Peptides 126 (2020) 170249

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Melanin-concentrating hormone (MCH) in the median raphe nucleus: Fibers, receptors and cellular effects

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ARTICLE INFO

Keywords:
Serotonin
Hypothalamus
Peptide
Sleep
Depression
REM

ABSTRACT

Serotonergic neurons of the median raphe nucleus (MnR) and hypothalamic melanin-concentrating hormone (MCH)-containing neurons, have been involved in the control of REM sleep and mood.

In the present study, we examined in rats and cats the anatomical relationship between MCH-containing fibers and MnR neurons, as well as the presence of MCHergic receptors in these neurons. In addition, by means of *in vivo* unit recording in urethane anesthetized rats, we determined the effects of MCH in MnR neuronal firing.

Our results showed that MCH-containing fibers were present in the central and paracentral regions of the MnR. MCHergic fibers were in close apposition to serotonergic and non-serotonergic neurons. By means of an indirect approach, we also analyzed the presence of MCHergic receptors within the MnR. Accordingly, we microinjected MCH conjugated with the fluorophore rhodamine (R-MCH) into the lateral ventricle. R-MCH was internalized into serotonergic and non-serotonergic MnR neurons; some of these neurons were GABAergic. Furthermore, we determined that intracerebroventricular administration of MCH induced a significant decrease in the firing rate of 53 % of MnR neurons, while the juxtacellular administration of MCH reduced the frequency of discharge in 67 % of these neurons. Finally, the juxtacellular administration of the MCH-receptor antagonist ATC-0175 produced an increase in the firing rate in 78 % of MnR neurons. Hence, MCH produces a strong regulation of MnR neuronal activity. We hypothesize that MCHergic modulation of the MnR neuronal activity may be involved in the promotion of REM sleep and in the pathophysiology of depressive disorders.

1. Introduction

The serotonergic neuronal system is involved in highly relevant functions such as sleep and the regulation of mood [1–3]. Serotonergic neurons with anterior or ascendant projection lie in the median raphe nucleus (MnR, also called nucleus centralis superior or B8 and B5), as well as within the dorsal raphe nucleus (DR) [1,4–8]. In the rat MnR, there are approximately 1100 serotonergic neurons according to Jacobs and Azmitia (1992), and 4100 according to Vertes and Crane (1997). In the cat, the number oscillates between 7000 and 7400 according to Jacobs and Azmitia (1992) and Wilkund et al. (1981). Serotonergic neurons are located mainly along the midline or central region of the MnR [6,8]. The MnR also contains GABAergic and glutamatergic neurons, which are distributed both in the central and paracentral (lateral) region of the nucleus [6,8]. The firing pattern of the MnR neurons has a close relationship with hippocampal theta rhythm in anesthetized animals, and the activity of these neurons is turned-off during REM sleep [1,9].

Melanin-concentrating hormone (MCH) is a neuromodulator peptide synthesized in neurons of the lateral hypothalamus and incerto-hypothalamic area, that project diffusely throughout the central nervous system (CNS) [10–12]. MCHergic neurons have been involved in physiological processes such as energy homeostasis, mood regulation and sleep [13–20].

MCH biological function is mediated by two G-protein coupled receptors (GPCR) known as MCHR-1 and MCHR-2 [21,22]. While MCHR-2 is functional in humans, primates and carnivores, it is either lacking or non-functional in rodents and lagomorphs [21,23]; this receptor is present (gene ID: 101086149) and probably functional in cats. In rodents, it has been shown that the activation of the MCHR-1 has an inhibitory effect at presynaptic and postsynaptic regions [24,25].

MCH-containing fibers are present in the MnR [10,12,26]. Furthermore, functional experiments performed by Lopez Hill et al. (2013) have shown that microinjections of MCH into the MnR of the rat,

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https://doi.org/10.1016/j.peptides.2019.170249
Received 9 May 2019; Received in revised form 11 November 2019; Accepted 26 December 2019
Available online 03 January 2020
0196-9781/

produces a specific and dose-dependent pro-depressive effect, as evaluated in the forced swimming test [26]. However, the cellular effect of MCH on the MnR neurons is still unknown.

In the present study, we examined by immunohistochemistry, the anatomical relationship between MCHergic fibers and serotonergic neurons of the rat MnR. In addition, in order to determine the presence of MCH receptors within the MnR we employed the methodology introduced by Devera et al. (2015). Accordingly, we perfused MCH conjugated with the fluorophore rhodamine (R-MCH) into the lateral ventricle of cats and rats. Finally, we explored the electrophysiological effects of MCH by means of MnR unit recordings in urethane-anesthetized rats. Although we have performed similar experiments in the DR [27], we consider important to know if there are morphological (MCHergic fibers and receptors) and functional (effect of MCH on neuronal activity) differences between both serotonergic nuclei.

2. Materials and methods

Two adult cats (≈ 3.5 Kg) and fifty Wistar rats (250–310 g) were used in this study. The animals were maintained with food and water available *ad libitum,* and kept under controlled conditions (temperature 22 ± 2 ˚C, 12-h day–night cycle, lights on at 7:00 A.M.). 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), EC Directive for animal experiments (86/609/EC) and approved by the Institutional Animal Care Commission (N° 070153-000841-18). Adequate measures were taken to minimize pain, discomfort or stress of the animals, and all efforts were made to use the minimal number of animals necessary to produce reliable scientific data.

2.1. Microinjection of rhodamine-labeled MCH

Tissues sections from animals utilized in Devera et al. (2015) (2 cats and 4 rats) were re-analyzed. Coronal sections of the brains processed in this study, were stored in dark recipients at -20 ˚C in an anti-freezing solution until re-analyzed for the present report; in such conditions, fluorescence remained stable for as long as 2–3 years. Briefly, the experimental procedures were the following (for details, see [27]).

Cats. Experiments were performed in two anesthetized animals. The animal's head was positioned in a stereotaxic frame and the skull was exposed. A hole, 5 mm in diameter, was drilled in the skull overlying the right lateral ventricle to provide access for drug administration. R-MCH (Phoenix Pharmaceuticals Inc., FR-070-47; 1.2 μg/μl) was diluted in DMSO 20 % and distilled water 80 %. In these animals, intracerebroventricular (i.c.v.). microinjections of R-MCH (20 μl) were performed for a period of 3 min with a Hamilton microsyringe; the needle was left in position for 20 min in order to avoid leakage during the withdrawal procedure. Four hours after microinjection, the animals were euthanized with an overdose of sodium pentobarbital. Thereafter, the animals were perfused with fixatives, and the brain was frozen and serially sectioned in the coronal plane at 14 μm, and stored in the abovementioned conditions.

Rats. Two animals were anesthetized and then placed in a stereotaxic frame (for details, see [27]). A small hole was drilled in the skull in order to place the tip of a Hamilton microsyringe in the left lateral ventricle. The microinjection of R-MCH (1.2 μg/μl, 10 μl, i.c.v.) was performed for a period of 3 min; the syringe needle was left in position for 20 min. Two and four hours following R-MCH administration the rats were euthanized with an overdose of sodium pentobarbital and perfused with fixatives. Thereafter, the brain was frozen and serially sectioned in a coronal plane at 14 μm, and stored as previously commented. Control experiments were performed in two additional rats. In these animals, we microinjected rhodamine-B (0.1 mg/ml, 10 μl, i.c.v., Sigma-Aldrich) without MCH, and then we followed the same procedures as described before.

2.2. Immunofluorescence procedures

Single immunostaining procedures were performed in brain sections from the rats and cats. All antibodies and procedures were utilized in previous studies of our group [12,27–30]; in these reports the specificity of these antibodies was assessed by omission and/or pre-absorption tests. The procedures are concisely described below (for details, see [27,30]).

Cats. In order to recognize serotonergic neurons, sections were incubated with goat polyclonal anti-serotonin antibody (Incstar, Stillwater, MN; 1:10.000). Thereafter, they were incubated with a donkey anti-goat antibody conjugated with fluorescein isothiocianate (FITC, Jackson ImmunoResearch Laboratories Inc., 1:200).

Anti-GAD67 antibodies were used to detect GABAergic neurons. Sections were exposed to mouse anti-GAD67 serum (Chemicon International; 1:1000). Thereafter, the sections were incubated in donkey anti-mouse IgG conjugated with FITC.

MCH immunoreactivity was also examined; rabbit polyclonal anti-MCH antibody (Phoenix Pharmaceuticals; 1:2000) and donkey anti-rabbit IgG conjugated with FITC were employed.

Rat. Tissues from animals utilized in [30] were also analyzed (3 rats). In these tissues, double immunohistochemistry for serotonin and MCH fibers were performed. Free-floating brain sections were simultaneously incubated with a polyclonal rabbit anti-MCH (1:5000; Phoenix Pharmaceuticals Inc., USA) and a polyclonal goat anti-serotonin antibody (1:500; ImmunoStar, USA) during 48 h at 4 ˚C. After that, sections were simultaneously incubated with a donkey anti-rabbit IgG conjugated to Alexa Fluor-488 and donkey anti-goat conjugated to Cy3 (both diluted at 1:1500; Jackson ImmunoResearch, USA) for 1.5 h at room temperature.

