Melanin-concentrating hormone in the *Locus Coeruleus* aggravates helpless behavior in stressed rats

Jessika Urbanavicius[1], Sara Fabius[1], Aline Roncalho[2], Samia Joca[3], Pablo Torterolo[4] and Cecilia Scorza[1*]

[1]Departament of Experimental Neuropharmacology, Instituto de Investigaciones Biológicas Clemente Estable. [2]Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil. [3]Department of Physics and Chemistry, School of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, Brazil; [4]Department of Physiology, School of Medicine, Universidad de la República, Montevideo, Uruguay

*Corresponding author: Dr. Cecilia Scorza, Department of Experimental Neuropharmacology, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo. E-mail: cscorza@iibce.edu.uy; scorzacecilia@gmail.com

Abstract

Animal studies have shown that antagonists of receptor 1 of Melanin-Concentrating Hormone (MCH-R1) elicit antidepressive-like behavior, suggesting that MCH-R1 might be a novel target for the treatment of depression and supports the hypothesis that MCHergic signaling regulates depressive-like behaviors. Consistent with the evidence that MCHergic neurons send projections to dorsal and median raphe nuclei, we have previously demonstrated that MCH microinjections in both nuclei induced a depressive-like behavior. **Even though MCH neurons also project to *Locus coeruleus* (LC), only a few studies have reported the behavioral and neurochemical effect of MCH into the LC.**

We studied the effects of MCH (100 and 200 ng) into the LC on coping-stress related behaviors associated with depression, using two different behavioral tests: the forced swimming test (FST) and the learned helplessness (LH). To characterize the functional interaction between MCH and the noradrenergic LC system, we also evaluated the neurochemical effects of MCH (100 ng) on the extracellular levels of noradrenaline (NA) in the medial prefrontal cortex (mPFC), an important LC terminal region involved in emotional processing.

MCH administration into the LC elicited a depressive-like behavior evidenced in both paradigms. Interestingly, in the LH, MCH (100) elicited a significant increase in escape failures only in stressed animals. A significant decrease in prefrontal levels of NA was observed after MCH microinjection into the LC.

Our results demonstrate that increased MCH signaling into the LC triggers depressive-like behaviors, especially in stressed animals. These data further corroborate the important role of MCH in the neurobiology of depression.

Keywords: forced swimming test, learned helplessness, noradrenaline, prefrontal cortex

1. Introduction

MCH is a neuropeptide synthesized by neurons that are located mainly in the lateral hypothalamus and the incerto-hypothalamic area [1]. MCH exerts its effects through the G protein-coupled receptors, MCH-R1 [2-3] and MCH-R2 [4]. In rodents, only MCH-R1 is functional [5]. MCH has been usually related **with** the control of different functions such as food intake, energy balance, body weight and the sleep-wake cycle [6-7]. **However, in the last decades a new role related to the modulation of mood and emotional states has been** assigned to MCH [8-10]. Pharmacological and neuroanatomical studies support this statement. Pharmacological studies have shown that acute or chronic administration of MCH-R1 antagonists in rodents **elicit** antidepressant and anxiolytic effects [11-14] demonstrating for the first time the potential use of MCH1-R antagonists in the treatment of depression and/or anxiety. On the other hand, it has been reported that MCH neurons send projections to important structures associated with emotional processing such as the prefrontal cortex, hippocampus, amygdala and the nucleus accumbens [1]. Accordingly, monoaminergic nuclei such as dorsal (DR) and median raphe (MnR) nuclei, and the ***Locus Coeruleus*** (LC), which play a significant role in the neurobiology of depression [15-16], also receive MCHergic innervation [17-19]. All this evidence supports the role of the MCHergic signaling in the regulation of depressive-like behaviors. However, additional research is needed to elucidate how MCH functionally interacts with those brain circuits and thus elicits behavioral outcomes related to mood and emotion.

We have previously reported that the acute microinjection of MCH into the rat DR and MnR **elicit** a depressive-like effect evaluated in the forced swimming test

(FST) [20-22]. This behavioral response **is** mediated by MCH-R1, since **the MCH-R1 antagonist ATC-0175** was able to prevent the effect of MCH [22]. This behavioral outcome was quite similar to **that induced** by fluoxetine, since the pre-treatment with this selective serotonin reuptake inhibitor (SSRI) prevented the decrease in immobility time induced by MCH [20]. **Additionally, using** an *in vivo* microdialysis approach we observed that MCH was able to decrease extracellular levels of serotonin (5-HT) in DR. These results suggest that **the** direct inhibitory action (possibly through MCH-R1) on 5-HTergic neurons explain the development of the depressive-like behavior induced by MCH into the DR [19]. In addition to that, a noradrenergic (NAergic) mechanism seems to participate in the MCH depressive-like action, since the pre-treatment with nortriptyline (noradrenaline reuptake inhibitor) was also able to block the depressive-like effect induced by MCH into the DR [22]. Accordingly, it has been recently reported that MCH injection into the LC increased learned immobility in the forced swimming test, considered a depressive-like behavior. This effect was blocked **by the MCH-R1 antagonist SNAP-94847** [23]. **It is possible that the pro-depressive effect of MCH into the LC is associated to changes in the extracellular NA levels.**

