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Subchronical treatment with Fluoxetine modifies the activity of the MCHergic and hypocretinergic systems. Evidences from peptide CSF concentration and gene expression

Bruno F. Calegare [a], Alicia Costa [b], Leandro Fernandes [a], Ana L. Dias [a], Pablo Torterolo [b,*], Vânia D' Almeida [a]

[a] Department of Psychobiology, Universidade Federal de São Paulo, São Paulo, Brazil
[b] Department of Physiology, School of Medicine, Universidad de la República, Montevideo, Uruguay

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ABSTRACT

In the postero-lateral hypothalamus are located two neuronal systems that utilize the neuropeptides melanin-concentrating hormone (MCH) and hypocretins (also called orexins) as neuromodulators. These systems have reciprocal connections between them, and project throughout the central nervous system. MCH has been involved in the generation of sleep, mainly REM sleep, while hypocretins have a critical role in the generation of wakefulness.

MCHergic activity is also involved in the pathophysiology of major depressive disorder (MD). In this regards, intracerebral administration of MCH promotes pro-depressive behaviors (i.e., immobility in the forced swimming test) and REM sleep hypersomnia, which is an important trait of depression. Furthermore, the antagonism of the MCHR-1 receptor has a reliable antidepressant effect, suggesting that MCH is a pro-depressive factor. Hypocretins have been also involved in mood regulation; however, their role in depression is still on debate.

Taking these data into account, we explored whether systemic subchronical treatment with Fluoxetine (FLX), a serotonergic antidepressant, modifies the concentration of MCH in the cerebrospinal fluid (CSF), as well as the preproMCH mRNA expression. We also evaluated the hypocretinergic system by quantifying the hypocretin-levels in the CSF and the preprohypocretin mRNA expression.

Compared to control, FLX increased the levels of preprohypocretin mRNA without affecting the hypocretin-1 CSF levels. On the contrary, FLX significantly decreased the MCH CSF concentration without affecting the preproMCH gene expression. This result is in agreement with the fact that MCH serum level diminishes during the antidepressant treatment in MD, and supports the hypothesis that an increase in the MCHergic activity could have pro-depressive consequences.

1. Introduction

Major depressive disorder (MD) is a serious, recurrent, heterogeneous, and disabling psychiatric illness, that will affect one out of five people in their life-time and is the leading cause of disability worldwide; however, the current knowledge about the mechanisms associated with the pathogenesis of this disease is still limited, and current treatments remain ineffective in a large subset of patients [2,15,22].

In accordance with the monoamine theory of MD, the main classes of antidepressants are directed to elevate the synaptic levels of monoamines in the brain. However, these drugs are associated with several limitations, which include limited clinical efficacy, therapeutic lag with high risk of suicide and morbidity during latent period and treatment resistant cases [32,36]. Therefore, in order to improve the understanding of MD and its treatment, the study of the contribution of other neuromodulatory systems in this pathology is warranted.

The hypothalamus is considered the highest hierarchical structure in the control of homeostasis, and the postero-lateral region has been considered an integrative area involved in mediating different behaviors and processes that are critical to this function. Within this and adjacent regions of the hypothalamus, there are neurons that utilize the neuropeptides melanin-concentrating hormone (MCH) or hypocretin-1 and 2 (also called orexin A and B, respectively) as neuromodulators [4,27]. Both groups of neurons project throughout the central nervous system [4,27].

* Corresponding author.
E-mail address: ptortero@fmed.edu.uy (P. Torterolo).
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Interestingly, while hypocretins have mainly an excitatory synaptic action, MCH has the opposite effect, and whereas the MCHergic system tends to conserve energy, the hypocretinergic system is considered to have catabolic survival functions [7,28]. Moreover, whereas the hypocretinergic neurons are involved in the generation and maintenance of wakefulness and degeneration of these neurons produces narcolepsy, a sleep pathology, the MCHergic system promotes sleep, mainly rapid eyes movement (REM) sleep [33,34].

The hypocretinergic system has been involved in mood regulation and reward; however, the precise role of hypocretins in behavioral and neurophysiological impairments observed in depression is still unclear. The fact that both hypoactivity and hyperactivity of the hypocretinergic system have been found to be associated with depression (see [24] for a comprehensive review), may reflect the heterogeneous nature of MD.

Borowsky et al. [5] have demonstrated in rats that the MCH-R1 antagonist SNAP-7941 has an antidepressant-like profile in the forced-swim test (FST, a widely used experimental paradigm for screening antidepressant activity), suggesting that MCH is a pro-depressive neuromodulator. This finding was confirmed by preclinical studies [6,8,11,12,19,35,38], but the mechanism by which the MCHergic system participates in mood regulation is still unknown. However, recent studies showed that MCH suppresses the activity of presumed serotonergic neurons of the dorsal raphe nucleus (DRN) [9], and decreases the release of serotonin within this nucleus [37]; by this means, MCH might promote a depressive mood [19].

