DIURNAL VARIATION
OF THE MELANIN-CONCENTRATING HORMONE
LEVEL IN THE HYPOTHALAMUS

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Melanin-concentrating hormone (MCH), the neuropeptide produced mainly in the hypothalamus, plays an operative role in regulating food intake and the sleep/wake cycle. Considering that these physiological functions pursue diurnal variations, we checked whether the total hypothalamic MCH level depends on the time of the day. The aggregated MCH peptide content of the whole MCH neuron population was significantly higher at the end of the sleeping period (lights on), than at the end of the active period (lights off). This result, together with earlier observations, indicates that in contrast to the MCH gene expression, the level of MCH peptide is object of circadian variation in the hypothalamus.

Keywords: Neuropeptide – circadian rhythm – energy homeostasis – sleep

INTRODUCTION

The hypothalamus holds the light-driven, genetically defined biological clock of mammals, as well as key structures necessary for the integration of environmental factors, such as food availability, social and meteorological conditions [8, 19].

It is particularly important to handle energy homeostasis during different levels of food availability. A complex hypothalamic neural network, including melanin-concentrating hormone (MCH) and orexin producing neurons, two discrete but spatially overlapping neuron populations contribute to the regulation of energy homeostasis on a large scale. Interestingly, the very same neurons are partakers of the circuit which influence the sleep–wake cycle.

In the mammalian brain MCH producing neurons occur within a rostro-caudally restricted region of the hypothalamus, first of all in the lateral hypothalamic area (LHA) and in the zona incerta (ZI) [3, 17]. Mapping of the distribution of MCH neuron perikarya and their projections in the rat [1, 2, 5] and mouse brain [3, 4] revealed the presence of MCH neuron somata in the lateral hypothalamic area, including the perifornical region, the rostromedial zona incerta, the posterior hypothalamic nucleus, and the capsule of the ventromedial nucleus, as often as not breaching the cyto-

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architectonic borders. Notwithstanding this restricted localization of the perikarya, MCH conveying projections reach a wide variety of target areas within the CNS and on the periphery. Regions affected include, among others, those involved in regulating feeding behaviour (e.g. lateral septum, nucleus accumbens) and those promoting arousal state (e.g. tuberomammillary nucleus, basal forebrain, locus coeruleus, ventrolateral periaqueductal gray, dorsal raphe), making this neuropeptide a potential candidate to constitute functional link between physiological processes [16, 22].

Two MCH neuron subpopulations are present in the hypothalamus: neurons containing MCH as the only neuropeptide – classified as type A MCH neurons – and those expressing another neuropeptide, the cocaine- and amphetamine regulated transcript (CART) and the neurokinin 3 receptor as well, denoted as type B MCH neurons. The two subpopulations are partially segregated within the hypothalamus: laterally and caudally the majority of the MCH neurons belong to type A, while type B MCH neurons are predominating medially and in the rostral ZI. Due the imperfectness of their spatial segregation, immunohistochemical identification is needed to tell the perikarya of the two subpopulations apart. The projections of type A and type B MCH neurons are principally directed towards the brainstem and the telencephalon, respectively [4, 5]. The functional importance of the coexpression of MCH and CART is unknown for the time being.

MCH has for long been known as a potent orexigenic neuropeptide, influencing both feeding behaviour and energy expenditure [13, 14, 16]. Fasting is known to increase the expression of MCH mRNA [18]. MCH is also a central player in the promotion and stabilization of sleep [7, 10–12, 21]. It has been suggested that MCH is affecting the pathophysiology of depression as well [21].

Considering the circadian rhythm of these physiological processes, it is plausible to suppose diurnal variations in the functioning of the MCHergic system. Nevertheless, available data are scanty.

Harthoorn et al. [9] analysed individual MCH neurons in the rat hypothalamus. They detected constant levels of MCH mRNA and peptide content at ZT2 and ZT14. (ZT stands for Zeitgeber time – "time giver" in German language. ZT0 traditionally corresponds to turning the lights on.) Similarly, statistically significant correlations were not found between the periodic expression of MCH and orexin genes with food intake in mouse hypothalamus [20].