LC is involved in a wide number of physiological functions including stress response [24-25]. Although the relationship between MCH signaling and stress has been previously explored [26-27], there is conflicting evidence about the role of MCH in stress response. Chronic mild stress in mice increases MCH-receptor expression in **the** hippocampus [28] and repeated restraint stress produced depressive-like behaviors in mice associated with an increased expression of MCH transcripts in the basolateral amygdala [29]. MCH activates the hypothalamic-pituitary-adrenocortical (HPA) axis while MCH-R1 antagonists blocked MCH-induced release of **corticotrophin-releasing factor** [30]. In contrast, MCH blocked stress-induced plasma adrenocorticotropic hormone (ACTH) levels in other studies [31-32]. **Besides these contradictory data,**

there is a limited amount of studies that assess the behavioral effect of MCH into the LC and its association with stress conditions.

In the present study we investigated whether the LC is involved in the regulation of MCH-induced behaviors in animals subjected to two different behavioral tests widely used to study depressive- or anti-depressive-like behaviors: the FST and the learned helplessness [33]. We also evaluated the neurochemical effects of MCH on NA release in the medial prefrontal cortex (mPFC), an important LC terminal region involved in emotional processing [34-35], to characterize the functional interaction between MCH and the NAergic system.

2. Materials and Methods

2.1. Animals

Adult male Wistar rats weighing 290-320 bred in IIBCE animal facilities (Montevideo) were used in the study. All animals were housed in groups of 5 in plastic cages (50 cm × 37.5 cm × 21 cm) and kept under controlled conditions (temperature 22 ± 2°C, 12-h day-night cycle, lights on at 7:00 am) with **rat chow** and water available *ad libitum*. All procedures were carried out in accordance to the Bioethics Committee guidelines of IIBCE **(number of approved protocol 010/11/2016)** and following the National Institutes of Health guide for the care and use of Laboratory animals; NIH Publications N° 8023, revised 1978) and current ethical regulations under animal experimentation law N° 18.611. Adequate measures were taken into account to minimize pain, discomfort or stress of the animals, and all efforts were made to use the minimal number of animals necessary to obtain reliable scientific data.

2.2. Surgical procedures to cannula implantation in the LC

Surgical procedures were performed as previously described by Biancardi and colleagues (2014) with modifications [36]. Briefly, animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (5 mg/kg) and placed in a stereotaxic frame (David Kopf Instruments, USA). Once the skull was exposed, a hole was drilled above the right LC. A stainless steel guide cannula (25 G; 0.02 mm OD, 20 mm length) was unilaterally implanted in the right LC with the incisor bar positioned at -3.3 mm and the stereotaxic bar set with an angle of 14° (postero-anterior direction) to avoid sinus lesion. The coordinates were taken according to the atlas of Paxinos and Watson (2005) [37], AP: -11.8 mm from Bregma, L: +1.3 mm from midline and DV: -4 mm from duramater (2 mm above the final coordinate). The guide cannula was attached to the skull with three stainless-steel screws and acrylic cement. After recovering from anesthesia, the rats were returned to their home cages in groups of three to four until the day of the experiment.

2.3. Drug administration

Five days after surgery, the animals were randomly assigned to each experimental group to receive **0.9 %** saline or MCH (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) and also randomly tested to avoid the influence of diurnal variations on behavior. Animals were gently immobilized and an infusion cannula (33G, 22 mm length) was inserted through the guide cannula to reach the LC (final position DV: -6 mm). MCH was diluted in saline to obtain a final concentration of 1.0 and 2.0 μg/μl (100 and 200 ng, respectively). These doses were selected according to previous studies performed in LC [38]. Saline or MCH (100 and 200 ng) were perfused using an infusion

pump (Harvard Apparatus, Instech USA) set at a flow rate of 0.4 µl/min during 15 s (0.1 µl final volume) and 15 additional seconds to allow drug diffusion.

2.4. Behavioral analysis

2.4.1. Forced Swimming Test (FST): the procedure used for the FST was performed as previously described [22]. The apparatus consisted in a transparent cylindrical tank (50 cm height and 20 cm diameter) filled with water (24-25 °C) to a depth of 34 cm (in order to allow rats to swim or float without touching the bottom of the tank with their paws). Two experimental swim sessions with a 24 h interval were applied. In the first pretest session, the animals were placed in the tank for 15 min, immediately dried and returned to their home cage. During the test session, the total time spent immobile (making only the movements necessary to remain afloat), swimming (horizontal movements throughout the cylinder) and climbing (vigorous and upward-directed movements of the forepaws along the cylinder wall) were scored by an experimented researcher in real time during 5 min. Sessions were videotaped for later analysis or confirmation when necessary [39-41]. Animals received unilateral intra-LC injections of saline, MCH 100 or 200 ng (0.1 µl) and 30 min after were exposed to the FST test during 5 min [21-22].

