

# Light-dark influence on enkephalinase activity in hypothalamus and pituitary

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## Abstract

**OBJECTIVES:** Enkephalins functions are partly modulated by enkephalinases especially membrane-bound alanyl aminopeptidase (EC-3.4.11.2) considered as the major enkephalin-degrading enzyme in brain. The analysis of its activity in standard and non-standard light/dark conditions may help the understanding of the regulatory mechanisms of enkephalins.

**METHODS:** Enkephalinase activity was determined fluorometrically, using an arylamide derivative as substrate, in hypothalamus and pituitary of adult male rats, in a standard 12:12 h light/dark cycle; samples were collected at 10:00 h, 13:00 h and 16:00 h of the light period (light on from 7:00 to 19:00 h) and at 22:00 h, 01:00 h and 04:00 h of the dark one (light off from 19:00 h to 07:00 h). For comparison, the enzymatic activity was also measured in the same locations at the same time-points but under constant light conditions.

**RESULTS:** In standard light/dark conditions, the results demonstrated an opposite daily rhythm in hypothalamus and pituitary. While the highest levels of AlaAP or enkephalinase activities were measured in hypothalamus during the dark period, they were the highest in the pituitary during the light one. In contrast, the lowest levels of activity were observed in the light period in the hypothalamus whereas they were in the dark one in the pituitary. A similar pattern was observed under constant light. The differences were however higher in hypothalamus and lower, but still significant, in pituitary.

**CONCLUSION:** These results may reflect the behaviour of the endogenous substrates of enkephalinase and consequently be involved in their functions. This observation may affect the chronotherapeutic strategies.

## INTRODUCTION

Enkephalins are involved in multiple neuroendocrine and behavioural functions. They act as neuromodulators regulating hypothalamic neurohormones and pituitary hormones (Bodnar 2018). Importantly, these neuropeptides and hormones as well as their functions are under a circadian pattern influenced by environmental light/dark conditions (Urbanski 2011). Enkephalins are also under a circadian rhythm (Kumar *et al.* 1982) which, as a consequence, may influence the factors and functions regulated by them. Disruptions of these rhythms may have important physiologic consequences (Valdés-Tovar *et al.* 2015). The functional role of enkephalins is regulated in part by enkephalinases which may also be under a circadian pattern of activity. As it is possible to affect this enzymatic activity through activators or inhibitors (Bonnard *et al.* 2016), it is important to search for a possible daily rhythm for this activity in standard and non-standard conditions. This aspect has not yet been analysed. The purpose of our study was to determine enkephalinase (EC 3.4.11.2, AlaAP, APM) activity through a standard 12h/12h light/dark cycle (07 h to 19 h light/ 19 h to 07 h dark) and also under constant light conditions. This enzyme is considered the major enkephalin-degrading enzyme in the brain its distribution being similar to the one of the enkephalin receptors (Hersh 1985). In addition to enkephalins, this enzyme also hydrolyzes endorphins (Danziger 2008), angiotensin III (Zini *et al.* 1996) and the artificial substrate alanyl- $\beta$ -naphthylamide (Hersh *et al.* 1987).

## MATERIALS AND METHODS

Seventy two adult male Wistar rats weighing 200–250 g (at reception) were used. The animals were separated in twelve groups (n=6 each) and housed in cages under controlled temperature (25 °C) with free access to water and food during at least 24 days until sacrifice. Six groups were under a regular 12:12 h light:dark cycle, with lights on from 07 to 19 h and off from 19 to 07 h. The other six groups were under constant light during such period of time. In each of the experimental conditions (light/dark or constant light) six separated experiments were performed using one different group for each of the six time-points studied: 10:00, 13:00, and 16:00 h of the light period and 22:00, 01:00 and 04:00 h of the dark period. The same time-points were analysed in the groups under constant light. The experiments were performed in summer. All the experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC and approved by the bioethics committee of the University of Jaén. The animals of each group were individually anaesthetized with equithensin (2 ml/kg body weight) (42.5 g/l chloralhydrate dissolved in 19.76 ml ethanol, 9.72 g/l Nembutal®, 0.3961/l propylenglycol and 21.3 g/l magnesium

sulphate in distilled water). The brains, perfused with saline through the left cardiac ventricle, were quickly removed (less than 60 s) and cooled in dry ice. For the dark period study, the animals were perfused under a dim red light. At the time of the analysis, brains were individually defrosted and sliced by hand with a blade. The hypothalamus was identified in the slices and dissected according to the stereotaxic atlas of Paxinos & Watson (1998). The selected area (pooled left and right) was between 7.7 mm and 3.7 mm anterior to the interaural line with approximately 2 mm of thickness from midline and 3 mm height from the base. The pituitary gland (pooled anterior and posterior) was also dissected and cooled in dry ice. The membrane-bound fraction and the fluorometric measurement of enkephalinase activity using L-alanyl-beta-naphthylamide as substrate was previously described in details (Ramírez *et al.* 2011). All enzymatic measures were performed on the same day. Proteins were quantified in triplicate by the method of Bradford (1976) using BSA as a standard. Specific enkephalinase activity was expressed as nanomoles of L-alanyl-beta-naphthylamide hydrolyzed per minute per milligram of protein. The data were analysed with an ANOVA table as previously described in details (Domínguez-Vías *et al.* 2017). Significant results were declared at the 5% level. The analysis was performed with Stata 14.0 software.