In a recent comprehensive study, Dias Abdo Agamme et al. [6] measured the level of the MCH peptide by ELISA in the cerebrospinal fluid, along with the expression of the MCH gene (Pmch) in the hypothalamus, and the expression of the MCH receptor gene in the frontal cortex and the hippocampus, during active and sleep phases. Under undisturbed conditions all parameters were independent from the phases, excepting the MCH receptor expression, which was significantly higher when sleep predominated. Disturbances of the sleep–wake cycle, on the other hand, induced day/night differences in CSF levels of MCH and in Pmch gene expression.

In the present study, we have undertaken to test directly the MCH levels in the mouse hypothalamus at the end of the active and sleep phases by immunohistochemical assessment of the total MCH peptide content.

MATERIALS AND METHODS

Animals and tissue preparation

Young adult (30–34 g) Crl:NMRI BR male mice (Toxi-Coop Zrt, Hungary) were used during the experiments.

Mice were kept under standard conditions, with water and rodent chow *ad libitum*. A 12/12 h dark/light cycle was applied (lights on at 8:00 AM and off at 8:00 PM). Animals were divided into two groups: ZT0 and ZT12 group member mice were exterminated immediately after the end of the dark period, and immediately after the end of the light period, respectively.

Transcardial perfusion was done under anaesthesia with glutaraldehyde supplemented Zamboni's fixative (4% paraformaldehyde, 0.4% glutaraldehyde, 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4). After removal from the skull, brains were further incubated overnight at +5 °C in the same fixative without glutaraldehyde. A 50 μm coronal sections were cut with a vibratome.

The study was conducted in accordance with the 2010/63/EU Directive, with written permission of the institutional Animal Health and Welfare Committee.

MATERIALS

Primary antibody

Anti-MCH: polyclonal, raised in rabbit (Sigma-Aldrich, M8440). Immunogen: keyhole limpet hemocyanin (KLH)-coupled synthetic peptide corresponding to human MCH.

Techniques

Detection of MCH immunoreactivity

Sections containing the hypothalamus were processed for immunohistochemistry free-floating. All incubations and washings were carried out at room temperature (20–23 °C).

Blocking of the non-specific binding sites with 20% normal donkey serum (Life Technologies) was followed by overnight incubation with anti-MCH (Sigma-Aldrich, dilution: ×10,000). Biotinylated anti-rabbit IgG secondary antibody (Amersham, dilution: ×100) and avidin-biotin-horseradish peroxidase (VECTASTAIN Elite ABC HRP Kit, Vector laboratories, Cat. No: PK-6100) were applied according to the manufacturer's instructions. The 3,3'diaminobenzidine (DAB) was used as chromogen. To assure the comparability of the results, the immunodetection procedure for all mice (ZT0 and ZT12 collectively) has been performed in parallel, as a single series. Complete rostro-caudal series of MCH neuron containing sections were considered

for further densitometric and statistical evaluation. Incomplete series (with failing or damaged sections) have been disregarded. At the end, three series of sections from each ZT group have been evaluated.

Densitometric evaluation

Immunolabelled sections were monitored and photographed by a Leica DM750 microscope equipped with a Leica DMC2900 camera. On each section left and right sides of the hypothalamus were separately photographed with standardized illumination by using ×10 magnification. Photos (2048×1536 pixels) were converted into 8 bit grayscale for the purpose of the densitometric evaluation, done with the Scion Image Programme, version v4.02β for Windows. Thresholding to the optical density level of the background staining was performed. A constant sized reference frame has been positioned onto each photo to include all immunopositive neurons. The resulting data (positivity in arbitrary units) reflect the immunopositive area within the reference frame (Fig. 1). The number of data varied between 34 and 42 per brain. These data were used as counts in the statistical analysis.

Statistical analysis

Statistical analysis was done by using the Stata13 software (StataCorp LP, USA). Unpaired *t*-tests have been performed at 95% confidence level. To check incidental consequences of slightly oblique section planes, density counts of left and right sides were compared. No significant differences have been found. Aggregate density counts at ZT0 and ZT12 (122 and 121) counts, respectively, as well as the means of density counts of the individual animals within and between the groups, have also been evaluated by *t*-test.