2.4.2. Learned helplessness (LH): the procedure used for the LH was performed as previously described **by** Joca et al. (2003) with minor modifications [42]. The experiment was carried out in an automated shuttle box with two compartments of equal size (30 × 33 × 54 cm) made of Plexiglas, separated by a wall with a central open door and equipped with a stainless-steel grid floor through which the scrambled shocks were delivered (Model EP 111, Insight) and with transparent covers. Two experimental sessions with a 24 h interval between them were performed. During the

pretest session, the animals were randomly assigned to the stressed group (S) or the non-stressed group (NS). The animals in the S group were individually exposed to 40 inescapable foot shocks (0.8 mA, 10 s duration) given according to a variable schedule range from 30-90 s. On the other hand, animals from the NS group were placed into the same apparatus for 30 min, but no shock was delivered. During the test session, animals of both groups were placed individually into the shuttle box and submitted to 30 escapable foot shocks (0.6 mA, 10 s duration, 30-90 s interval), which were preceded by a tone (60 dB, 670 Hz) that started 5 s before each shock and lasted until its end. Animals can avoid shock during the sound emission or interrupt their presentation by crossing to the opposite side of the chamber. The absence of one of these behaviors was considered an escape failure. During this session, the number of escape failures, and the number of inter-trial crossings were automatically recorded. Animals that showed more than ten (10) escape failures during the test were considered to be helpless [43]. Animals received unilateral intra-LC injections of saline, MCH 100 or 200 ng and were immediately exposed to escapable foot shocks (LH test session) during 30 min.

2.4.3. Open Field Test (OFT): to discard that the behavioral changes in the FST had been associated to alterations in the animal motor activity induced by MCH, an independent group of implanted animals were treated with MCH or saline and subjected to an OFT session. The apparatus consisted of an acrylic square box (45 x 45 cm) with walls of 50 cm high, homogeneously illuminated (35 lux). Each rat was placed in the center of the arena and allowed to explore the field freely. Total distance traveled (as an index of locomotor activity) was registered immediately after saline or MCH (100 ng) administration for 30 min. In order to know the effect of MCH on the locomotion related with the animal performance during the FST, the first 5 min was shown separately. In addition, time spent in the central area **(20 x 20 cm)**; as an index

of anxiety behavior) was recorded. The behavioral activity was automatically recorded and analyzed using a by a camera connected to a computer equipped with a video tracking software Ethovision 7.0 (Noldus, Wageningen, The Netherlands). After recording the animal behavior, the OF was cleaned with alcohol 30 % before placing the following rat. In all the experiments rats were naive to the OF and were used only once. All the experiments were performed between 9:00 and 14:00 h.

2.5. In vivo intracerebral microdialysis procedure

Taking into account that a relationship between the LC neurons activity and NA dialysate levels in the PFC has been described [34; 44], we collected prefrontal NA extracellular levels as a measure of changes in the flow activity of NA neurons under MCH microinjection in LC. In an independent group of animals, a guide cannula was implanted in the LC (following the same procedure described in item 2.2.) while a concentric microdialysis probe (Cuprophan, 4 mm membrane length, 18 kDa molecular cutoff, 210 µm outer diameter) was also implanted in the ipsilateral mPFC (coordinates: AP: -3.2 mm rostral to Bregma, L: +0.8 mm lateral to midline and DV: - 5.4 mm from dura [37]. After surgery, rats were housed individually with free access to food and water. The microdialysis experiments were performed using freely moving rats on the day following the surgery. On the experimental day, each animal was perfused through the microdialysis probe placed in the mPFC with artificial cerebrospinal fluid (CSF; 125 mM NaCl, 2.5 mM KCl, 1.26 mM CaCl2 and 1.18 mM MgCl2) containing 1 µM nortriptyline at a flow rate 1.5 µl/min (CMA/100 microinjection pump, Sweeden). After a 120 min stabilization period, 4 to 5 fractions were collected to obtain baseline values before the administration of saline or MCH 100 ng into the LC (under the same experimental conditions followed to the saline or MCH microinjection described above). Successive dialysate samples were collected each 20 min (30 µl) and NA

concentration in samples was immediately determined by High-Performance Liquid Chromatography (HPLC; Waters 2465) equipped with an electrochemical detector Epsilon e5P at + 0.65 V (glassy carbon working electrode versus an Ag/AgCl reference electrode). The column (C18, 3 µm particle size) was kept at 30 °C and the mobile phase consisted of citric acid (0.15 M), sodium octil sulphate (0.9 mM) and tetrahydrofuran 1.5 % at pH = 3 and a flow rate of 0.3 ml/min. At the end of the experiments, brains were processed for histological procedures to determine the placement of the dialysis probes and administration cannula.