Photomicrographs were obtained either by an epifluorescent microscope (Nikon Eclipse E600 FN) or a confocal laser-scanning microscope (Olympus FV300) and Fluoview 5 software (Olympus, Japan). Images were exported to Photoshop (Adobe Systems, USA) for contrast and brightness adjustments.

2.3. Unit recordings

Standard unit recording were performed in 43 urethane-anesthetized rats (1.5 g/kg, i.p.) following the same procedures as in [27]. Briefly, the rats were positioned in a stereotaxic frame and a small hole was drilled in the skull in order to place a recording electrode in the MnR. Electrodes were lowered at an angle of 20° to avoid the sagittal vein (AP, -8 mm; L, 2.6 mm; H, 7 − 9 mm; from Bregma) [31]. A guide cannula was also implanted into the lateral ventricle (AP, 1.0 mm; L, 2.0 mm; H, 3.4 mm) for microinjections of drugs.

Two screw electrodes were placed in the frontal and parietal cortices, and two twisted nichrome electrodes were placed in the dorsal hippocampus (coordinates: AP, -4.4 mm; L, -2.6 mm; H, 3.4 mm) to record the electrocorticogram (ECoG).

Extracellular recordings were carried out using standard procedures with glass micropipettes of 10 − 25 MΩ, filled with 2 M NaCl or with 2 % Neurobiotin (Nb, Vector Laboratories) in 0.5 M NaCl solution. Homemade double micropipettes were also used for recording and juxtacellular application of drugs. The drug-filled micropipette tip had a diameter of 5 − 20 μm and was separated from the recording electrode by 50 − 150 μm.

Neuronal signals were amplified by an AC-coupled amplifier (Dagan 2400A), filtered between 0.3 − 10 kHz and digitized at 20 kHz, 16 bits. Single unit activity in the MnR was acquired and processed by Spike 2 software (Cambridge Electronic Design, UK). In order to visualize the ECoG, the signal was amplified (×1000), filtered (0.1–100 Hz), acquired (512 Hz, 16 bits) and processed with Spike 2.

The baseline discharge of MnR neurons was recorded for at least 5 min before drug application. Then, 5 μl of MCH (1 μg/μl, Phoenix Pharmaceuticals, Inc., Burlingame, CA; #070-47) or saline (NaCl 0.9 %)

were microinjected into the lateral ventricle with a Hamilton microsyringe, which was connected to the injection cannula. Juxtacellular MCH (0.125 μg/μl or 0.25 μg/μl), dissolved in saline or ATC-0175 chlorhydrate (1 mM, a non-peptide MCHR-1 antagonist from Tocris Biocience, Mi, USA) dissolved in DMSO 0.1 %, were applied by pressure pulses of 5–20 PSI for 200–300 ms. DMSO 0.1 % or saline were used as control. The doses of MCH and ATC-0175 were similar than the dosage employed by Devera et al. (2015), and Urbanavicius et al. (2014), respectively.

The averaged waveforms of the action potentials (AP) were analyzed. The APs were mostly biphasic; the duration of the AP was considered as the sum of the duration of both phases [32]. The basal pattern of discharge was analyzed off-line by frequency, coefficient of variation as well as interval and autocorrelation histograms.

The mean firing rate is presented as the mean ± SEM. The effects of the drugs were evaluated by comparing the firing rate pre, and post drug administration in each neuron (windows of 1 − 5 min depending on the firing rate of the analyzed neurons), by means of Mann–Whitney test [27,33,34]. The means of the different groups were compared by Wilcoxon or Mann-Whitney tests (following Shapiro Wilks normality test). The chi square test was used to compare proportions between groups. The criterion chosen to discard the null hypothesis was $P < 0.05$.

In order to identify the recorded neuron, Nb was applied by iontophoresis (5 nA, 200 ms on/off for 3 min [27]). To identify Nb-labeled cells, 30 μm serial sections were cut along the pontomesencephalic tegmentum with a cryostat, incubated with Alexafluor 555-conjugated streptavidin (1:5000, Molecular Probes) and mounted on slides (using 70 % glycerol for coverslipping); the sections were analyzed by epifluorescence microscopy. In order to localize the tip of the recording micropipette (and then the recording site) in experiments without Nb labeling, at the end of each experiment the tip of the electrode was cut and left in the brain. The animals were perfused with 4 % PFA, the brain was removed, and the brainstem was cut in coronal sections of 150 μm with a vibratome.

3. Results

3.1. MCH-containing fibers in the MnR

MCH + fibers were detected both in the central and paracentral regions of the MnR of the rat (Fig. 1A). Double immunostaining for serotonin and MCH revealed that MCH + fibers were intermingled with serotonin + neurons in the central MnR region; some of them showed close proximity to serotonergic soma and dendrites (Fig. 1A and B). An example of MCH + varicosities in close apposition to a serotonergic soma is shown through orthogonal views of z-stacks images in Fig. 1C.

3.2. MCH conjugated with rhodamine was internalized by MnR neurons

Following i.c.v. administration of R-MCH in rats and cats, the fluorophore was detected within the soma of MnR neurons, both in the central and paracentral regions of the nucleus. Fig. 2 shows examples of R-MCH labeled neurons in the central MnR of the rat. An irregular grainy pattern of R-MCH labeling was mainly localized in the cytoplasm. No clear differences were observed between the rats euthanized one or four hours following i.c.v. microinjections, either in the quantity of MnR neurons that internalized R-MCH or in the degree of the fluorescence density (data not shown).

R-MCH was also incorporated into MnR neurons of the cat. Fig. 3 presents photomicrographs of a representative MnR coronal section showing R-MCH positive neurons (Fig. 3A, red), serotonin immunoreactivity (Fig. 3B, green), and a merge of both photomicrographs (Fig. 3C). It is interesting to note that some of the serotonergic neurons incorporated R-MCH (large arrows). Furthermore, non-serotonergic neurons within the MnR also internalized R-MCH (arrowheads). A topographic view of the central MnR showing R-MCH and serotonin-labeled neurons is exhibited in Fig. 3D; small arrows point to serotonergic neurons that did not incorporate R-MCH. Immunohistochemical studies for the detection of glutamate decarboxylase-67 (GAD67) were also carried out in cats. Because GAD accumulates principally in axon terminals, the administration of colchicine (that blocks axonal transport) is required to reveal the entire population of GAD-immunoreactive neurons [30]. However, even without this pre-treatment, we were able to observe a small number of GABAergic somas as well as GABAergic fibers in the MnR [27]. GABAergic neurons also

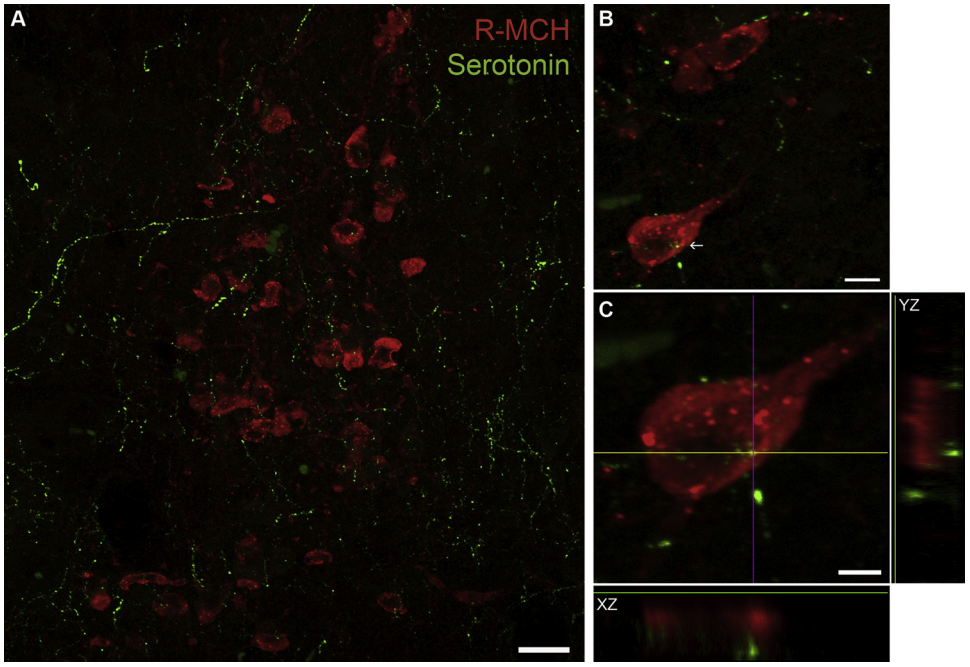

Fig. 1. Images of dual-immunostaining for serotonin and MCH in the MnR of the rat. Coronal sections were double labeled to visualize serotonin (red) and MCH-immunoreactivity (green). A. Photomicrograph exhibiting MCH + fibers intermingled with serotonin + neurons in the MnR (at Bregma -7.5 to -7.9 mm). Note that while the serotonergic neurons are present only in the midline region, MCHergic fibers reach both the median and paracentral (lateral) regions. B. At high magnification MCH + varicose fibers appear in close proximity to serotonergic neurons. C. Zoom of the neuron indicated by a small arrow in B. Orthogonal views (xz and yz) show apposition between MCH + fibers and serotonergic neurons. The image in C is comprised of 16 optical sections of 0.5 μm thickness. Calibration bars: A, 20 μm; B, 10 μm; C, 5 μm.

