (Mexico), in order to minimize animals suffering and to reduce the number of animals used in the experiments, following the proposal of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3. Functional pinealectomy

In order to inhibit the endogenous MEL synthesis, the rats were housed in individual cages under a temperature-controlled (23 ± 1 °C) room and were exposed to a continuous white fluorescent light (WFL) during 15 days (<50 lux).

3. Experimental procedures

3.1. Experiment 1. Quantification of tissue content of endogenous opioid peptides in the rat brain during 24-h light-dark cycle period

In order to analyze the Met-Enkephalin (ME), Leu-Enkephalin (LE) and Synenkephalin (SYN) concentration in the hypothalamus and hippocampus of the rat brain; control and experimental rats were sacrificed by decapitation every 4 h along the 24-h light-dark cycle period ($n = 5$/group).

3.2. Experiment 2. Darkness effect over functional pinealectomy

WFL animals ($n = 5$/group), were exposed to 4 and 6 h under dark environment. Exposure to dark environment has been shown to restore the endogenous synthesis of MEL as previously shown by Reiter (1993), avoiding thus, unnecessary pharmacological or surgical procedures that could bias the tissue content of opioid peptides assessed in both hippocampal and hypothalamic areas of the rat brain along the regular 24-h light-dark cycle period (see below). Each group of animals was exposed to different conditions as follows: (1) a *naive* control group was exposed to the regular 12:12-h light-dark cycle (2) a WFL-treated group (3) a WFL-treated group was exposed to a 4 h period of darkness (WFL + 4 h); and (4) a WFL-treated group was exposed to a 6 h period of darkness (WFL + 6 h). After each treatment, all animals were sacrificed at 13:00 h and brains and blood were recovered as described above. Dim red lighting was used at all times to allow visualization and manipulation of animals.

3.3. Experiment 3. Melatonin and luzindole administration

Melatonin: 25 WFL treated rats ($n = 5$/ group) received a single injection of MEL (10, 100, 150, 300 and 600 μg/kg s.c.) dissolved in a final volume of 100 μl of 0.1% ethanol.

*Luzindol*e: 12 WFL rats ($n = 4$/group) received a single injection of LZ (187.5, 375, 750 μg/kg i.p.). After 30 min the rats were injected with MEL (150 μg/kg s.c.). Control rats received a similar subcutaneous injection of the vehicle solution. In both groups, either the experimental and control rats were kept under regular vivarium light for 4 h (09:00–13:00 h) followed by animal sacrifice.

3.3.1. Tissue dissection and preparation of a whole peptide fraction from the rat brain

The hypothalamus and hippocampus from rat brains were dissected according to standard procedures previously reported (Glowinsky and Iversen, 1966; Engel and Sharpless, 1977). Both brain regions were boiled in 8 ml of 0.1 N HCl for 15 min, homogenized, and centrifuged at 50,000×*g* for 1 h/ 4 °C. Supernatants were loaded to Amberlite XAD-2 columns (8 × 0.7 cm) for solid phase peptide extraction. The flow rate was held constant at 0.5 ml min^{-1} and elution of a whole peptide fraction was carried out using a continuous gradient with pure methanol. Eluted samples were lyophilized and resuspended in 2 ml of distilled water and finally stored at −20 °C for further quantification of IR-Enkephalin by radioimmunoassay (RIA) technique.

3.3.2. Radioimmunoassay

Cross-reactivity for Met-Enkephalin, Leu-Enkephalin and Synenkephalin antisera used in the RIA, have been previously described (Asai et al., 1998; Saravia et al.,1998; Padrós et al., 1995). Quantitative determination of enkephalins and SYN were expressed as pmol of immunoreactive (IR)-peptide mg^{-1}.

3.3.3. Quantification of melatonin in serum

Quantitative determination of MEL in rat serum was performed by standard solid phase antibody capture ELISA assay (ICN Biomedicals Research Products. Cat. 193596), and optical density read at 405 nM in a FL6000 microwell plate fluorescence reader (Bio-Tek).

3.3.4. Protein determination

Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard reference.