2.6. Histological assays

To confirm the location of the microinjection site in the LC and the microdialysis probe located in the mPFC, rats were deeply anesthetized with urethane (2.4 g/kg, i.p.) and perfused transcardially with saline solution followed by buffered 4 % **formaldehyde**. Brains were dissected out and cryoprotected in sucrose 30% in PBS for 48 h and then frozen on dry ice. Coronal brain sections (30 µm) were obtained using a cryostat (Leica Microsystem CM 1900, USA) and mounted onto gelatin-coated slides. Sections were stained with Cresyl violet (0.25 %). The placement of the microdialysis probe and the microinjection site of MCH or saline were assessed following the Paxinos and Watson atlas [37]. Images were captured using a digital camera (Nikon Coolpix E995, USA) attached to a stereoscopic microscope (Nikon Inc. SMZ750, USA) equipped with a plan 1x lens or to a microscope with a 4x lens (Nikon Inc. E800, USA). Animals, in which the microdialysis probe or the guide cannula was misplaced out of mPFC or LC, were not included in the study.

2.7. Statistical analysis

Data from FST and OFT experiments are presented as mean ± standard error of the mean (SEM). The results were analyzed by One-way ANOVA for independent measures (treatment) followed by a post-hoc Newman-Keuls multiple comparison test or by Student *t*-test. Data from LH experiments are presented as the median with interquartile ranges. The results were analyzed by the Kruskal-Wallis test followed by Dunn's multiple comparison test. Data from the microdialysis experiments were analyzed by Two-way ANOVA for repeated measures (treatment and time) followed by post-hoc Bonferroni test. In all cases, the statistical significance was set at $P < 0.05$.

3. Results

3.1. Behavioral effect of MCH intra-LC evaluated in the FST and OFT

Figure 1 shows the behavioral effect of MCH on the LC evaluated in the FST. One-way ANOVA showed a significant effect for treatment [$F_{(2, 21)} = 6.93$, $P < 0.01$] and the post hoc analysis revealed a dose-dependence effect of MCH. Thus, MCH (100) was the only effective dose that induced a significant increase in the immobility time compared to the control group ($P < 0.05$) and compared with the higher dose of MCH ($P < 0.01$). For swimming time, One-way ANOVA did not reveal a significant effect for treatment [$F_{(2,21)} = 2.43$, $P = 0.11$]. Thus, this active behavior was not significantly altered by MCH. In contrast, One-way ANOVA showed a significant effect of treatment for climbing time [$F_{(2,21)} = 7.33$, $P < 0.01$]. The Newman-Keuls test showed that climbing behavior was significantly decreased in the MCH (100)-treated rats respect to control group ($P < 0.01$) and MCH (200; $P < 0.01$).

Table 1 shows the behavioral effects induced by MCH (100) recorded in the OFT. Student *t*-test revealed that the distance moved was unchanged in comparison with saline-treated animals during the first 5 min ($t_{12} = 1.39$, $P = 0.18$), suggesting that

the depressive-like effect of MCH observed in the FST is not due to changes in the locomotor activity. Also, when we considered the total distance traveled (30 min), no significant changes were observed in animal locomotion (t_{12} = 0.11, P = 0.91). The time spent in the center did not significantly change at the first 5 min (t_{12} = 0.72, P = 0.48) or the whole period (t_{12} = 1.72, P = 0.11).

3.2. Behavioral effect of MCH intra-LC evaluated in the LH

Figure 2 shows the behavioral effect of MCH in LC on the number of escape failures evaluated in the LH in non-stressed (A) and stressed animals (B). Kruskal-Wallis analysis showed that the administration of MCH 100 or 200 ng did not significantly change the number of escape failures in non-stressed animals in comparison with the saline (control) group (H = 0.352; P = 0.83, N = 9-13). The numbers of escape failures (Mean ± SEM) for saline, MCH 100 and MCH 200 ng were: 7.23 ± 2.14; 11.17 ± 3.38 and 10.67 ± 3.88, respectively. However, in stressed animals, Kruskal-Wallis analysis revealed that MCH significantly increased the number of escape failures at the dose of 100 ng in comparison with the saline group (H = 8.399, P < 0.05, N = 8-12) indicating that the effect of MCH was sensitive to stress exposure. The numbers of escape failures (Mean ± SEM) for saline, MCH 100 and MCH 200 ng respectively were: 15.17 ± 3.14; 25.91 ± 1.70 and 22.25 ± 3.76.

Figure 3 shows the number of inter-trial crossings recorded in the LH paradigm. Comparing MCH 100 or 200 ng with saline-treated rats, Kruskal-Wallis analysis did not show any effect on the number of inter-trial crossings either in non-stressed (H = 0.563, p = 0.7, N = 9-13) or stressed animals (H = 3.109, p = 0.2, N= 8-12). The number of inter-trial crossing (Mean ± SEM) for saline, MCH 100 and MCH 200 ng in the non-stressed animals was 7.38 ± 1.5; 9.25 ± 1.7; and 8.77 ± 2.6, respectively, while in stressed animals was 3.75 ± 0.7; 2.27 ± 0.7; and 2.12 ± 0.9, respectively.