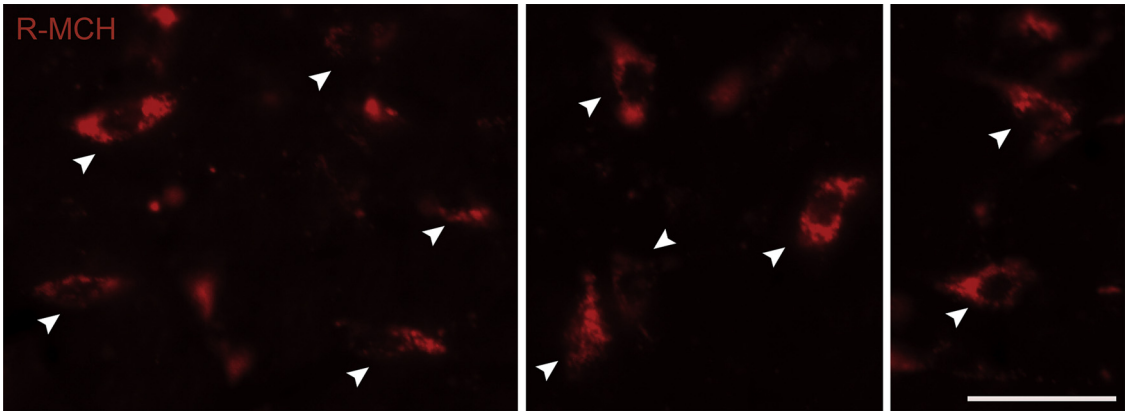

Fig. 2. MCH-rhodamine is internalized by MnR neurons in the rat. The photomicrographs illustrate rhodamine fluorescence within MnR neurons (examples are indicated by arrowheads). These neurons are located in the medial region of the MnR. Calibration bar: 50 μm.

incorporated R-MCH (two examples are shown in Fig. 4, arrows). A non-GAD immunoreactive neuron that incorporate R-MCH is also shown in Fig. 4A (arrowhead).

As it was previously shown for the DR [27], following R-MCH administration into the lateral ventricle, a subtle MCH immunoreactivity was evident in MnR neurons that internalized R-MCH. This fact is shown in Fig. 5, where weak immunoreactivity for MCH is readily observed in a representative MnR neuron that internalized R-MCH; this neuron is in close proximity to a varicose MCH-containing axon (Fig. 5 B and C, arrowheads). It is important to note that in previous studies we showed that MnR neurons do not show MCH immunoreactivity in control conditions (*i.e.* without R-MCH perfusion) [12,28,35]. We also performed control experiments by i.c.v. administration of rhodamine (without MCH). In contrast to the specific internalization observed for R-MCH, rhodamine staining was only present in the ependyma and associated with vessels (Supplementary Fig. 1).

3.3. Electrophysiological characteristics of the MnR neurons

Unit recordings were obtained from neurons that were confirmed to be located within the limits of the MnR of the rat, by Nb staining (Fig. 6A) or by means of the reconstruction of the micropipette tract (Fig. 6B). In some experiments the iontophoresis of Nb produced staining in two to three neurons (Fig. 6A, bottom); in these cases, although the recorded neuron was not identified, it was possible to recognize the recording site. Fig. 6C, shows the location of the recorded neurons, and a summary of the effect produced by the injection of MCH (i.c.v. and juxtacellular), MCH antagonist (juxtacellular) or vehicle (control; i.c.v. and juxtacellular effects of saline, as well as DMSO administration are shown in the same section).

The entire population of recorded neurons (n = 78) displayed the following electrophysiological characteristics. The action potential (AP) had an average duration of 2.3 ± 0.09 ms, the basal firing rate was 9.0 ± 1.2 Hz (0.2–57.5 Hz) and the coefficient of variation (CV) was 0.42 ± 0.04. No differences were observed in these parameters between neurons recorded in the central (n = 24) and paracentral (n = 54) regions of the MnR. 82 % (n = 64) of these neurons exhibited unimodal interval histograms (IH) and 44.8 % (n = 35) had a rhythmic pattern or a predominant interval in the autocorrelation histogram (ACH). A burst firing pattern, *i.e.* doublets or triplets with intervals < 20 ms with a diminution in the amplitude of lower order spikes, was observed in eighteen neurons (23 %).

A summary of the recorded neurons and its treatments is shown in Table 1. No significant differences were found in the duration of the AP, basal frequency or CV between the group of neurons treated with drugs (MCH and MCHR-1 antagonist; n = 61), and the vehicle-treated group

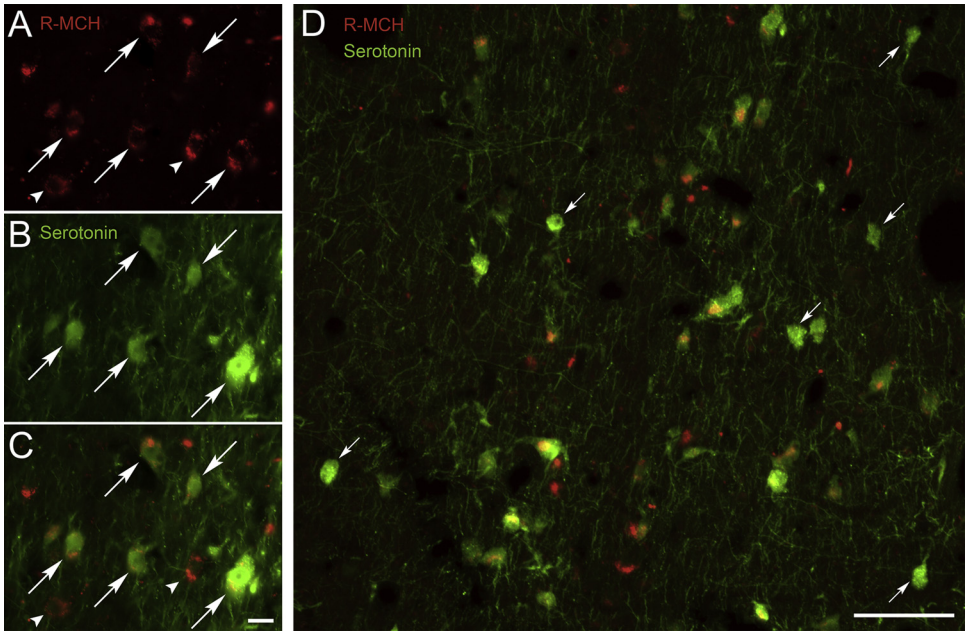

Fig. 3. MCH-rhodamine is internalized by serotonergic neurons. A. Photomicrograph showing that R-MCH (red) was internalized by MnR neurons of the cat. Photomicrograph in B (FITC, green labeling) depicts neurons with serotonin immunoreactivity. Superimposition of both photomicrographs is shown in C. It is readily observed that R-MCH is present in serotonergic (arrows) and non-serotonergic neurons (arrowheads). D. Topographic photomicrograph showing R-MCH and serotonin immunoreactivity in the median region of the MnR. R-MCH was internalized in serotonergic and non-serotonergic neurons. Interestingly, R-MCH was not present in some serotonergic neurons (small arrows). Calibration bars: A–C, 20 μm; D, 100 μm.