3.3.5. Statistics

Levene homogeneity of variances test was performed in all cases and square root or natural logarithm transformations was done when variances were heterogeneous. Two-way analysis of variance (light treatment and hour) was used to analyze tissue content of opioid peptides along 24 h-circadian rhythm. One-way analysis of variance was used to analyze the effect of exogenous MEL and Kruskall–Wallis test in the case of hippocampus Synenkephalin analysis. Tukey or Mann–Whitney U tests were used for *post hoc* comparisons between control and experimental groups, and between hour-day, treatment and groups of animals.

4. Results

4.1. Experiment 1. Circadian rhythm

Tissue content of ME-IR in both hippocampal and hypothalamic structures of the rat brain exhibited contrasting differences in the peptide-circadian rhythm in control and experimental animals as depicted in Fig. 1a and b. As shown in Fig 1a, the ME-IR tissue content in the hippocampus of the control group remains constant during the light-phase. However, during the dark-phase ME-IR concentration began to increase until 01:00 h, when the maximal value was reached ($F_{(5,52)} = 9.11$, $p = 0.000$). At 05:00 h ME-IR levels were similar to those observed during the light-phase. When compared 01:00 h vs. different time-points along the 24 h circadian rhythm. *Post hoc* Tukey test showed significant differences (09:00 h, $p = 0.005$, 13:00 h, $p = 0.049$, 17:00 h, $p = 0.044$, 21:00 h, $p = 0.048$, 05:00 h, $p = 0.008$). In contrast, WFL animals displayed an opposite pattern of the ME-IR circadian

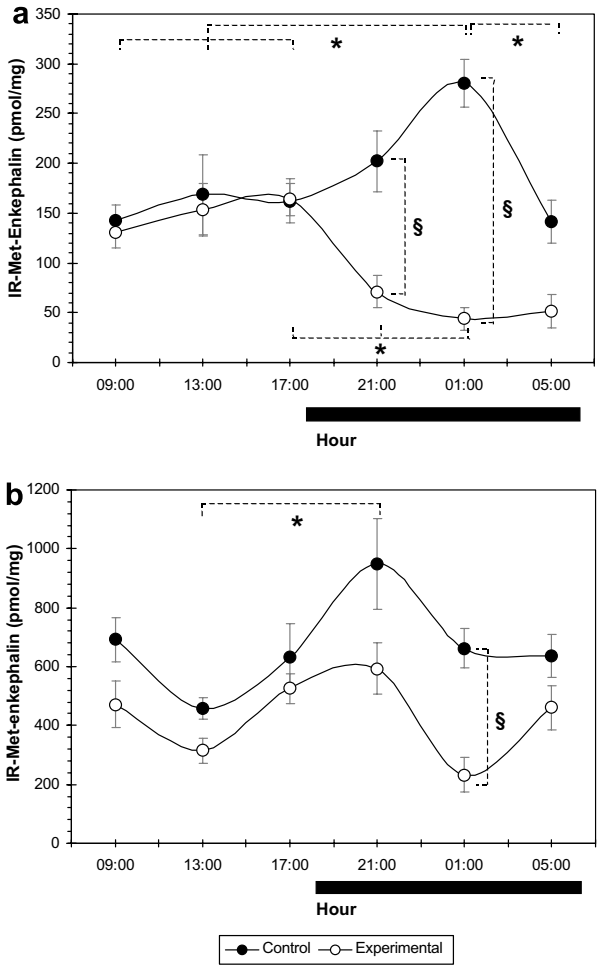

Fig. 1. Circadian variations of IR-Met-Enkephalin in the hippocampus (panel a) and hypothalamus (panel b). Control group (●), white fluorescent light (WFL) group (○). Black area represents the dark period during a 24-h cycle. Statistical analysis: ANOVA followed by Tukey test. $^{*}p < 0.05$ between hours, $^{\S}p < 0.05$ between treatments.