3.3. Neurochemical effects of MCH intra-LC on NA extracellular levels in mPFC

Figure 4 shows the effect of MCH on extracellular levels of NA in the prefrontal cortex. In the control group, the mean of NA levels in basal samples before saline administration into the LC was 21.5 ± 2.5 pg/30 µl and this value remains stable along the experimental session. In turn, in MCH-treated animals, the NA basal levels before MCH administration into the LC was quite similar to the control group (16.8 ± 4.7 pg/30 µl). However, after MCH (100 ng) microinjection into LC, two-way ANOVA revealed a significant effect of treatment [$F_{(1,9)} = 18.74$, $P < 0.0001$], time [$F_{(9,80)} = 2.53$, $P < 0.05$], and time x treatment interaction [$F_{(9,80)} = 2.73$, $P < 0.01$]. Post-hoc analysis showed that MCH microinjection induced a significant decrease in the NA levels ($P < 0.05$) in comparison with saline-treated animals. This effect was evidenced 40 min after the administration of MCH (sample 6) and lasted for 60 min. After that, the levels of NA started to return to the basal level (Fig. 4).

3.4. Evaluation of cannula placement in LC and mPFC

Figure 4 shows representative images of the localization of cannula placement and microinjection sites in LC (Fig. 4 B), and microdialysis probes in mPFC (Fig. 4 C). Only those animals with a correct anatomical localization were included in the data analysis. **Rats that showed cannula misplacement were excluded in behavioral studies (N = 18; from the LC in the LH, FST and OF experiments) and microdialysis experiments (N = 3; one from the mPFC and two in LC).**

4. Discussion

In the present study, we showed that the acute administration of MCH into the rat LC elicits a depressive-like behavior evaluated in two behavioral paradigms, the FST and the LH. Moreover, and particularly in the LH paradigm, we found that the depressive-like phenotype induced by MCH was evoked only in previously stressed animals, suggesting that an acute stress response could trigger a physiological condition which can boost the MCH action. We also demonstrated that the administration of MCH into the LC, at the effective dose used in FST and LH paradigms, induced a decrease in the extracellular NA levels in the mPFC.

The behavioral effect induced by MCH when injected into the LC agrees with previous studies for MCH administration in other brain regions [14; 20; 22; 45-46]. Moreover, we were able to replicate the effect induced by MCH microinjected into the LC, which was recently reported by Ye and colleagues (2018) using the FST and the sucrose preference test [23]. Overall, these results support the important role of MCHergic system in the regulation of behaviors related to depression, and demonstrate that the LC is a relevant substrate in the neural network regulated by MCH.

While the underlying molecular mechanisms by which MCH induces depressive-like behaviors in the LC remain under study, our neurochemical results provide important information that helps to explain this effect. Prefrontal extracellular NA levels significantly decreased after the microinjection of MCH into LC. This neurochemical effect could be directly associated **to a decrease of** LC neuronal activity, since it has been reported that prefrontal NA levels correlates with changes in LC-noradrenergic neurons discharge rates [34]. Additionally, morphological data have shown that mPFC receives the highest density of NA varicosities in comparison with other cortical regions [47-48]. Loss of NA neurons in LC and the concomitant decrease in NA concentrations in PFC and hippocampus have been associated with the expression of a depressive-like behavior in the FST [49]. Consistent with all these data,

the depressive-like profile observed in the MCH-treated animals may be explained by a decrease in NA levels in LC. Accordingly, and based on the inhibitory actions of MCH through the MCH-R1 **(inhibitory intracellular signaling) [50-52],** we had previously demonstrated that MCH decreased the extracellular levels of 5-HT in the DR [19], an effect which was associated to the pro-depressive effect induced by MCH after the DR microinjection [20; 22]. Both results additionally suggest that a functional relationship between MCHergic and monoaminergic systems (5-HTergic and NAergic) seems to be essential in the expression of depressive-like behavior induced by MCH.

It was pointed out that the LC-noradrenergic system plays an important role in several physiological functions including the stress-coping response [25; **53**]. In the present study, the action of MCH was studied in two models in which we evaluated coping strategy to an acute inescapable stress [41; **54**]. However, in the learned helplessness model two sets of animals were used, the non-stressed and stressed ones. Animals are initially exposed to inescapable stress (foot-shocks) and after an acquisition period, animals are exposed to the same environment, but now escape is possible [41; **55**]. Stressed animals developed the helplessness behavior (i.e., no attempts to escape) offering the chance to compare the effect of MCH both in stressed and non-stressed rats. **In contrast, in the FST animals are trained in a pre-test session, which is considered an acute stress precondition**. **It is** known that behavior in the FST is a reaction to the acute stressful stimulus of being placed in a container without an escape route [39; 42; **54**]. **Thus, trough the LH paradigm, it** became evident that MCH evoked the helplessness behavior only on those animals which were stressed in relation to non-stressed animals. This result agrees with previous studies, which have reported a close but still unclear association between the MCHergic system and stress response [26; 29-30; 32; **56-58**]. Our data provide strong evidence in support to that an acute stress exposure is a vulnerability condition able to

impair the stress-coping behavioral response observed after an increased MCH signaling into the LC.