Recently, Schmidt et al. [30] showed that MCH serum level decreases in patients with MD during antidepressant treatment. In the present study, with the hypothesis that antidepressant pharmacological treatment decreases MCHergic activity, we analyzed the MCH concentration in the cerebro-spinal fluid (CSF) as well as the hypothalamic expression of the preproMCH (*Pmch*) gene in rats, following subchronical treatment with Fluoxetine (FLX), an antidepressant drug of the selective serotonin reuptake inhibitor (SSRI) group [40]. In order to know if FLX also modulates the hypocretinergic system, hypocretin-1 levels in the CSF and prepro-hypocretin (*Hcrt*) gene expression were also analyzed.

2. Methods

Twenty Wistar adult male rats (280–300 g) were used in this study. The animals were maintained with food and water ad libitum, and kept under controlled conditions (temperature 22 ± 2 °C; 12:12-h light-dark cycle). All of the experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academy Press, Washington DC, 2010) and approved by the Institutional Animal Care Commission. Adequate measures were taken to minimize pain, discomfort and stress of the animals. All efforts were made in order to use the minimal number of animals necessary to produce reliable scientific data.

2.1. Experimental procedure

Fluoxetine hydrochloride was kindly donated by Gador Laboratories (Montevideo-Uruguay) in powder form. Animals (n = 10) were treated with three doses of FLX (each of 20 mg/kg, i.p.) 23 h, 5 h and 1 h before CSF extraction and euthanasia. This treatment was chosen because it reversed the pro-depressive effect of MCH [19]. Control group (n = 10) received vehicle (saline) injections under the same schedule.

As in a previous study, CSF taps from the cisterna magna were performed under ketamine (90 mg/kg) and xylazine (5 mg/kg) anesthesia using a 1-ml syringe connected to a 27.5-G needle [10]. The taps were carried out during the light phase (8 h after the light was on). CSF limpid aliquots (100–150 μL) were frozen immediately over dry ice and stored at −80 °C until used. Thereafter, euthanasia was performed by decapitation. CSF samples with blood were discarded.

2.2. CSF analysis

CSF levels of MCH and hypocretin-1 were measured by means of fluorescent immunoassay kits (Phoenix Pharmaceuticals, CA, USA) [10]. These fluorescence immunoassay kits were designed to detect very specifically MCH or hypocretin-1 in a range of 0–10.000 pg/ml based on the principle of "competitive" enzyme immunoassay. The concentrations of the samples were within the linear range of the standard curve provided by each kit; the results we obtained displayed high accuracy and reproducibility.

Both hypocretin-1 and MCH were quantified in the same CSF sample. In order to determine the concentration of one of these neuropeptides, 50 μL of CSF were assayed in duplicates; the mean of both measures was considered the concentration of the peptide for the corresponding animal. The samples were incubated in an immunoplate with 25 μL of rabbit anti-MCH or anti-hypocretin-1 antibodies at 4 °C for 20 h. Next, 25 μL of the corresponding biotinylated peptide was introduced and the samples were incubated at room temperature for 1.5 h. The immunoplates were washed four times with 200 μL of assay buffer. Subsequently, 100 μL of streptavidin-horseradish peroxidase was applied, and the samples were incubated at room temperature for 1 h. After incubation, the immunoplates were washed four times with 200 μL of assay buffer. Next, 100 μL of substrate solution was applied and the samples were incubated at room temperature for 20 min. The reaction was terminated by adding 100 μL of stop solution. The fluorescence product was detected (excitation 325 nm; emission 420 nm) using a SpectraMax M2 fluorometer (Molecular Devices, Sunnyvale, CA, USA). The fluorescence readings were corrected using blanks, and the results were compared to their respective standard curves.

2.3. Gene expression analyses

Gene expression was analyzed as in a previous study of our group [10]. After euthanasia, the brains were immediately removed and the hypothalamus were harvested, frozen immediately over dry ice, and stored in microtubes at −80 °C until used. RNA was extracted using Brazol reagent (LGC Biotecnologia). The quantity and quality of the RNA extracted was measured using the Nanodrop 8000 spectrophotometer (Thermo Scientific). Total RNA (1 μg) was used to synthesize the complementary DNA (cDNA) using ImProm-II Reverse Transcriptase (Promega).

Diluted cDNA sample was used as template for real-time PCR amplification using 2X Maxima SYBR GREEN/ROX qPCR Master Mix (Thermo Scientific) and the respective primers for *Pmch* (NCBI Gen Bank accession number: *Pmch* mRNA, NM_012625.1) and *Hcrt* (NCBI Gen Bank accession number: *Hcrt* mRNA, NM_013179.2). Amplification and detection were performed using an Applied Biosystems 7500 Real-Time PCR system. A two-step cycling protocol was used.

Target mRNA levels were normalized for each well to endogenous control; beta actin (NCBI Gen Bank accession number: beta actin mRNA, NM_031144) and alpha tubulin 1a (NCBI Gen Bank accession number: Tuba 1 mRNA, NM_022298.1) were used to confirm the results. PCR products were subjected to a heat dissociation protocol (gradual increase of temperature from 60 to 95 °C) for melting curve analyses.

The relative gene expression was calculated using the comparative Ct (2 -deltadelta Ct) method [20].