RESULTS

Density count data, proportional with the level of immunopositivity have been obtained by densitometric evaluation of the immunolabelled brain sections, as illustrated in Figure 1. Density count data appear on an arbitrary scale. Comparability of the data has been guaranteed by careful standardization of all experimental conditions. Total hypothalamic MCH levels of individual mice have been assessed by the addition of density count data in all coronal hypothalamic sections depicting MCH neurons. Statistical comparison of aggregate density counts of all sections obtained from ZT0 and ZT12 mice has been performed. Box and whiskers plots (Fig. 2) indicate that the distribution of density readings is highly skewed. Even if the data do not follow normal distribution, due to the large number of data (121 and 122, respectively), the *t*-test (with equal variances) could be used for statistical comparison.
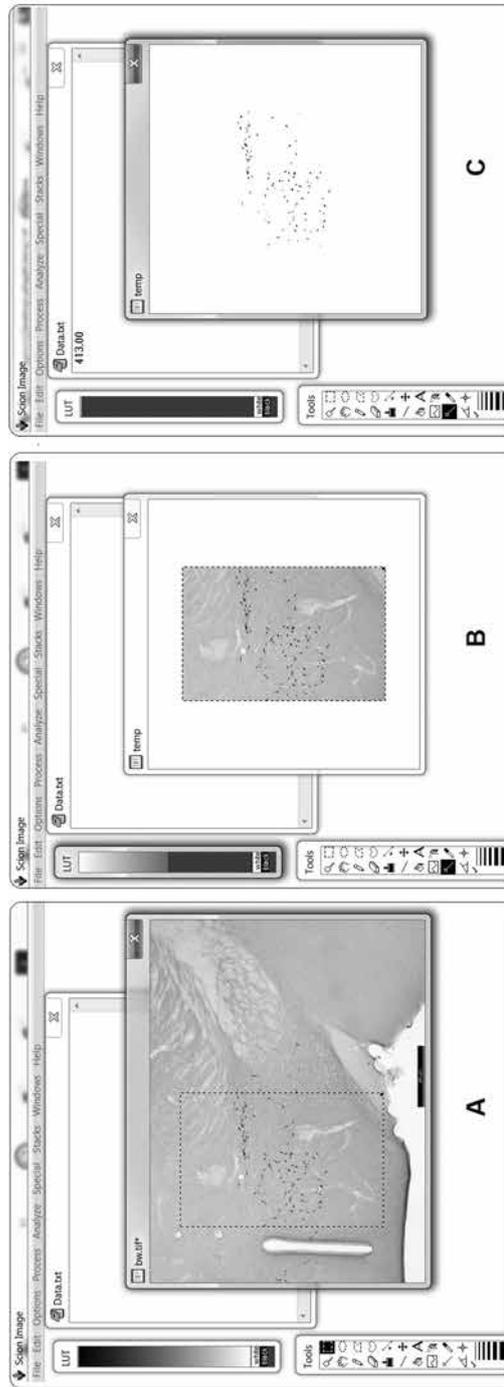
Fig. 1. Densitometric assessment of MCH immunoreactivity level. Immunohistochemistry was applied to detect MCH immunoreactivity in coronal brain sections (for experimental details, cf. "Materials and methods"). The course of the densitometric process is outlined here on a randomly chosen immunolabelled section. (A) Input to the Scion Image programme: immunolabelled section, converted to grayscale. A reference frame of predefined area is positioned over the immunopositive cells. The same frame has been used throughout the complete rostro-caudal series. (B) Marking of immunopositive areas for densitometry by subtracting the background ("thresholding": immunopositive structures are originally highlighted with red on the screen). (C) Calculation and display of the density count value in Data.txt window
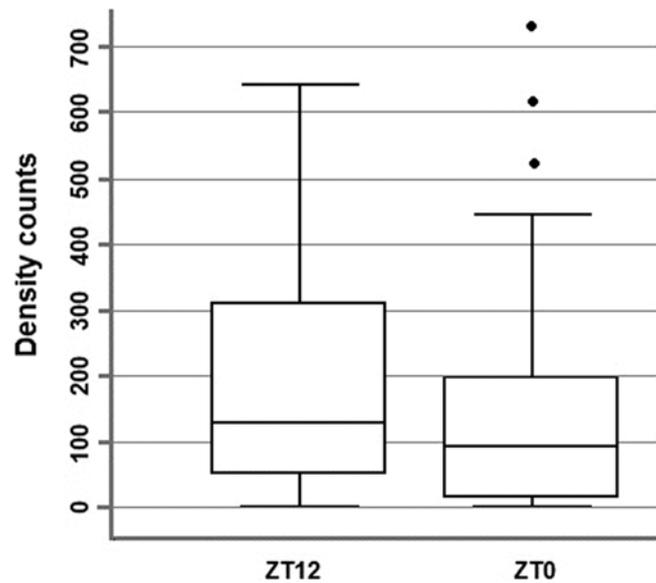
Fig. 2. Box and whisker plots of density count data demonstrate significantly lower MCH level in the hypothalamus at the end of the active period (ZT0) than at the end of the sleeping period (ZT12)