rhythm, in which light exposure during the dark-phase induced a significant decrease of Met-Enkephalin, reaching the lowest concentration at 21:00 and 01:00 h (*Post hoc* Tukey test $p = 0.016$ and $p = 0.000$, respectively). As shown in Fig. 1b, tissue content of ME-IR in the hypothalamus in control animals, displayed a gradual but significant increase of IR-peptide from 13:00 to 21:00 h, reaching its highest immunoreactive concentration at 21:00 h ($p = 0.003$) and decaying slightly to lower immunoreactive levels from 01:00 to 05:00 h. *Post hoc* Tukey test analysis showed a significant effect for the hour of the day ($F_{(5,52)} = 5.67$, $p = 0.000$) as well as between hour and treatment in both groups of animals ($F_{(1,52)} = 26.75$, $p = 0.000$). WFL treated animals showed a similar ME-IR concentration during the light-phase when compared with the control group. However, the IR-peptide content decreased during the dark-phase. The most significant reduction of tissue content of Met-Enkephalin occurs at 01:00 h ($p = 0.024$).

In Fig. 2a we showed the circadian fluctuation of the tissue content of LE-IR in the rat hippocampus. In the control group we found two main concentration peaks along 24 h circadian rhythm, the first one was detected at 13:00 h, and the second at 01:00 h. After the dark-phase this circadian peak decayed gradually, reaching its lowest concentration in tissue at 05:00 h. In contrast, WFL treated animals exhibited a very slight, but mostly linear decay in LE-IR occurring from 17:00 h to 05:00 h. ANOVA showed a significant effect between hour-day and treatment on LE-IR concentration ($F_{(5,52)} = 3.80$ $p = 0.005$; $F_{(1,52)} = 9.22$, $p = 0.004$, respectively) but no interaction was observed between hour and treatment in both groups ($F_{(5,52)} = 1.03$, $p = 0.407$). *Post hoc* Tukey test analysis reveals a significant difference between treatments at 01:00 h ($p = 0.05$).

In the hypothalamus (Fig. 2b), we found that both control and WFL treated rats, displayed a closely simi-

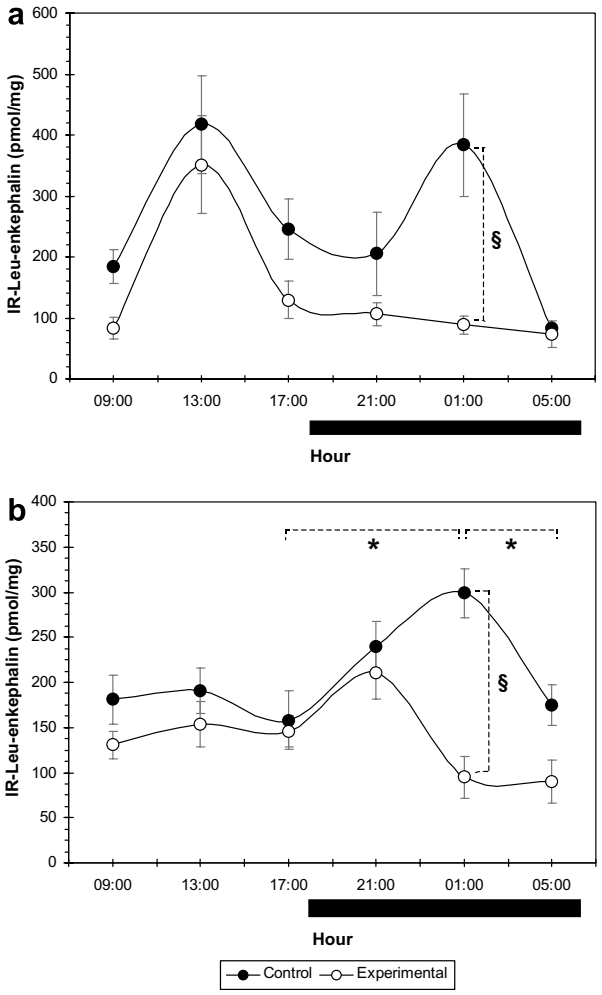

Fig. 2. Circadian variations of IR-Leu-Enkephalin in the hippocampus (panel a) and hypothalamus (panel b). Control group (●), white fluorescent light (WFL) group (○). Black area represents the dark period during a 24-h cycle. Statistical analysis: ANOVA followed by Tukey test. $^{*}p < 0.05$ between hours, $^{\S}p < 0.05$ between treatments.

lar LE-IR concentration during the light-phase and even until 21:00 h. After this point, control group reached its maximal tissue content at 01:00 h (Tukey test $p = 0.015$) and decayed to basal immunoreactive levels at 05:00 h ($p = 0.05$). ANOVA showed a significant effect between hour-day and treatment on LE-IR concentration ($F_{(5,52)} = 3.44$, $p = 0.009$; $F_{(1,52)} = 22.89$, $p = 0.000$, respectively) and a significant interaction between hour and treatment in both groups of animals ($F_{(5,52)} = 3.74$, $p = 0.006$) as well.