## RESULTS

Under standard light/dark (12:12 h) conditions, enkephalinase activity showed in hypothalamus a peak at 13:00 h of the light period, differing significantly from 10:00 h ( $p < 0.001$ ) and 16:00 h ( $p < 0.01$ ). In the dark period, enkephalinase activity increased progressively until a maximum at 04:00 h. At 22:00 h, the activity differed significantly from 10:00 h ( $p < 0.001$ ) and 16:00 h ( $p < 0.001$ ) without difference with 13:00 h. At 01:00 h of the dark period, the activity differed significantly from 10:00 h ( $p < 0.001$ ), 13:00 h ( $p < 0.05$ ) and 16:00 h ( $p < 0.01$ ). At 04:00 h enkephalinase activity differed significantly from 10:00 h ( $p < 0.001$ ), 13:00 h ( $p < 0.01$ ), 16:00 h ( $p < 0.01$ ) and 22:00 h ( $p < 0.05$ ). In the pituitary, enkephalinase activity fell to its lowest levels in the three time-points of the dark period, all of them differing significantly ( $p < 0.001$ ) from all the time-points tested in the light one. Enkephalinase activity was always significantly higher in hypothalamus during both light and dark period than in pituitary with a high  $p < 0.001$  level except at 16:00 h where it was slightly lower at  $p < 0.01$  (Figure 1).

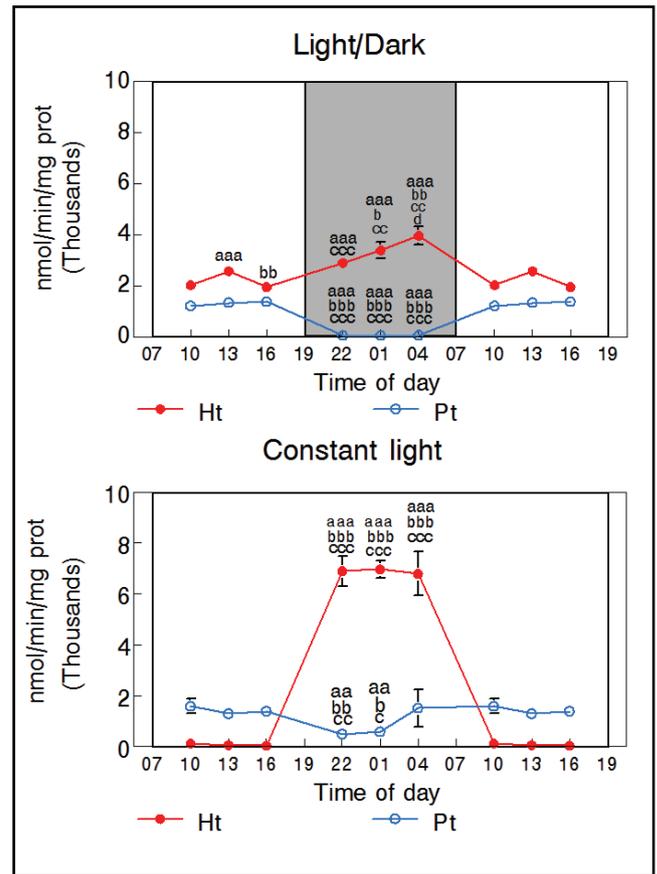
Under constant light conditions, the activity in hypothalamus was low at 10:00 h, 13:00 h and 16:00 h (without differences between these time-points). At 22:00 h, 01:00 h and 04:00 h the activity increased significantly ( $p < 0.001$ ) in comparison with the three previous time-points. In pituitary, there were no differences between 10:00 h, 13:00 h and 16:00 h. At 22:00 h, the enkephalinase activity was significantly lower ( $p < 0.01$ ) than

the three previous time-points. At 01:00 h, the activity was lower than at 10:00 h ( $p<0.01$ ), 13:00 h ( $p<0.05$ ) and 16:00 h ( $p<0.05$ ). At 04:00 h, no differences were observed in comparison with any time-point. In contrast to the light/dark conditions, at 10:00 h, 13:00 h and 16:00 h the activity was higher ( $p<0.001$ ) in pituitary than in hypothalamus. However, the contrary occurred at 22:00 h, 01:00 h and 04:00 h (Figure 1).