The means, CI (95%) and medians for ZT0 and ZT12 were 122.57 (98.70–146.4), 87 and 182.04 (153.6–210.5) and 128, respectively. The difference between the data at the two diurnal time points, ZT0 and ZT12 was significant ($p = 0.0017$, CI (95%): 22.54–96.40). Non-parametric tests (Kolmogorov–Smirnov and Shapiro–Wilk) also showed significant difference between ZT0 and ZT12 (data not shown).

Although the observed difference is highly significant, extension of the experiments to a higher number of animals is planned.

DISCUSSION

MCH neurons are known to be involved in circuits influencing feeding behaviour and sleep-wake rhythm. Therefore, it seemed to be highly conceivable that their peptide content followed circadian pattern.

Former studies addressed this point by diverse approaches. Harthoorn et al. [9] selected 25 MCH neurons in the rat hypothalamus and found that their MCH content did not depend on the diurnal time point. Likewise, the expression profile of the Pmch gene did not exhibit particularly strong circadian expression profile in mice [20], similarly to several other genes regulating food intake. Dias Abdo Agamme et al. [6] also observed absence of day/night differences in Pmch expression in the rat hypothalamus under undisturbed conditions.

It is worth noting, however, that the time of the day has been found to influence the response of the MCHergic system to abnormal conditions, e.g. fasting [19] or sleep disruption [6].

The aim of the work presented here was to compare the amount of MCH peptide present in the whole MCH neuron population of mice at the end of the active (dark) and the sleeping (light) periods. MCH peptide levels were assessed by densitometric evaluation of immunohistochemically labelled sections. MCH content of the whole hypothalamic MCH neuron population was calculated by adding up the MCH content of all coronal sections containing MCH neuron perikarya. Type A and type B MCH neuron subpopulations were not distinguished. The MCH level calculated this way has proven to be significantly lower at the end of the active period (ZT0) as compared to the end of the sleeping period (ZT12). This result implies that the replenishment and/or the depletion of the MCH pool depends on the time of the day. Depletion of the hypothalamic MCH pool is supposed to depend mostly on the activity dependent transport of the neuropeptide to its multiple target sites. For the time being, the rate and regulation of this transport process is unidentified. To address this issue, the possible diurnal variation of the MCH level in the various target areas should be studied as well. As far as the replenishment of the pool is concerned, earlier results indicated that the level of Pmch gene expression, as characterized by the MCH mRNA level, does not depend on the time of the day [6, 20]. It is to be considered, that beyond transcription, formation of mature MCH involves translation of the mRNA to pre-proMCH and the posttranslational proteolytic cleavage of the emerging prohormone [15, 23] as well. The possible circadian rhythm of these processes was not addressed yet.

As a conclusion, the observed diurnal variation of the MCH peptide level in the hypothalamus is verisimilarly of physiological importance. Further investigations are needed to ascertain the underlying mechanisms.

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