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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

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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.

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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].

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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 preprohypocretin (*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].

2.4. Statistics

The data are reported as the means ± S.D. All the parameters were tested concerning normality (Kolmogorov-Smirnov's test) and homogeneity (Levene's Test). While CSF data showed a parametric distribution, gene expression data were non-parametric; so, these data were normalized using the Z-score to allow the utilization of parametric tests. The significance of the mean between the peptides concentrations, the MCH/hypocretin-1 CSF ratio (that was calculated for each CSF sample) or mRNA levels following vehicle and FLX treatments, was analyzed by the two-tailed unpaired Student *t* test. The level of significance was set at $p < 0.05$.

3. Results

In control condition (vehicle treatment) the concentration of MCH in the CSF was 146.6 ± 60.5 pg/ml, while the concentration of hypocretin-1 was 218.5 ± 53.8 pg/ml. As it is shown in Fig. 1(A), FLX treatment decreased the CSF level of MCH (69.8 ± 31.4 pg/ml, 47.6% of the control, p=0.004, t=3.34). FLX did not modify the hypocretin-1 concentration (206.5 ± 51.7 pg/ml, p=0.63, t=0.48) (Fig. 1B). We also analyzed for each CSF sample the MCH/hypocretin-1 ratio. FLX tended to decrease the MCH/hypocretin-1 ratio (0.73 ± 0.48 in control group Vs. 0.36 ± 0.20 in FLX group, p=0.057, t=2.07).

The expression of *Pmch* gene in the hypothalamus did not changed with FLX treatment (p=0.55, t=−0.61) (Fig. 2A). In contrast, FLX treatment increased the level of the *Hcrt* mRNA (p=0.02, t=−2.49; Fig. 2B).

4. Discussion

In the present study, we found that MCH concentrations in the CSF of normal rats decrease following subchronical treatment with

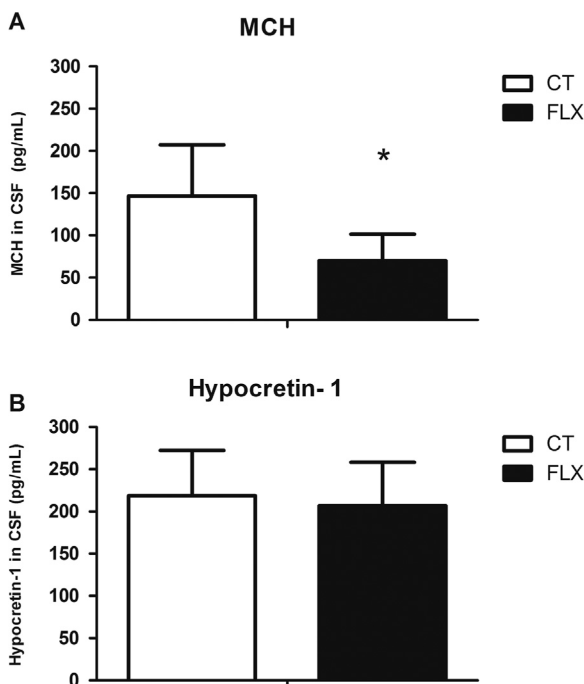

Fig. 1. Bar chart that shows the MCH (A) and hypocretin-1 (B) CSF concentration following vehicle (control, CT) or fluoxetine (FLX) treatments. Results are presented as mean ± S. D. *, p =0.004, two-tailed, unpaired Student's *t* test.

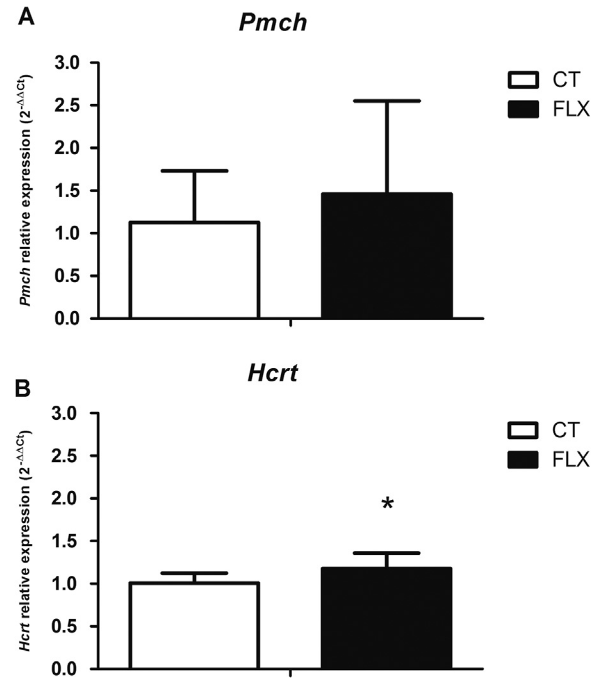

Fig. 2. Bar chart that shows the *Pmch* (A) and *Hcrt* (B) gene expression following vehicle (control, CT) or fluoxetine (FLX) treatments. Results are presented as mean ± S.D. *, p=0.02, two-tailed, unpaired Student's *t* test.