In contrast, WFL treated rats were unable to maintain this peptide increase after 21:00 h as the control group, and showed a significant decrease during the following hours of the dark phase, with a lowest value at 01:00 h. As shown in Fig. 2b, during each time-point assessed along 24 h circadian rhythm, the LE-IR content was invariably lower in the WFL group when compared to the control group. *Post hoc* test analysis reveals a significant difference in peptide content at 01:00 h ($p = 0.000$).

Circadian fluctuations on the tissue content of SYN in the hippocampus, in both control and WFL animals, showed a similar concentration during the light-phase (Fig. 3a). However, during the darkness the control group displayed a gradual but significant increase of IR-peptide from 17:00 to 01:00 h and a slightly decay to lower immunoreactive levels at 05:00 h. In contrast, WFL treated animals displayed a peptide tissue content decrease during the dark-phase when compared to the control group. In spite of this, no significant changes were found in the oscillatory fluctuations of this peptide along the 24-h circadian rhythm. On the other hand, Mann–Whitney U-test showed significant differences between groups ($p = 0.01$), specifically at 13:00 h and 01:00 h ($p = 0.009$) during the light and dark-phase periods, respectively.

In the hypothalamus the tissue content of SYN displayed different circadian fluctuations in both groups of animals as shown in Fig. 3b. Control group displayed two immunoreactive peaks with similar tissue concentration detected during the light and dark-period (13:00 h and 01:00 h, respectively). WFL treated group showed differences in the oscillatory profile when compared with the control group. As shown, the WFL animals displayed a single immunoreactive peptide peak at 13:00 h, attaining same peptide concentration in the tissue as the control group. However, a fast decay in peptide content was observed from 17:00 to 05:00 h, with the lowest concentration occurring at 09:00 h. ANOVA analysis between hour-day and treatment showed significant changes on the SYN concentration ($F_{(5,52)} = 8.07$, $p = 0.000$; $F_{(1,52)} = 24.93$, $p = 0.000$, respectively), in both, control and WFL treated groups. However, no significant interaction was detected between hour and treatment in both groups of animals ($F_{(5,52)} = 1.65$, $p = 0.162$).