It is well known that an increase in NA release in the brain is the response to acute and repeated exposure to stress [**59-60**]. Activation of LC and release of NA for exciting neurons throughout the brain has been recognized as part of the response to stress-induced depression [**61**]. In contrast, clinical observations indicated that drugs that depleted NA and other monoamines (e.g., 5-HT) impair mood, while agents (antidepressants) that enhanced the availability of brain monoamines are able to improve mood and reverse the symptoms of depression [**62**]. Despite that, many animal studies indicated that low NA levels have been related to the stress-induced helplessness response in the LH [**63-64**] and restored NA and 5-HT levels were involved in the reversion of the helplessness behavior induced by antidepressant treatment [**64-65**]. Monti and colleagues (2015) have shown that acute unilateral administration of MCH into the LC (at the same doses used in this study) induces an increase in the time spent in REM sleep in rats [38]. LC-NA neurons are REM-off and inhibition of this neuronal population facilitates REM sleep occurrence, supporting an inhibitory action of MCH on NA neurons [38; **66**]. Taking into account all these data, in our experimental protocol of LH MCH may inhibit NA neurons in the non-stressed animals, while in an additive way, MCH in addition to an acute stress condition (i.e., in stressed animals) may facilitate its inhibitory action reaching a threshold required to evoke a depressive-like behavior. Further studies will be required to address this hypothesis.

In addition to the relevant role of NA in stress response, alternative mechanisms involving other neuromodulators should not be ruled out. Stress activates the HPA axis. A plethora of molecules, i.e., neurotransmitters, steroid hormones, and neuropeptides are released to drive adaptive responses to stress [**67**]. The hypothalamic corticotropin-releasing hormone (CRH), its related peptides, the urocortins (1-3) and their respective

receptors constitute a central peptidergic system responsible for the stress adaptation. **Urocortin 2 is expressed in LC, and the central administration of this peptide induces antidepressant and anxiolytic effects [68]. It is possible that MCH inhibits neurons expressing urocortin 2 in the LC, eliciting a disruption of CRH-family peptide signaling and leading to alter the stress-coping response [68-69].** As was mentioned above, it has been reported contradictory data about MCH and stress. Although the reason for these discrepancies remains unexplained, our data support a putative mechanism linking MCH, the stimulation of the HPA and the consequently induction of CRH and ACTH [30; **57**]. A limitation of the present study is the lack of plasma CRH, urocortins or ACTH assesment in MCH-pretreated stressed animals. Future experiments should consider this relevant issue.

Conclusion

Although different variables (genetic predisposition, biological and environmental factors) could explain the vast majority of complex neuropsychiatric disorders, stress has emerged as the most prominent environmental factor causally involved in the etiology of psychiatric disorders. Stress is a significant risk factor for the development of depression, and our work supports the hypothesis of a **role of the MCHergic system in the LC as a part of the pathophysiological process in depression.** Our data also show the influence of acute stress exposure on the behavioral action of MCH.

Conflict of interest

The authors report no conflicts of interest about this work.

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Figure captions

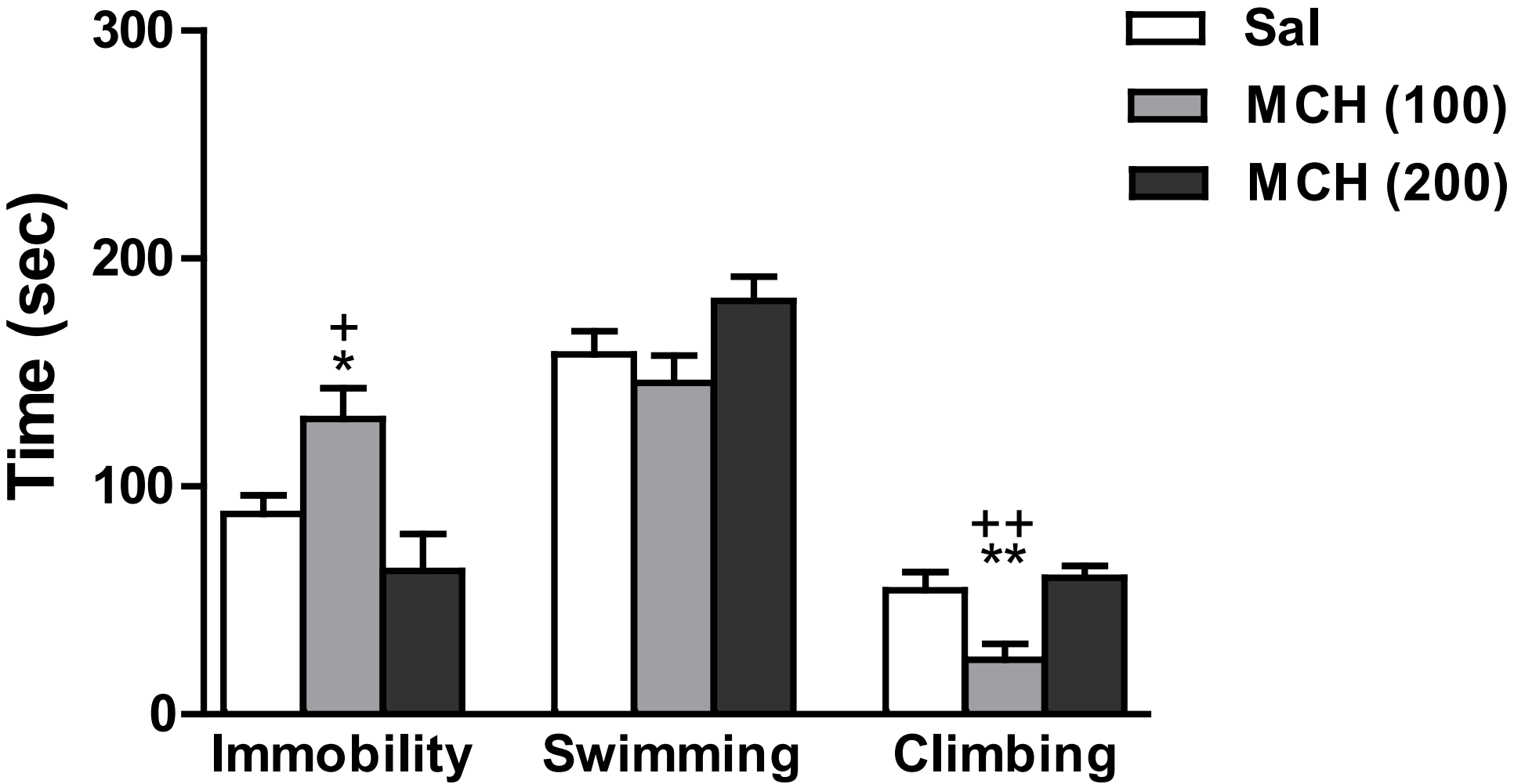
Figure 1. Effects of MCH into the LC on the time spent in immobility, swimming or climbing in the FST. Animals received unilateral intra-LC injections of saline, MCH 100 or 200 ng (0.1 µl) and 30 min after were exposed to the test session. Bars represent mean ± SEM. One-way ANOVA followed by Newman-Keuls test; * = vs. saline; + = vs. MCH 200 ng. *,+ = $P < 0.05$; **, ++ = $P < 0.01$. N = 6-8 per group.