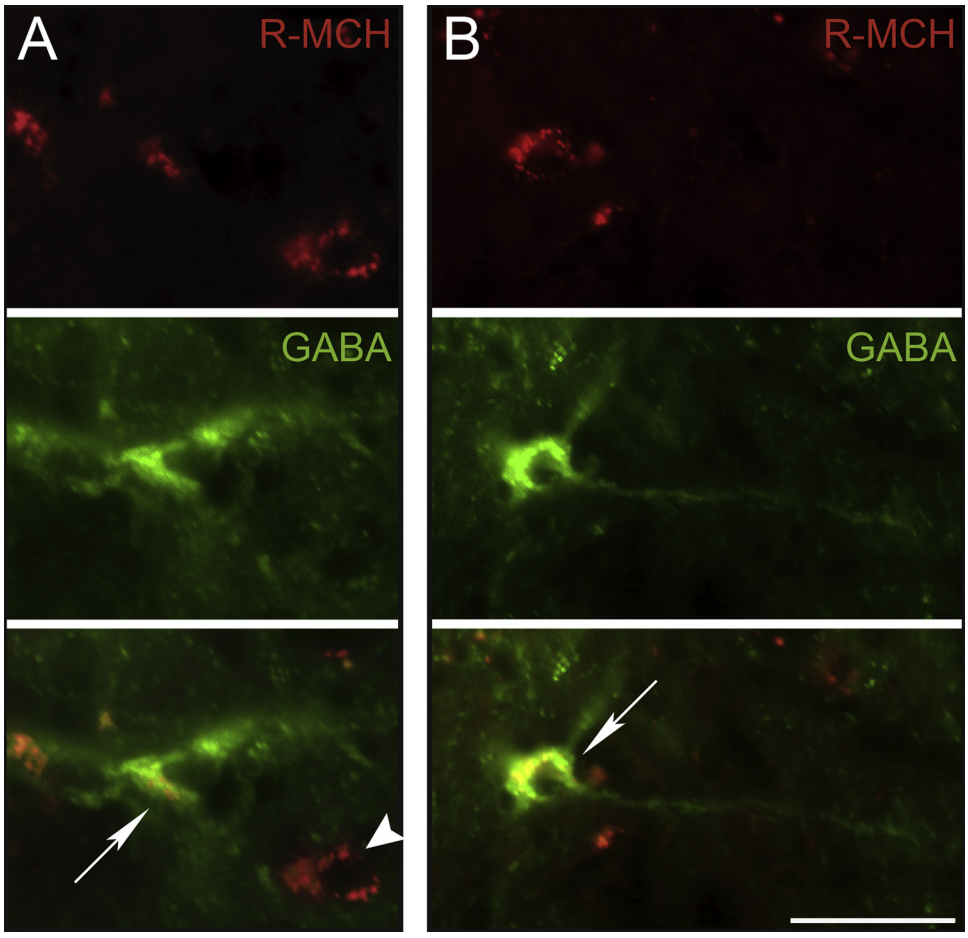

Fig. 4. MCH-rhodamine is internalized by GABAergic neurons of the MnR paracentral (lateral) region of the cat. In the examples depicted in A and B, the photomicrographs reveal that R-MCH (red, top) was internalized by MnR neurons. Middle. The photomicrographs show immunoreactivity for GAD67 (green). Both upper photomicrographs are merged in the photomicrographs of the bottom. Hence, R-MCH is present in GABAergic neurons (examples are indicated by arrows) of the MnR. In A, a non-GAD-immunoreactive neuron also internalized MCH-rhodamine (arrowhead). Calibration bar: 50 μm.

(saline + DMSO, n = 17; Mann Whitney).

3.4. Effects of i.c.v. administration of MCH

The effects of the administration of MCH (5 μg) into the lateral ventricle were studied. The i.c.v. infusion of MCH induced a significant decrease in the firing rate in 8 out of 15 (53 %) MnR neurons (Fig. 7A1). On average, MCH administration resulted in a decrease in the discharge rate of this group of neurons from 7.2 ± 1.9 Hz to 4.4 ± 1.9 Hz ($p = 0.012$, Wilcoxon, Fig. 7B1), with a latency of 157 ± 29 s; the effect lasted 390 ± 96 s. In four neurons, the reduction in the firing rate was followed by a small but clear rebound (significantly higher frequency compared to the basal recording). A significant increment in the firing rate was observed in only one neuron, while the rest of the recorded neurons (n = 6, 40 %) were not affected by the treatment. An example of the effects produced by the i.c.v. administration of MCH is shown in Fig. 8A. This neuron has a broad AP (3.4 ms), an irregular and slow basal firing rate (0.6 ± 0.3 Hz). The i.c.v. administration of MCH produced a decrease in the frequency of discharge, with a latency of ≈ 179 s and a duration of ≈ 800 s.

In contrast, the i.c.v. administration of saline did not modify the firing rate of the recorded neurons (n = 8). The frequency before and after saline administration was 7.6 ± 3.5 *vs.* 7.5 ± 3.3 Hz, respectively (Wilcoxon, $p = 0.61$, Fig. 7B1). An example of this lack of effect is shown in Supplementary Fig. 2A.

3.5. Effects of juxtacellular administration of MCH

Juxtacellular pressure injections of MCH 0.25 μg/μl were carried out in 27 neurons. In 18 neurons (67 %), MCH produced a significant decrease in firing rate, while it did not produce any effect in the rest of the neurons (n = 9). The neurons that decreased their frequency rate in response to MCH are shown in Fig. 7A2. On average, MCH decreased the firing rate in this group of neurons from 5.9 ± 1.2 Hz to 3.6 ± 0.9 Hz (Wilcoxon, $p = 0.0001$, Fig. 7B2), with an average latency of 29.1 ± 7.8 s; the diminution in the firing rate lasted 175.1 ± 50 s. A significant rebound in firing rate was observed in 8 neurons; two notable examples are shown in the Supplementary Fig. 3.

Fig. 8B presents a recording of a representative neuron with a basal firing rate of 3.8 ± 2.2 Hz and a rhythmic pattern in the ACH. It shows a bimodal IH with an AP duration of 1.9 ms. The juxtacellular administration of MCH produced a significant decrease in its discharge rate (0.7 ± 0.5 Hz, $p < 0.05$). The delay of the effect was about 6 s and its duration was approximately 130 s.

The effect of the repeated juxtacellular administration of MCH was consistent. An example is shown in Fig. 9. This neuron has a rhythmic and regular discharge pattern, with a unimodal IH and an AP with a duration of 2.2 msec. Following two successive microinjections of MCH, the frequency rate of this neuron decreased.

In order to explore a dose-response effect, a lower concentration of MCH was tested. MCH 0.125 μg/μl produced a decrease in the firing rate in 3 out of 10 neurons (30 %), while MCH 0.25 μg/μl reduced the firing rate of 67 % of the recorded neurons ($p = 0.046$, chi square test).

On the other hand, the juxtacellular administration of saline (n = 5) did not cause any effect on the firing rate of the recorded neurons ($p = 0.89$, Wilcoxon; Figure 7B2). An example is shown in Supplementary Fig. 2B.

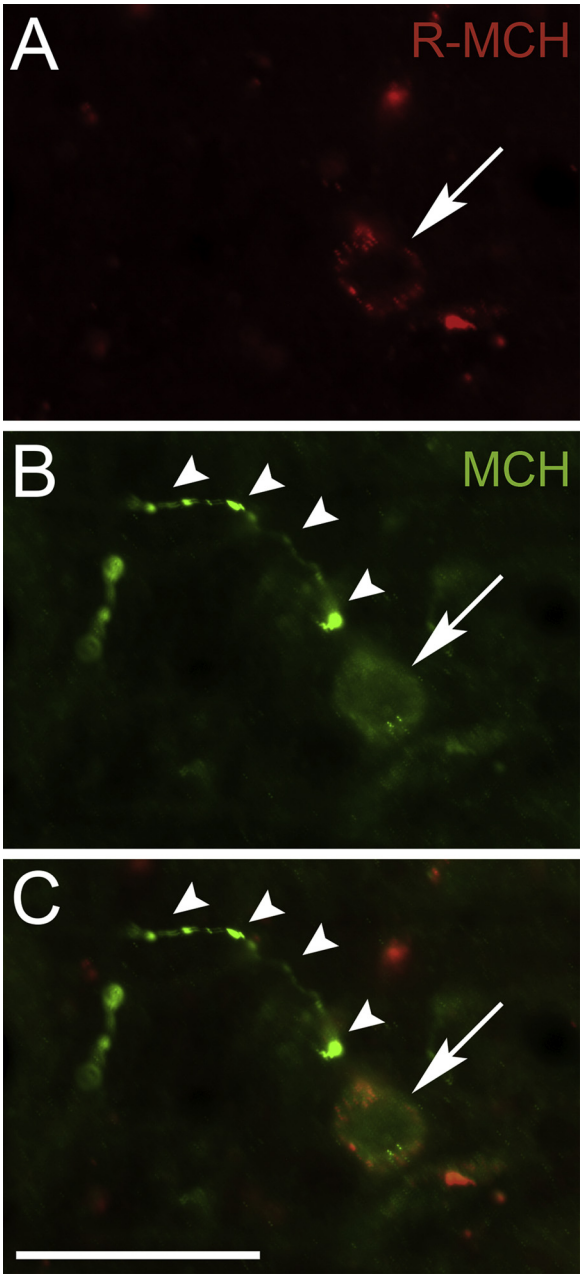

Fig. 5. MCH immunoreactivity is present in MnR neurons. In A, it is shown that R-MCH (red, arrow) is internalized by a MnR neuron. The photomicrograph in B (FITC-green labeling) depicts the same neuron presenting a subtle MCH immunoreactivity (arrow). A varicose MCHergic axon (arrowheads) is in close relationship with this neuron. In C, A and B photomicrographs were merged. Calibration bar: 50 μm.

3.6. Effects of MCH according to the firing profile of the MnR neurons

The effects of the i.c.v. plus juxtacellular administration of MCH 0.25 μg/μl were analyzed together, and related to the electrophysiological characteristics of the neurons (Table 2). The electrophysiological features described in the literature that characterizes serotonergic neurons are: broad action potentials (> 1.4 ms), slow (< 4.0 Hz), rhythmic and regular firing rate, as well as burst firing [1,32,36–38]. Of 26 neurons that were inhibited by MCH (applied either i.c.v. or juxtacellular), 23 of them had AP durations > 1.4 ms, 8 neurons exhibited a firing rate < 4 Hz, 13 neurons discharged rhythmically (or had a preferential interval) in the ACH and 4 presented a "burst" pattern of discharge. Seven neurons presented these three electrophysiological features; four of these seven neurons (57 %) were inhibited by MCH (Table 2).