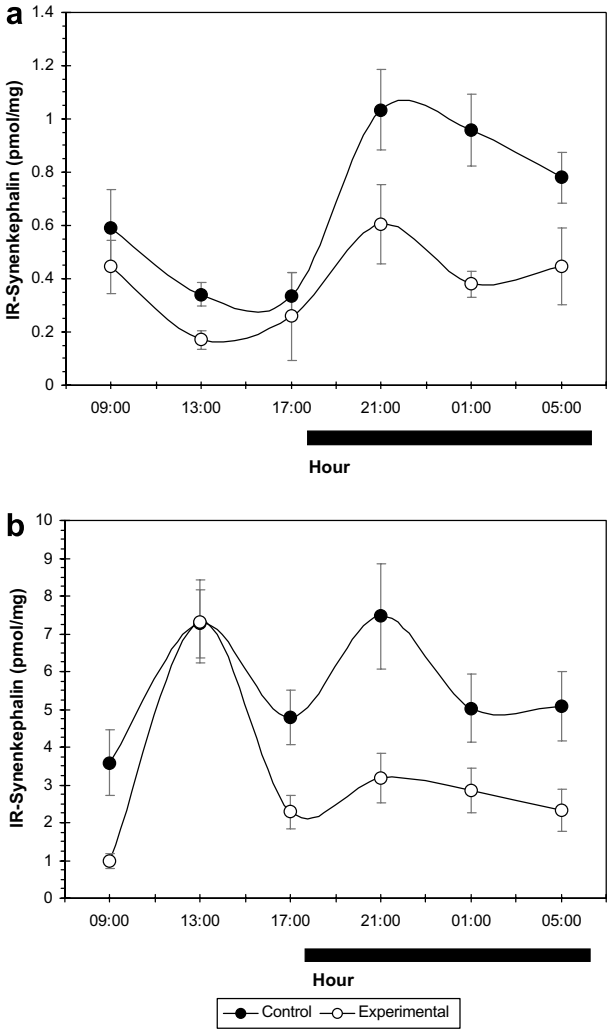

Fig. 3. Circadian variations of IR-Synenkephalin in the hippocampus (panel a) and hypothalamus (panel b). Control group (●), White Fluorescent Light (WFL) group (○). Black area represents the dark period during a 24-h cycle. Statistical analysis: a) Kruskall–Wallis followed by Mann–Whitney U test; b) ANOVA followed by Tukey test.

4.2. Experiment 2. Darkness effect over opioid tissue levels

Serum melatonin and tissue content of ME-IR and LE-IR in both hypothalamus and hippocampus after darkness exposition are shown in Table 1. ANOVA test showed significant differences between enkephalins concentration, treatment (time-exposure to darkness) and groups of animals as well ($F_{(3,16)} = 7.162$, $p = 0.003$). In the hippocampus the ME-IR tissue content showed a non significant decrease in WFL rats when compared to the control group. The darkness stimulus was able to induce a significant increase in the peptide tissue concentration after 6 h of exposition when compared with the other three groups ($p = 0.03$ vs. control, $p = 0.005$ vs. WFL and $p = 0.005$ vs. WFL + 4 h). In the hypothalamus the ME-IR concentration in WFL showed a

Table 1
Effect of darkness on IR-Me and IR-Le tissue content in the rat brain

	IR-Met-Enkephalin		IR-Leu-Enkephalin		MELATONIN
	Hippocampus	Hypothalamus	Hippocampus	Hypothalamus	
Control	187.2 ± 29	984.0 ± 121	101.0 ± 29	283.0 ± 29	68.5 ± 11
WFL	132.4 ± 21	384.0 ± 101*	62.4 ± 6	118.2 ± 26*	63.8 ± 9
WFL + 4 H	147.6 ± 14	827.0 ± 226&	316.4 ± 51*&	574.0 ± 102&	78.0 ± 12
WFL + 6 H	356.4 ± 66* & §	1173.0 ± 134& §	461.8 ± 60**&	941.0 ± 178** & §	113.4 ± 26

IR-Met and IR-Leu-Enkephalin tissue content in the hippocampus and hypothalamus of the rat brain, after 4 or 6 h of darkness in those animals subjected to white fluorescent light (WFL) during 15 days. Each value is the mean ± SEM of five rats and the concentration is expressed as IR-Enkephalin pmol/mg protein. Statistical analysis was carried out using one-way ANOVA followed by a Tukey Test. Melatonin values are expressed in pg/mL of sera.
* $p < 0.03$ vs. control.
** $p < 0.005$ vs. control.
& $p < 0.005$ vs. WFL.
§ $p < 0.005$ vs. WFL + 4H.

significant decrease when compared to control group ($p = 0.03$). The darkness effect produced a significant increase in the peptide tissue content after 4 and 6 h after darkness stimuli when compared with the WFL group ($p = 0.005$). WFL + 6 h group showed an increase over the WFL + 4 h group ($p = 0.005$). The LE-IR tissue content in both analyzed structures, showed a similar profile than those observed for ME-IR. In the hippocampus we found a clear tendency to reduce the peptide levels after functional pinealectomy treatment. In contrast, the darkness produced an overall tissue content increase in this structure. Statistical analysis reveals an increase around 500% after 4 h of stimulation ($p = 0.005$) and 700% after 6 h, ($p = 0.005$), when compared with the WFL group. There were not statistical differences between WFL + 4 h and WFL + 6 h groups. In the hypothalamus, the WFL group showed a tissue content significant decrease ($p = 0.03$), when compared to the control group. As we observed in the hippocampus, the darkness induced an increase in both WFL + 4 h ($p = 0.005$) and WFL + 6 h ($p = 0.005$) groups when compared to the WFL group. In this structure there was a significant difference between 4 and 6 h of darkness treatment ($p = 0.005$).