Figure 2. Effects of MCH into the LC on the number of escape failures in the LH model in animals habituated to the shuttle box (non-stressed, A) or subjected to inescapable foot shocks (stressed, B) during the pre-test session. Twenty-four hours later, both groups of animals received unilateral intra-LC injections of saline, MCH 100 or 200 ng (0.1 µl) and immediately after were exposed to escapable foot shocks (test session). Data are expressed as the median with interquartile ranges. Kruskal-Wallis followed by Dunn's multiple comparison test; * = $P < 0.05$ vs. saline. N = 8-13 per group.

Figure 3. Effects of MCH into the LC on the number of intertrial crossings in the LH model in animals habituated to the shuttle box (non-stressed, A) or subjected to inescapable foot shocks (stressed, B) during the pre-test session. Twenty-four hours later, both groups of animals received unilateral intra-LC injections of saline, MCH 100 or 200 ng (0.1 µl) and immediately after were exposed to escapable foot shocks (test session). Data are expressed as the median with interquartile ranges. There were not statistical differences among groups; Kruskal-Wallis followed by Dunn's multiple comparison test. N = 8-13 per group.

Figure 4. Effect of MCH microinjections intra-LC on mPFC NA extracellular levels. In A, MCH 100 ng (0.1 µl; closed circles) or saline (0.1 µl; open circles) was microinjected