There is an important relationship between the firing profile of MnR neurons and the hippocampal theta rhythm; presumed serotonergic neurons have a theta-off profile [9]. The relation with the hippocampal theta rhythm was studied in 7 neurons that were treated with MCH. Five of these neurons were theta-on and two neurons were theta-off (the firing pattern in relation to theta rhythm is shown in Supplementary Fig. 4). Four out of 5 theta-on neurons were inhibited with MCH and none of these two theta-off neurons were inhibited in response to the neuropeptide.

There were not significant differences in AP duration, basal frequency or CV between the neurons that were (n = 26) or were not (n = 16) inhibited by MCH (p = 0.22, 1 and 0.9, respectively; Mann-Whitney test). The percentage of neurons in the central and paracentral regions of the MnR that were inhibited by MCH were 71 and 66 %, respectively; there were not statistical differences in these proportions (p = 0.91, chi square test).

3.7. Effects of juxtacellular administration of MCHR-1 antagonist

The juxtacellular administration of the MCHR-1 antagonist (ATC-0175) caused an increase in the firing rate of 7 out of 9 neurons (78 %); the average firing of these 7 neurons is shown in Figure 7A3. ATC-0175 increased the frequency of discharge from 9.7 ± 3.3 Hz to 13.3 ± 3.8 Hz (Wilcoxon, p = 0.01, Figure 7B2). The latency of the effect was 24.5 ± 9.2 s and the duration 101.4 ± 17.9 s. A representative example of the effect of the antagonist is shown in Fig. 10. The administration of its vehicle (DMSO) did not modify the frequency of discharge of the tested neurons (n = 4).

4. Discussion

In the present study, we showed that MCHergic fibers reach the central and paracentral MnR and have close apposition with serotonin-containing neurons. Moreover, following the administration of R-MCH into the lateral ventricle of cats and rats, we were able to demonstrate its *in vivo* internalization into serotonergic and GABAergic neurons of the MnR; these results strongly suggest the presence of MCH receptors in these neurons. In addition, the i.c.v. and juxtacellular (0.25 μg/μl) administration of MCH produced a decrease in the firing rate of 53 and 67 % MnR neurons, respectively. Finally, the juxtacellular administration of a MCHR-1 antagonist produced the opposite effect. These anatomical and functional findings demonstrate that MCH regulates the activity of MnR neurons.

4.1. MCHergic fibers and receptors in the MnR

Previous studies established that MCH-containing neurons project toward the MnR [10,12,26]. In the present study, we demonstrated that MCH-containing fibers reach both the central region (where serotonergic neurons are mostly located) as well as the paracentral region where the majority of neurons are non-serotonergic [6,8]. In addition, we showed the existence of close apposition between MCH-containing varicosities and serotonergic neurons.

Hervieu et al. (2000) and Saito et al. (2001) studied the distribution of MCHR-1 mRNA in the CNS; MCHR-1 mRNA was detected in the DR but it was not mentioned its presence within the MnR [39,40]. Furthermore, by *in vivo* single cell transcriptomics, MCHR-1 mRNAs has been also found to be expressed in DR serotonergic neurons [41].

Hervieu et al. (2000) also described the presence of MCHR-1 immunoreactivity in the central region of the MnR. However, the study of the MCH receptor by immunohistochemistry in native tissues (*i.e.* CNS) has been problematic. In this regards, Chee et al. (2013) stated that: "Antibodies against the MCH receptor are not available despite efforts by both individual investigators and commercial companies to produce

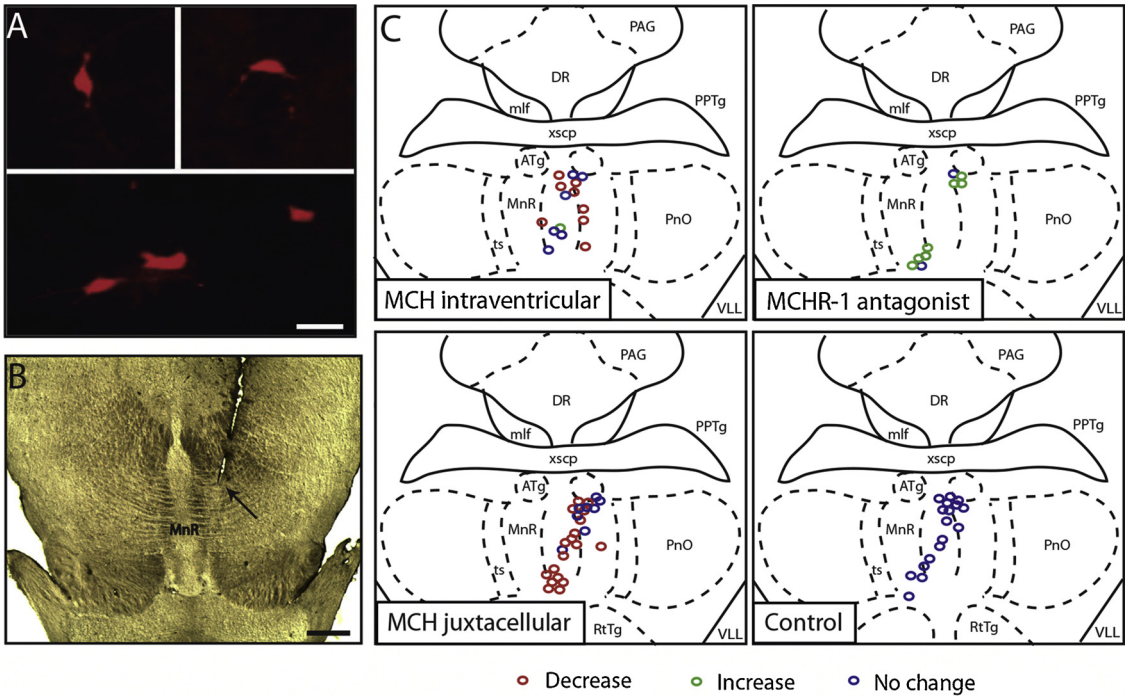

Fig. 6. Localization of the neurons recorded within the MnR. A. The photomicrographs show representative neurons labeled with Neurobiotin. B. Photomicrograph showing a representative example of the track left by the recording electrode (arrow). C. The localization of the recorded neurons following MCH (i.c.v. and juxtacellular 0.25 μg/μl), MCHR-1 antagonist (juxtacellular) or vehicle (saline or DMSO, Control) administration are shown. The recording sites were compacted in AP -7.8 mm section, according to Paxinos and Watson (2005). Abbreviations: ATg, anterior tegmental nucleus; DR, dorsal raphe nucleus; mlf, medial longitudinal fascicule; MnR, median raphe nucleus; PAG, periaqueductal grey; PnO, pontine reticular oral; PPTg, pedunculopontine tegmentum; RtTg, reticulotegmental; ts, tectospinal tract; VLL, ventral nucleus of the lateral lemniscus; xscp, superior cerebellar peduncle. Calibration bars: A, 20 μm; B, 1 mm.

Table 1
Summary of the recorded neurons and drug treatments.

Neuronal treatment	Number of neurons
All recorded neurons	78
MCH	52
MCH i.c.v.	15
MCH juxtacellular (0.25 μg/μl)	27
MCH juxtacellular (0.125 μg/μl)	10
MCHR-1 antagonist juxtacellular (ACT0175)	9
Control experiments	17
Saline i.c.v.	8
Saline juxtacellular	5
DMSO juxtacellular	4

them. Consequently, studies of MCH target neurons have been limited to analysis of MCHR1 mRNA distribution" [42]. However, it was recently demonstrated that MCHR-1 immunoreactivity is localized at primary cilia of CNS neurons in rats and mice [43,44]. Nevertheless, the neurochemical phenotype of MnR neurons that express MCHR-1 was not determined yet.

For that reason, in the present study we utilized R-MCH internalization as an indirect method to demonstrate the presence of MCH receptors in MnR neurons; a novel and useful technique that we have previously used with successful results [27]. We consider that the rhodamine fluorescent signal detected in the cytoplasm of MnR neurons corresponds to ligand-receptor complexes that were internalized after the i.c.v. administration of R-MCH, as was demonstrated *in vitro* by Saito et al. (2004) [45]. Serotonergic neurons and small-sized non-serotonergic neurons in the central and paracentral MnR internalized R-MCH both in rat and cats; some of these non-serotonergic neurons were recognized as GABAergic.