FLX. This result is in agreement with the decrease in MCH serum level following 4 weeks of antidepressant treatment in depressive patients [30]. The diminution of MCH within the CSF could be mediated by a reduction in the activity of the MCHergic neurons. In fact, FLX increases serotonin synaptic levels [40], and serotonin hyperpolarizes MCHergic neurons [39]. In contrast, the expression of *Pmch* was not modified. These results suggest that FLX could be mediating a reduction in the release of the peptide rather than a decrease in its synthesis; however, measurements of the hypothalamic pre-proMCH protein are needed to confirm this hypothesis.

In agreement with these results, preclinical studies have involved MCH with depression; for example, local administration of MCH into the DRN induced a depressive-like behavior evaluated in the FST [19,38]. This effect was prevented by systemic and subchronic treatment with either FLX and nortriptline (a noradrenergic antidepressant) [19,38]. Furthermore, MCH immunoneutralization (i.e. anti-MCH antibodies microinjected into the DRN) elicited an antidepressant behavioral response in the FST [19]. Microinjections of MCH into the DRN not only produce a depressive-like effect that is prevented by antidepressant, but also produce a REM sleep hypersomnia [17,18,38], that is a classical trait of MD [1,3,26]. Georgescu et al. (2005) also showed an increase in the immobility time in the FST induced by bilateral microinjection of MCH into the nucleus accumbens [13], demonstrating that other brain areas are also involved in depressive-like behaviors induced by MCH.

Katai et al. (2013) have recently explored the effect of acute antidepressant treatment (escitalopram, also a SSRI) on c-fos expression in MCHergic neurons during REM sleep rebound [14]. They demonstrated that escitalopram decrease the number of active (Fos positive) MCHergic neurons, which is in agreement with the reduction of MCH concentration in the CSF observed in the present study, and in accordance with the hypothesis that the MCHergic system might be involved in the pathophysiology of depression.

Although there is growing evidence that involve the

hypocretinergic system in the pathophysiology of depression, its role is still controversial [24]. A recent study reported that there is a decrease in depressive-like behavior in hypocretin receptor-1 knockout mice, whereas hypocretin receptor-2 knockout mice exhibit an increase in the depressive-related behavior, pointing to a differential role for both hypocretinergic receptors in MD regulation [31]. Further, Nollet et al. [23] demonstrated that mice subjected to unpredictable chronic mild stress displayed increased depressive-like behavior following the tail suspension test and presented elevated hypocretinergic neuronal activity. These authors were able to reverse this elevation in the hypocretinergic activity with six weeks of FLX treatment. In human studies, Salomon et al. [29] reported that hypocretin-1 CSF levels were higher in MD patients, and treatment with sertraline, an SSRI antidepressant, resulted in an attenuation of CSF hypocretin-1 levels. Under our experimental conditions, FLX did not modify hypocretin-1 concentration in the CSF. Interestingly, FLX increased the prepro-hypocretin mRNA level in the hypothalamus. These results suggest an eventual increase in the hypocretin synthesis without a perceptible effect on the release of the peptide toward the ventricular system. However, we cannot discard a local, slower and/or subtler effect upon hypocretin release that is not expressed in the CSF concentration. Since the hypocretinergic neurons are involved in reward and motivation [21], FLX might ameliorate the anhedonic component of depression by the modulation of the hypocretinergic function.

It has been suggested that the MCHergic and hypocretinergic systems reciprocally regulates several physiological functions such as wakefulness and sleep [16]. It is likely that other functions, like mood, are also controlled in a reciprocally way by these systems. In this regards, FLX tended to decrease the MCH/hypocretin-1 concentration ratio ($p=0.057$). It is likely that FLX move the balance between the MCHergic and hypocretinergic neuronal activity, down-regulating the first in favor of the hypocretinergic side of the equation.

Finally, it is known that acute treatment with ketamine increases the antidepressant effect of FLX [25]. However, in our experiments, ketamine anesthesia as well as taps and freezing procedures lasted no more than 20 min, a time that should not be enough to alter neither the peptides CSF concentrations nor the gene expression. In addition, this procedure was done both in control and experimental animals; hence we consider that ketamine did not modify the results.

5. Conclusions and future directions

The subchronical treatment with FLX decreased the concentration of MCH in the CSF. In agreement with the fact that MCH antagonists have antidepressant effects, the effect of FLX upon the MCHergic system is a new evidence that support the hypothesis that MCH is a pro-depressive factor.

New experiments are needed in order to confirm and dissect the effect of classical antidepressant on the MCHergic and hypocretinergic systems. A new series of experiments in animal models of depression (chronic mild stress, olfactory bulbectomy, etc.) are warranted [22]; we hypothesize that the effect of FLX should be even more pronounced in depressive individuals.

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