into the LC (arrow) and NA extracellular levels were collected in the mPFC. Bars represent mean ± SEM. Two-way ANOVA followed by Bonferroni test. * = $P < 0.05$ vs. saline group. N = 5 per group. MCH = melanin-concentrating hormone; NA = noradrenaline. In B, a representative localization of the administration cannula placed above the LC (arrows) and the site of injection into the LC (dotted circle). In C, representative localization of the dialysis probe into the mPFC. Both tracks were verified by histological assays. As a reference coordinates (from Bregma) of LC and mPFC are shown; diagrams from Paxinos and Watson atlas were provided. Cg = cingulate cortex; PrL = prelimbic cortex and IL = infralimbic cortex. Calibration bars were 100 µm and 1 mm in B and C, respectively.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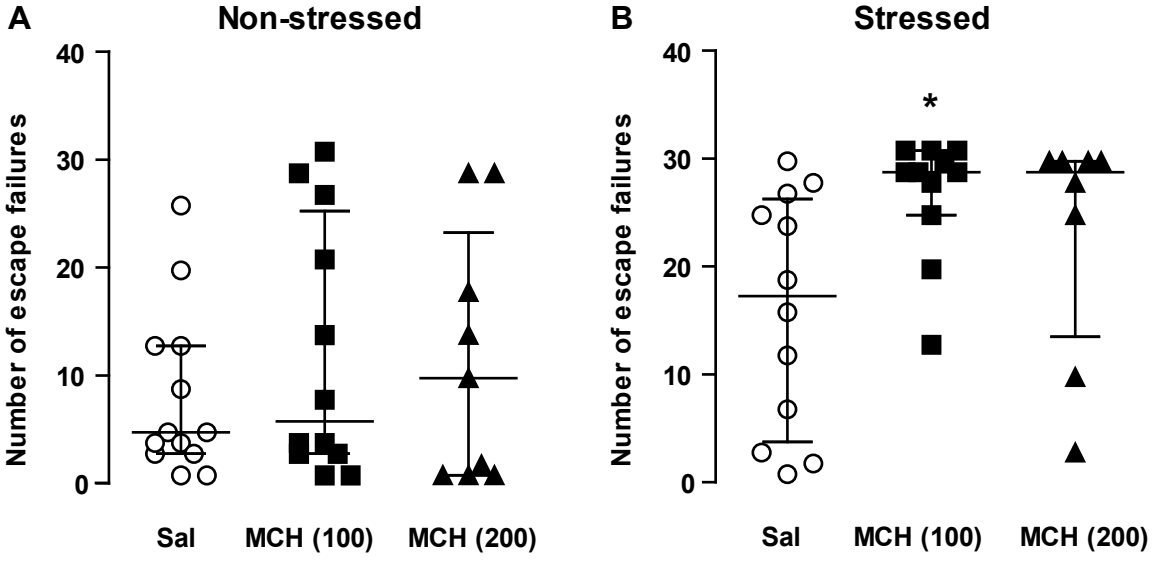
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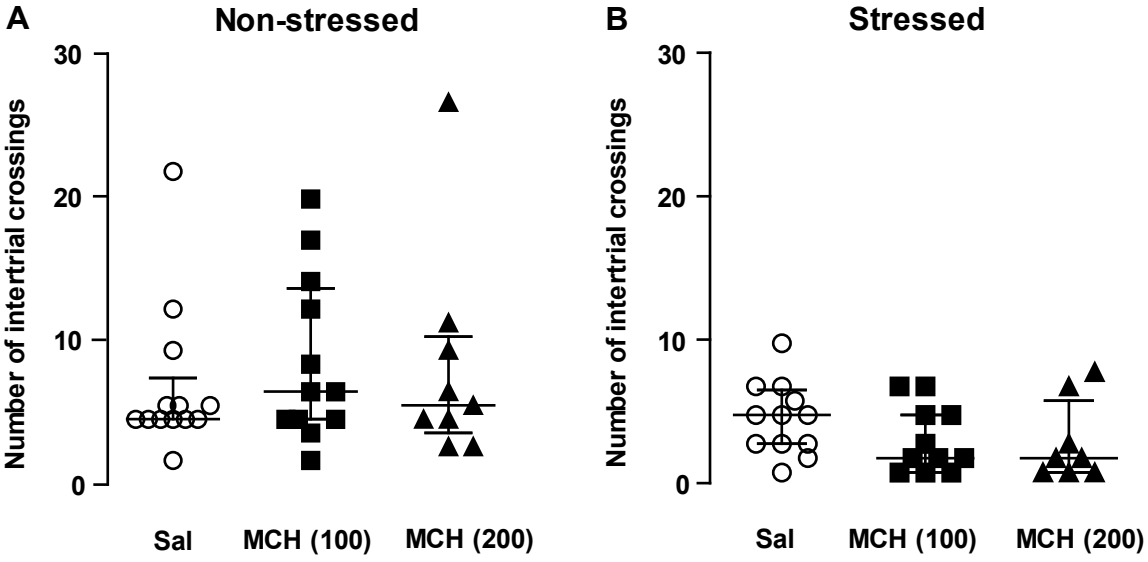
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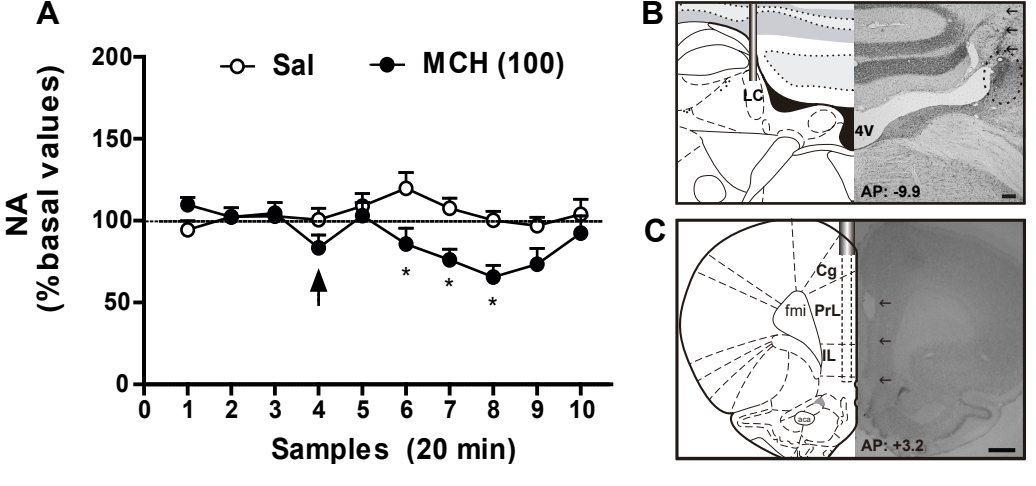
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