Supporting the fact that R-MCH internalization was receptor-dependent [27,46], we demonstrated that rhodamine (without MCH) does not internalize into neurons. In addition, preliminary results showed that R-MCH internalization was suppressed or reduced by phenylarsine oxide (PAO), an inhibitor of clathrin-mediated endocytosis, as well as by ATC-0175, the MCHR-1 antagonist used in this study [47]. Finally, as shown in the present study as well as by Devera et al. (2015), neuronal internalization of R-MCH was associated with the presence of a subtle MCH immunoreactivity in MnR neurons that previously were not described as MCHergic [12]. Hence, the preceding results strongly suggest that MCHR-1 is present in the MnR neurons of the rat; this is consistent with preliminary findings of our group that show that MCHR-1 immunoreactivity is present in the primary cilia of MnR neurons of the rat. However, new experiments are needed in order to know if one or both MCH receptors are involved in R-MCH internalization in MnR neurons of the cat.

4.2. MCH modulates the activity of MnR neurons

We recorded 78 MnR neurons; their basal electrophysiological characteristics are in accordance with previous studies [9,37,38,48]. As was observed in experiments performed in the DR [27], the MCH applied into the lateral ventricle of the rat reached MnR neurons after a latency of several seconds. MCH (i.c.v.) produced a clear decrease in the firing rate of 53 % of these neurons that lasted for several minutes. In addition to a direct effect of MCH on MnR neurons, extra-MnR circuits could also mediate this suppression of discharge. However, the juxtacellular application of MCH produced a local inhibitory (pre, and/or postsynaptic) response in 67 % of the recorded neurons (at the highest dose). The response was dose-dependent, stable and reproducible after repetitive administrations of MCH. The lack of effect of MCH on several recorded neurons agrees with the fact that some neurons within the MnR did not internalize R-MCH, and it is likely that these neurons do not express MCH receptors. This fact also suggests that the internalization of R-MCH is specific and receptor dependent.

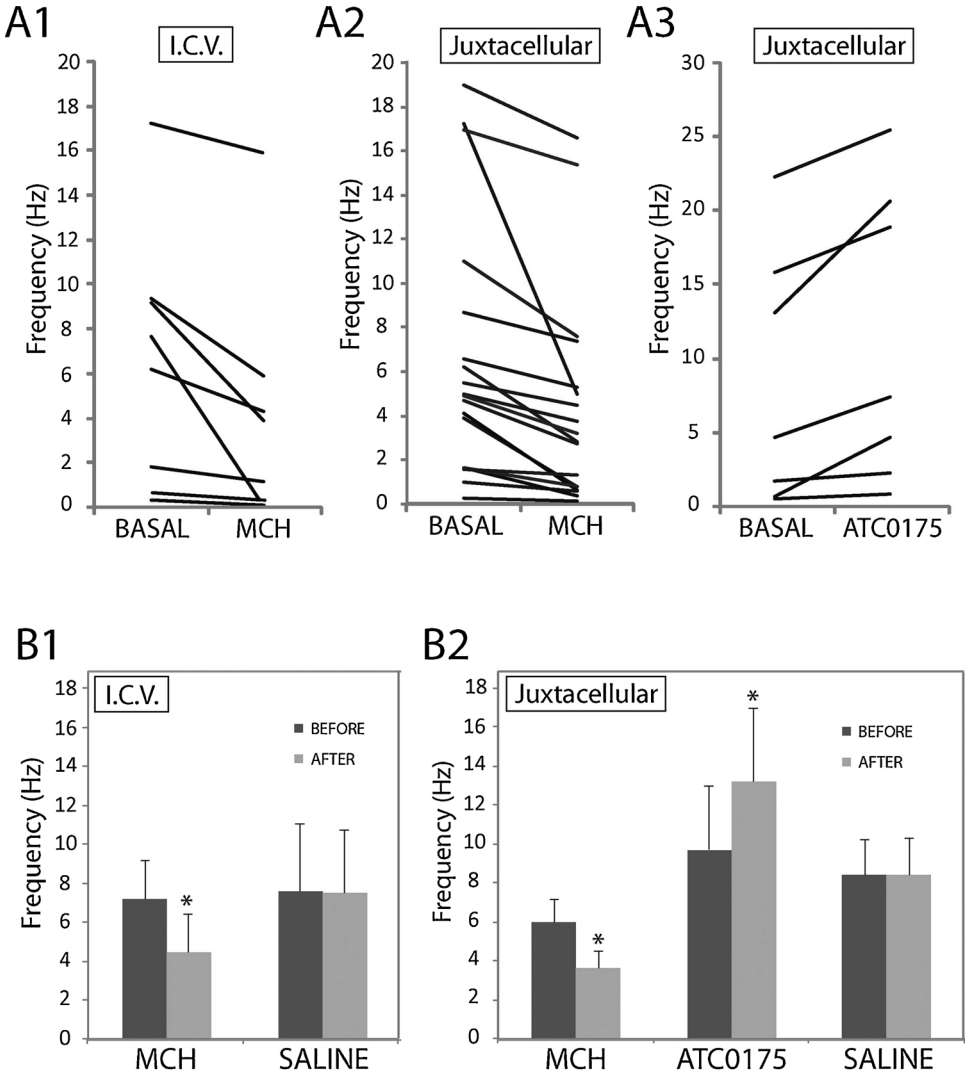

Fig. 7. Firing rate before (basal recording) and after the administration of MCH intra-cerebro-ventricular (i.c.v., A1), MCH juxtacellular (A2) or MCHR-1 antagonist juxtacellular (ATC-0175, A3). Only neurons that had a significant inhibitory (for A1 and A2) or excitatory (A3) effect are shown. The statistical significance for each neuron was assessed by the Mann-Whitney test. B1 shows the population mean and SEM of the firing rate before and after the i.c.v. administration of MCH and saline. B2 shows the same as B1 before and after the juxtacellular administration of MCH (0.25 μg/μl), ATC0175 and saline. *, $p < 0.05$, Mann Whitney test.

In some MnR neurons, as in some DR neurons [27], MCH induced-suppression of discharge was frequently followed by an increase in the firing rate or rebound (Supplementary Fig. 3). This effect could be produced by inwardly rectifying currents (which are present in serotonergic neurons) that resulted in a post-inhibitory rebound [49]; however, a circuit mechanism is also possible.

The effect produced by the juxtacellular administration of the MCHR-1 antagonist, implies specificity in the response produced by MCH. This result also implies that endogenous MCH exerts a tonic inhibitory effect. In this regards, previous studies of our group found that immunoneutralization of MCH in the DR of the rat, decreased the immobility time in the forced swimming test, which is considered an antidepressant effect (*vide infra*); this result was explained by the neutralization of endogenous MCH. The immunoneutralization of MCH in the DR also decreases REM sleep time [50].

As a control procedure, we performed the i.c.v. or juxtacellular administration of the vehicle for either MCH (saline) or the antagonist (DMSO). The lack of effect of both solutions (tested in 17 neurons, Table 1), also suggests the specificity of action of MCH, and discard a mechanical artefactual effect. The long latency of the firing suppression induced by MCH (expected by a neuromodulator), also reject a mechanical artefactual cause.

4.3. Effects of MCH on serotonergic and non-serotonergic neurons

As previously mentioned, MCH-containing fibers reach the central region of the MnR where most serotonergic neurons are located, as well as the paracentral MnR where there are a smaller number of serotonergic neurons [6,8]. In agreement with these results, R-MCH was internalized both in serotonergic and non-serotonergic neurons (some of them were identified as GABAergic). Hence, an effect of MCH on both serotonergic and non-serotonergic neurons was expected.

It is highly probable that MCH decreased the firing rate of MnR serotonergic neurons. This hypothesis is supported by the following evidences: a. R-MCH internalization strongly suggest that MCH receptors are present in the serotonergic neurons. b. MCH reduced the firing rate of neurons in the central region of the MnR (where serotonergic neurons are mostly located). c. A population of neurons with electrophysiological characteristics of serotonergic neurons (described in the Results section) decreased its firing rate after MCH administration. It is appropriate to consider that this classification underestimate (conservative approach) the number of serotonergic neurons, considering that neurons with atypical firing rates could also be serotonergic [38].