The increase of opioid peptide content in both brain areas analyzed, paralleled the increased serum levels of MEL in both WFL + 4 h and WFL + 6 h treated groups. As shown, WFL group showed a non-significant decrease of MEL when compared to the control group. However, both, WFL + 4 h and WFL + 6 h treated animals showed increased levels of MEL in 12.2% and 39.6%, respectively, and reached similar or even higher values when compared to the control group (ANOVA test $p = 0.05$).

4.3. Experiment 3. Melatonin and luzindole administration

The effect of subcutaneous injection of increasing doses of MEL (10,100, 150, 300, and 600 μg/kg) produced significant changes on the tissue content of ME-IR in the hypothalamus (ANOVA $F_{(6,25)} = 7.8$, $p = 0.000$) and hippocampus (ANOVA $F_{(6,25)} = 8.5$, $p = 0.000$) in both control and WFL treated rats, as shown in Table 2. In hypothalamus and hippocampus, ME-IR showed a tissue content decrease in WFL rats when compared to the control group ($p = 0.004$; $p = 0.05$, respectively). The lowest dose of exogenous MEL (10 μg/kg) given to functional pinealectomized rats produced a significant enhanced recovery on the tissue content of ME-IR in the hippocampus (ANOVA $p = 0.001$) and hypothalamus (ANOVA $p = 0.001$). Administration of higher doses of MEL (100, 150, 300 and 600 μg/kg) in the hypothalamus and hippocampus produced a significant opioid peptide increase when compared to the WFL rats ($p = 0.01$, $p = 0.04$, $p = 0.004$, $p = 0.004$, respectively).

Table 2
Effect of exogenous melatonin administration on IR-Met-Enkephalin tissue content in the rat brain

	IR-Met-Enkephalin	
	Hippocampus	Hypothalamus
Control	272.0 ± 28	927.5 ± 169
WFL	80.3 ± 6*	456.8 ± 56*
WFL + MEL 10 μg	117.0 ± 19	2614.0 ± 439**
WFL + MEL 100 μg	163.0 ± 24**	1420.0 ± 221**
WFL + MEL 150 μg	281.0 ± 52**	1325.0 ± 156** &
WFL + MEL 300 μg	189.3 ± 40**	1445.0 ± 276** &
WFL + MEL 600 μg	357.8 ± 79** & §	1644.0 ± 79** & §

IR-Met-Enkephalin tissue content after exogenous melatonin administration. Animals were injected with a single melatonin dose (10, 100, 150, 300, 600 μg/kg, s.c.). Control and WFL groups were injected with the equivalent volume of the used vehicle. After melatonin injection, the rats were kept in the same room with fluorescent light during 4 h. Each value is the mean ± SEM of five rats and the concentration is expressed as IR-Met-Enkephalin pmol/mg protein. Statistical analysis was carried out using one-way ANOVA followed by a Tukey Test.
* $p < 0.05$ vs. control.
** $p < 0.01$ vs. WFL.
& $p < 0.05$ vs. MEL 100.
§ $p < 0.05$ vs. MEL 300.

Table 3
Effect of luzindole on IR-Met-Enkephalin tissue content in the rat brain

	IR-Met-Enkephalin	
	Hippocampus	Hypothalamus
Control	228 ± 51	756 ± 59
WFL	104 ± 34*	235 ± 42*
WFL + MEL 150 μg	185 ± 36	802 ± 55**
WFL + LZ 187.5 μg + MEL 150 μg	45 ± 20*	241 ± 27*
WFL + LZ 375 μg + MEL 150 μg	107 ± 21	635 ± 81** §
WFL + LZ 750 μg + MEL 150 μg	144 ± 18	538 ± 37** §

IR-Met-Enkephalin tissue content after exogenous luzindole (LZ) administration. WFL animals were injected with a single luzindole dose (187.5, 375, 750 μg/kg, i.p.) and after 30 min they were injected with Melatonin (150 μg/kg s.c.). Control and WFL groups were injected with the equivalent volume of the used vehicle. After the injections, animals were kept in the room with white fluorescent light, and were sacrificed 4 h after MEL administration. Each value is the mean ± SEM of four rats and the concentration is expressed as IR-Met-Enkephalin pmol/mg protein. Statistical analysis was carried out using one-way ANOVA followed by a Tukey Test.