## DISCUSSION

There is a daily regulation of neurohormone and hormone profiles in which the hypothalamus-pituitary axis (Urbanski 2011) and the pineal gland (Johnston & Skene 2015) play a central role. Multiple pathologies involve circadian disruptions of the physiological rhythms and chronotherapeutic strategies are being developed for their management (Smolensky *et al.* 2016). Daily fluctuating levels of Met-Enkephalin were reported in hypothalamus showing low levels in the morning and reaching a peak at midnight. Pinelectomy reduced or shift such increment observed at midnight (Kumar *et al.* 1982). Melatonin regulates the circadian rhythm of opioids and also modulates their synthesis in brain (Miguel Asai *et al.* 2007). The absence of melatonin, induced by constant light, led to a significant decrease of enkephalin in hypothalamus. Our present results showing an increase of enkephalinase activity in hypothalamus (high level of enzyme activity/low level of substrate) agree with these results. Pyroglutamyl peptidase I, an aminopeptidase involved in the control of TRH, which in turn is modulated by hypothalamic enkephalins (Tapia-Arancibia & Astier 1983), is directly influenced by specific light-dark schedules (Sanchez *et al.* 1996). The present data also clearly indicate a direct effect of light and dark conditions on enkephalinase activity and, consequently, on its endogenous substrates and the functions they control.

With regard to the role of enkephalins in analgesia, our knowledge about the chronobiological aspects of pain is currently still partial (Junker & Wirz 2010). Disruptions of the standard light/dark cycle such as constant light conditions demonstrated that the responsiveness to pain and the antinociceptive effects of morphine increased in comparison with the standard light/dark condition (Oliverio *et al.* 1982). The use of opiates is an essential tool in the treatment of pain. In order to prolong their effect, some agents are being developed to prevent their enzymatic inactivation as it is the case for specific enkephalinase inhibitors (Bonnard *et al.* 2016). The immune system is also under a circadian pattern (Labrecque & Cermakian 2015) and daily rhythm alterations have important effects on the immune response through hormonal changes (Roberts 2000). Disruptions of these rhythms clearly alter the immune response (Polidarová *et al.* 2017) as endogenous opioids modulate the immune system processes (Finn *et al.* 2004).



**Fig. 1.** Diurnal variation (upper figure) of enkephalinase activity in hypothalamus (Ht) and pituitary (Pt) of adult male rats under a 12:12 h light:dark cycle (light on from 07 h to 19 h) during at least 24 days. The rats were sacrificed at 10:00 h (n=6), 13:00 h (n=6) and 16:00 h (n=6) of the light period and at 22:00 h (n=6), 01:00 h (n=6) and 04:00 h (n=6) of the dark one. Similar analysis with the same time-points and tissue collection were performed in rats under constant light conditions (lower figure) during also at least 24 days. Each point represents the mean  $\pm$  SEM levels of enkephalinase activity expressed as nmol of alanyl-beta-naphthylamide hydrolyzed per min per mg of proteins. (a) indicates a significant difference versus 10 h (b) indicates a significant difference versus 13 h (c) indicates a significant difference versus 16 h. (d) indicates a significant difference versus 22 h. Single letter ( $p<0.05$ ); double letter ( $p<0.01$ ); triple letter ( $p<0.001$ ).

The rhythmic pattern for enkephalinase activity observed in the present work could explain in part the results of the studies reported above as an example and other processes in which enkephalins participate. Moreover, it is necessary to consider that this enzyme also metabolizes Ang III which is the main hypertensive peptide of the renin-angiotensin system in brain (Zini *et al.* 1996). Therefore, the use of inhibitors of this AlaAP/Enkephalinase in hypertensive subjects should consider a possible daily rhythm for this activity. In this sense, it is necessary to recall the existence of circadian rhythms for blood pressure (Hermida *et al.* 2013) and for various components of the renin-angiotensin system (Lemmer *et al.* 2000). Such circadian rhythms

should therefore also be considered in antihypertensive therapeutic strategies (Hermida *et al.* 2013; Domínguez-Vías *et al.* 2017).

In conclusion, enkephalinase activity showed a significant opposite daily rhythm in hypothalamus and pituitary in standard light/dark conditions which change under non-standard conditions such as constant light. This may reflect the behaviour of its endogenous substrates and be involved in the circadian rhythm of hormones, neurohormones and their functions. This information improves our knowledge on enkephalin regulation and may be important for our present chronobiological strategies for several therapies.

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