Neurons from the paracentral MnR region, as well as neurons

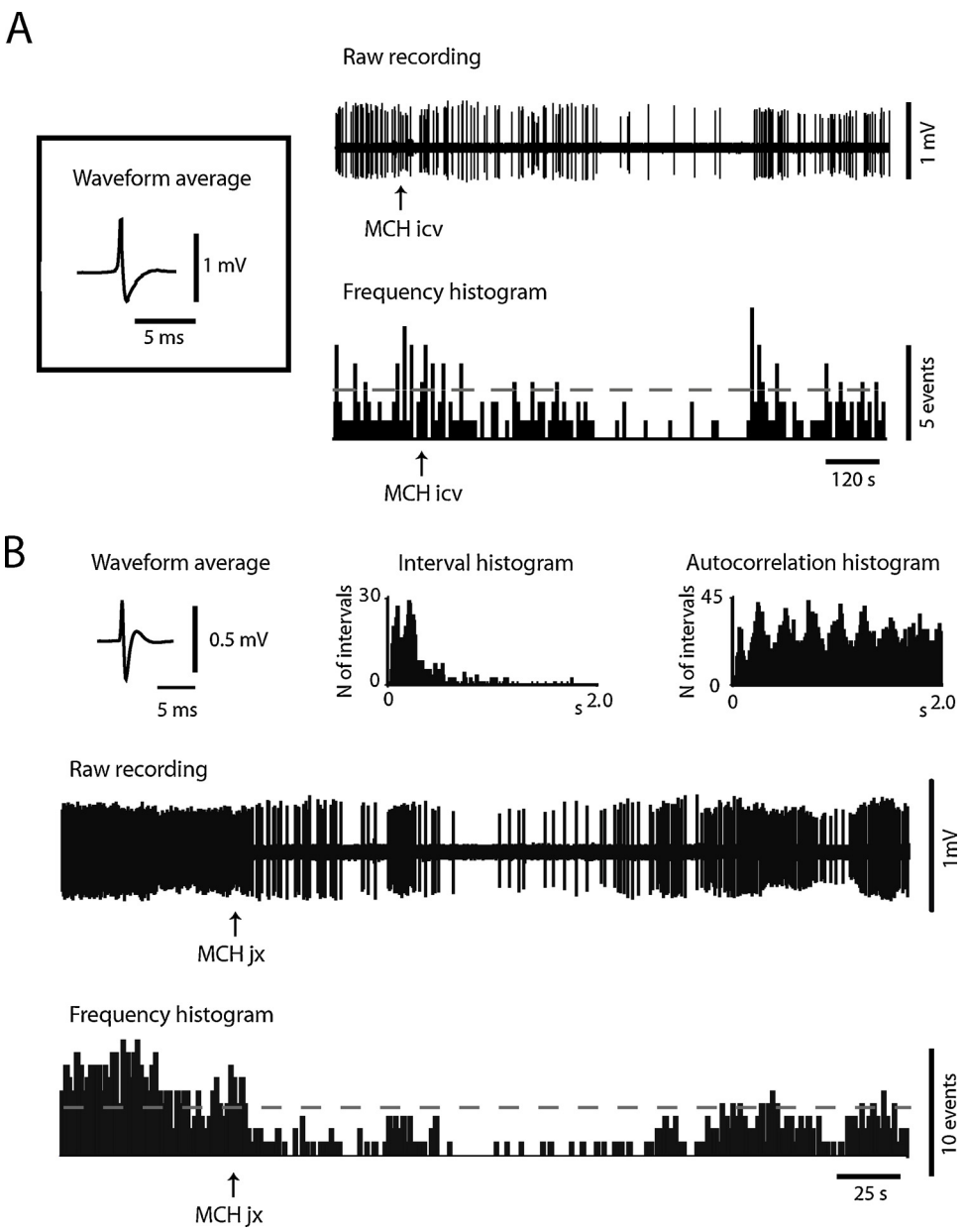

Fig. 8. A. Intracerebroventricular (i.c.v.) administration of MCH reduces the activity of a representative MnR neuron. The raw recording and the frequency histogram are shown. Following i.c.v. administration of MCH there is a decrease in the firing rate. The action potential average of the neuron is shown in the box. The basal mean firing rate is marked by a dashed line. B. Juxtacellular (jx) administration of MCH decreases the firing rate of a representative MnR neuron. The action potential average, interval and autocorrelation histogram are presented. The effect of the juxtacellular administration of MCH is shown in the raw recording and the mean frequency histogram. The basal mean firing rate is marked by a dashed line.

without the classic serotonergic firing profile, were also inhibited by MCH; these neurons could be either GABAergic or glutamatergic [6,8]. Additionally, MCH also reduced the activity of theta-on neurons that were probably non-serotonergic [9]. In fact, R-MCH was internalized in small-sized non-serotonergic neurons; some of them were GABAergic both in the MnR (present study) as well as in the DR [27].

Since MCH is an inhibitory neuromodulator, we expected a direct (postsynaptic) inhibitory action of MCH on MnR neurons; however, an excitatory effect was observed in one neuron. This neuron had an AP of short duration (< 1.4 ms) and a fast (30 Hz) irregular firing pattern; hence it is likely non-serotonergic [36,37]. Because MCH decreases the release of GABA in the hypothalamus [24,25], a presynaptic inhibitory effect on GABAergic neurons produced by MCH could explain the fact that MCH increased the firing rate of this neuron (disinhibition effect). Interestingly, in addition to the presence of R-MCH on the soma of MnR neurons, punctuate marks of R-MCH were observed throughout the MnR, suggesting the presence of internalized R-MCH into presynaptic structures. Further experiments are needed to understand the fine-tuning of MCH regulatory actions with respect to neuronal networks within the MnR.

It is important to note that the electrophysiological criteria used to identify presumed serotonergic and non-serotonergic neurons are appropriate as a first approach for classifying raphe neurons; however, it fails to identify subpopulations with atypical discharge profiles [6,38,51–53]. Thus, it would be important to study the effect of MCH on serotonergic, GABAergic and glutamatergic MnR neurons whose neurochemical phenotype were confirmed by immunohistochemistry.

4.4. MCH suppress the activity of dorsal and median raphe nuclei

MCH produces a decrease in the firing rate in both MnR (present study) and DR neurons [27]. The same doses of MCH (juxtacellular and i.c.v.) decreased the activity of 62 % MnR neurons, and 57 % (4/7) of the neurons with serotonergic electrophysiological patterns (Table 2). MCH decreased the firing rate of 70 % DR neurons, and from 80 % (8/10) of presumed serotonergic neurons. The effect of MCH perfusion into the DR was also analyzed by intra-DR microdialysis with quantification of serotonin levels; perfusion of a low dose of MCH into the DR decreases serotonin release [30]. Hence, these data suggest that MCH restricts serotonergic activity in both mesopontine raphe nuclei.

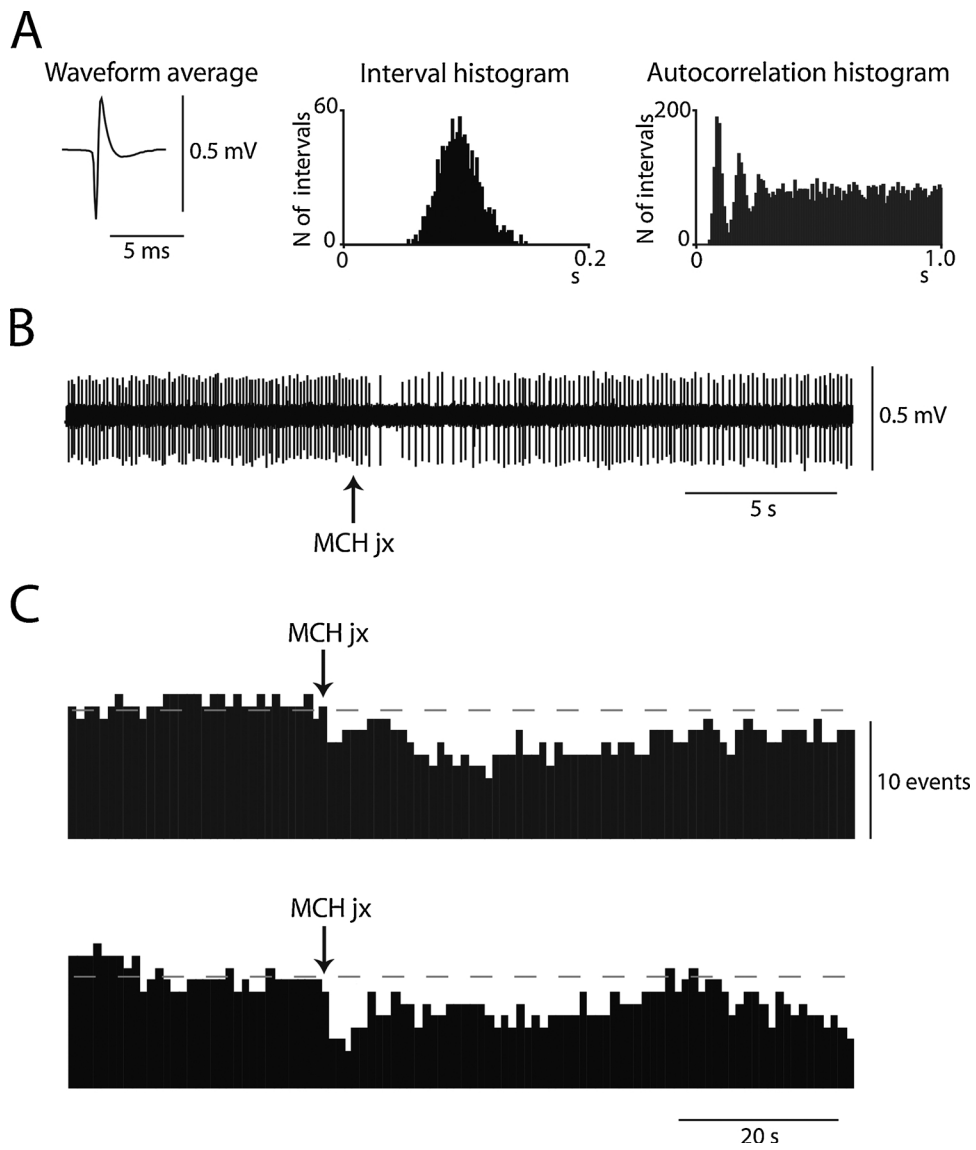

Fig. 9. Effect of the repeated juxtacellular administration of MCH. A. The action potential average, the interval and the autocorrelation histograms are shown. B. Raw recording of the neuron shows that the firing rate decreases after the administration of MCH. C. The frequency histograms before and following the juxtacellular (jx) administration of MCH is shown. MCH was injected several times in this neuron, with similar results; the Figure shows two examples. The arrow indicates the administration of MCH.