* $p < 0.05$ vs. control.

** $p < 0.01$ vs. WFL.

§ $p < 0.05$ vs. luzindole 187.5.

Serum levels of MEL were highly increased after exogenous hormone administration (ANOVA $p = 0.005$). Serum hormone concentration oscillates from 63.9 pg/ml in WFL rats to 12,345 pg/ml after 600 μg/kg of MEL injection. In Table 3, we showed the effect of the MEL receptor antagonist over the Met-Enkephalin content. As we shown in Tables 1 and 2, melatonin administration in WFL-treated rats induced a significant ME-IR tissue content increase in the hypothalamus and hippocampus when compared to the WFL rats. The lower dose of luzindole (187.5 μg/kg) was unable to inhibit significantly the opioid peptide tissue content increase induced by melatonin administration. Different results were obtained with higher doses (375, 750 μg/kg) of luzindole. In hypothalamus, we found a ME-IR tendency to reach similar concentration when compared to the WFL + 150 μg/kg MEL. However, in the hippocampus, there were not significant changes in the peptide concentration with any of the LZ doses used.

5. Discussion

The present work reveals that functional pinealectomy not only disrupts the Endogenous Opioid System (EOS) circadian rhythm, but also decreased the tissue content of Met-Enkephalin, Leu-Enkephalin and Synenkephalin. This effect was reverted by exogenous MEL administration and darkness exposition. Both stimulus were able to induce a significant increase of opioid peptides content in the hypothalamus and the hippocampus of the rat brain.

Previous studies have shown that EOS exhibit a circadian-rhythm, showing that the tissue concentration of opioid peptides (i.e., Met-Enkephalin. Leu-Enkephalin and Synenkephalin) (Kumar et al., 1982; Asai et al., 1988), peptide release (Asai et al., 1998) and expression of specific cell-surface receptors (Naber et al., 1981), reach maximum concentrations during the dark-phase period under a regular 24-h circadian rhythm. In extension to these works, our results show that the specific tissue content of ME-IR LE-IR and SYN-IR, in control animals, appear to be increased mainly during the dark phase (21:00–01:00 h) period along the 24-h circadian rhythm. Despite of the differences observed on the circadian fluctuations in controls animals, this EOS circadian rhythm was disrupted after exposing animals to functional pinealectomy. Moreover, the tissue content of ME-IR, LE-IR and SYN-IR in the WFL-treated animals, were significantly reduced during the dark-phase period, particularly from 21:00 h to 01:00 h.

Functional pinealectomy has been shown to disrupt the endogenous synthesis of MEL in pinealocytes, which leads to a lack of endogenous MEL; enhancing thus, the altered oscillatory pattern of the EOS-circadian rhythm, as shown for the enkephalins in the present study. In mammals, MEL synthesis occurs at night, where the highest levels of the endogenous indolamine have been reported to occur between 00:30–01:00 h during a regular 24-h circadian rhythm (Reiter, 1993). To support the above observations, we found that in the control animals the highest concentration of melatonin was of 4.1 nM at 01:00 h and the minimal of 0.92 nM at 13:00 h; whereas in the WFL-treated animals, serum levels of MEL were reduced by five times (0.3 nM) at 01:00 h and 14 times (0.2 nM) at 13:00 h, respectively.

One crucial advantage of functional pinealectomy over other invasive intervening procedures is that the former one may be reverted without disrupting the anatomical structure of the brain and maintaining its full integrity.