Table 2
Electrophysiological characteristics of the neurons inhibited by MCH (intracerebroventricular plus juxtacellular 0.25 μg/μl).

Eletrophysiological characteristics	Number of neurons	Number of neurons inhibited by MCH (%)
All recorded neurons	42	26 (62)
Action potential > 1.4 ms	39	23 (59)
Firing rate < 4 Hz	18	8 (44)
Rhythmical pattern in the ACH	16	13 (81)
Burst discharge	7	4 (57)
Three or more features	7	4 (57)

ACH, autocorrelation histogram.

4.5. Biological significance of the effects of MCH in the mesopontine raphe nuclei

MnR and DR neuronal projections are different; while there is an important DR projection directed toward the prefrontal cortex, the MnR projects more densely to medial structures including the septum and hippocampus [6,54,55]. Most neurons of the DR have been classically described as 'clock-like' neurons (alluding to the striking regularity in its activity pattern), and display slow firing rate (1–4 Hz) and broad action potentials [32,37]. On the contrary, the MnR displays a more diverse neuronal pattern of activity [6,9,38]. An important fact is that MnR neurons, but not DR neurons, control hippocampal activity [7]; activation or suppression of MnR desynchronize or synchronize, respectively, the hippocampal theta rhythm [56]. From the behavioral point of view, it has been proposed that DR neurons would be involved in stress/conflict anxiety-related processes relevant for anxiety and mood disorders, while MnR neurons would be related to tolerance and coping with aversive stimuli, which could be important for mood disorders like depression [57]. All these structural and functional differences suggest that the functional impact of MCH applied on each raphe nuclei should be different.

In this regards, microinjections of MCH into the DR of the rat, induce a pro-depressive behavior in the forced swimming test [29,58]. This effect is blocked either by the intra-DR administration of MCH antagonists or by systemic pretreatment either with serotonergic

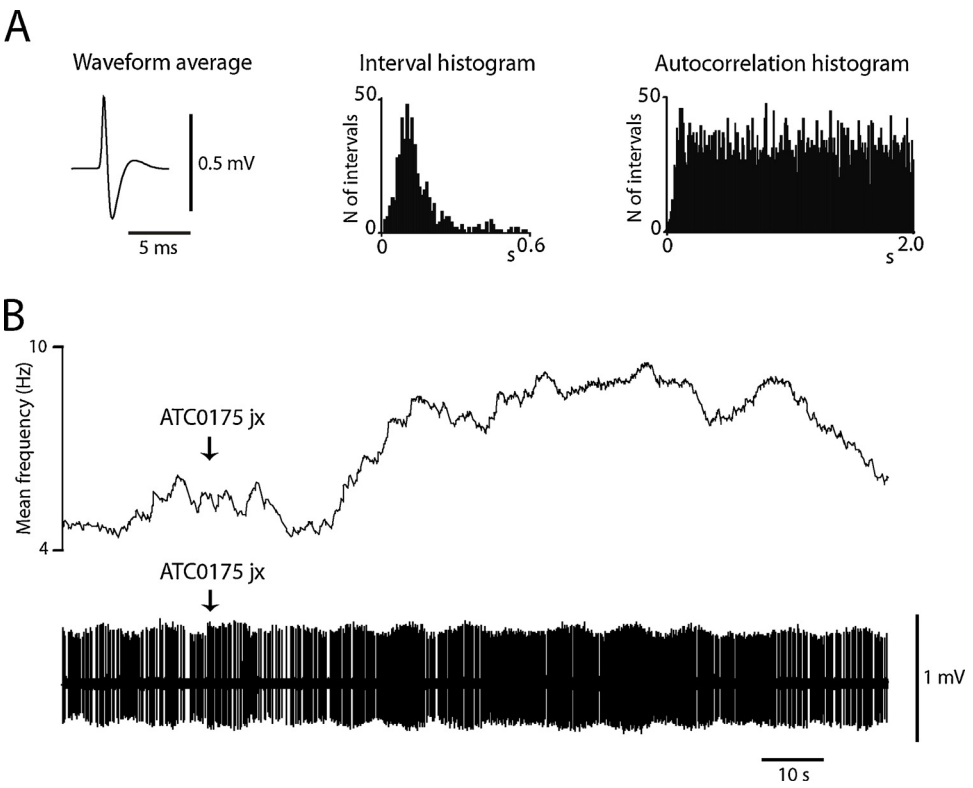

Fig. 10. Juxtacellular administration of the MCHR-1 antagonist (ATC0175) increases the activity of a representative MnR neuron. A. The action potential average, a discharge burst, as well as the interval and the autocorrelation histogram are shown. B. The mean frequency and raw recording are exhibited before and following the pressure injection of the drug. Note that ATC0175 increases the firing rate of this neuron. The arrows indicate the juxtacellular (jx) administration of the drug.

(fluoxetine) or noradrenergic (nortriptyline) antidepressants. In addition, intra-DR immunoneutralization of MCH induces an antidepressant effect [29]. MCH microinjected into the MnR also produced a pro-depressive effect [26]; however, while a pro-depressive effect was induced by 50 ng of MCH in the DR, 100 ng was needed to induce this effect in the MnR. It is likely that the abovementioned subtle differences in the effects produced by MCH on unit firing (MCH produced an inhibition in a larger percentage of neurons in the DR), may account for the distinct effectiveness in these behavioral experiments.

The i.c.v. administration of MCH promotes REM sleep generation [59]. Accordingly, intra-DR microinjections of MCH also induces REM sleep, while intra-DR immunoneutralization of this neuropeptide suppresses it [50,60]. Preliminary results also show that MCH microinjections into the MnR promotes REM sleep (Pascovich et al., unpublished); however, MCH applied into the DR produced a lower but significant increase in NREM sleep time, that was not observed when microinjected into the MnR.

The pro-depressive and REM sleep promoting effect of MCH applied into the mesopontine raphe nuclei is likely produced by the reduction of DR [27] or MnR (the present study) serotonergic activity. Regarding REM sleep, serotonergic neurons of the MnR and DR are part of the reticular mesopontine formation, which is "necessary and sufficient" for the generation and maintenance of REM sleep [61]. These neurons exhibit a tonic discharge pattern during wakefulness, decrease their activity during non-REM sleep, and are almost inactive during REM sleep [62]. Since MCHergic neurons are active during REM sleep [63], and the microinjection of MCH into the DR promotes REM sleep [60], we hypothesized that by suppressing serotonergic activity MCH contributes to the generation of REM sleep.

The MnR projects to several regions that play critical roles in the generation of REM sleep and in the control of mood, such as the mesopontine and basal forebrain cholinergic regions, perifornical hypothalamus, medial and intralaminar thalamus and hippocampus; these projections complement the ones from the DR [4,64]. Therefore, in order to control REM sleep and mood, the MCHergic system may modulate, in tandem, the serotonergic (and non-serotonergic) neurons of the MnR and DR. We also hypothesize that the effects of MCH within these raphe nuclei may explain, in part, the tight link between REM sleep and depression [15,20,65].

5. Conclusions

Our anatomical and functional studies demonstrated that the MCHergic system regulates the activity of MnR neurons; the main effect of MCH is to reduce neuronal firing rate, including the activity of presumed serotonergic neurons. This inhibitory effect may contribute to the generation and maintenance of REM sleep. Moreover, an imbalance in this effect may be involved in the physiopathology of depression.

Conflicts of interest

None.

Authors contributions

Financial support. PT, PL, CS
Experimental design. PT, PL, CS
Experimental procedures. CP, AD, MR, PL, JU, PT
Analysis of the data. CP, PL, JU, CS, PT
Discussion and interpretation of the data. CP, PL, JU, AF, AC, XL, CS, PT
Wrote the manuscript. CP, PT
All the authors participated in critical revision the manuscript, added important intellectual content, and approved the final version.

Acknowledgments

This work is dedicated to the memory of our dear mentor Dr. Michael Chase, that regretfully passed away last year. Dr. Chase collaborated with us at the beginning of this work. The study was supported by grants from the Agencia Nacional de Investigación e Innovación (ANII) FCE-1-2011-1-5997, CSIC-Universidad de la

República and Programa de Desarrollo de Ciencias Básicas (PEDECIBA), Uruguay.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.peptides.2019.170249.

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