Functional pinealectomy induced a significant reduction of both ME-IR and LE-IR tissue content in the hypothalamus and hippocampus of the rat brain. Reversion of this effect was observed after exposing WFL-treated animals to a 4 or 6 h period to darkness (Table 1). Furthermore, this treatment induced an increased content of both enkephalins in the brain regions tested, which highly exceeded the peptide immunoreactive values detected in controls. These changes in the tissue content of enkephalins after exposure of WFL-treated animals to darkness, may be due to the enhanced synthesis and secretion of MEL from pinealocytes (Simonneaux and Ribelayga, 2003). Thus, the increased concentration of MEL in the brain may induce a dose-dependent response in restoring the immunoreactive levels of brain enkephalins above the values observed in control animals. This assumption is supported by the

results obtained after subcutaneous administration of increasing doses of exogenous MEL (10, 100, 150, 300 and 600 μg/kg). As shown in Table 2, the tissue content of ME-IR in both hippocampus and hypothalamus from injected animals were completely restored above the detected immunoreactive values of controls and WFL animals, showing as well, a dose-dependent effect of MEL on the increased levels of enkephalin immunoreactivity in the brain regions analyzed. Moreover, these results suggest not only that MEL and EOS are reciprocally linked, but also that opioid peptide concentration in the brain is highly sensitive to the amount of melatonin secreted along the 24-h circadian rhythm. This suggestion is in agreement with the data obtained in Table 2. The lowest MEL dose (10 μg/kg) produced a Met-Enkephalin tissue content increased over 700% when compared to the WFL-treated rats.

In the context of the aforementioned results, the MEL-inducing effects observed on the tissue concentration of enkephalins in the rat brain, may be linked to specific activation of MEL receptors, where MT_1 receptor is related with the modulation of the neural firing, whereas, the MT_2 has been involved in the circadian rhythm control (Dubocovich and Markowska, 2005). Moreover, several lines of evidence have demonstrate that MEL induces the expression of the Proopiomelanocortin gene in the *arcuate nucleus* (Yu et al., 2000) and prefrontal cortex (Kotler et al., 1998) of the rat brain, as well as in the rat immune system (Wajs et al., 1995). However, our results showed that different doses of luzindole were partially unable to inhibit the melatonin action over opioid peptide tissue content increase. This last observation may suggest that MEL is acting through direct interaction with transduction signal molecules after having crossed the plasmatic membrane, as has been suggested (Benitez-King, 2006).

The relation between opioid peptides and melatonin has several physiological implications particularly in the analgesia and in the immune system. Previous results have shown that MEL injected into rats induced a time-dependent effect on the analgesia responses, showing that maximum responses occur at night under a regular 24-h circadian rhythm. However, these responses were abolished after animals were exposed to a five-day period of continuous lighting (Golombek et al., 1991; Golombeck et al., 1993). In agreement with the above observations, recent data from our group have shown that MEL promotes the presynaptic release of enkephalins from rat brain-synaptosomes (manuscript in preparation). These unpublished results may provide further evidence of the functional role of MEL in modulating the neuronal release of endogenous opioid peptides, supporting thereby, the physiological interaction between MEL and EOS in the brain.

In addition, recent immunohistochemical studies have shown the expression of high amounts of Met- and Leu-Enkephalin immunoreactivity in the human pineal gland, suggesting a possible paracrine opioidergic control of MEL at this organ (Phansuwan-Pujito et al., 2005). Moreover, opioid peptide receptors such as μ and δ are expressed in the rat brain pinealocytes (Chuchuen et al., 2004).

These receptors have been shown to participate selectively in analgesia responses mediated by MEL, demonstrating that the δ-opioid receptor subtype, rather than the μ-opioid receptor, appears to mediate melatonin-dependent antinociceptive effects in the rat brain (Li et al., 2005). Nonetheless, several authors proposed that MEL induces its analgesic activity by increasing the cell-release of β-Endorphin, in addition to other endogenous opioid peptides (Shavali et al., 2005).

In conclusion, major circadian changes observed after functional pinealectomy on the tissue content of enkephalin peptides in both hippocampal and hypothalamic regions in the rat brain, occur mainly during the dark-phase period (at 01:00 h). MEL appears to be implicated in the physiological control of the EOS-circadian rhythm and regulating the synthesis of the opioid peptides in the rat brain.

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