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Melanin-concentrating hormone does not modulate serotonin release in primary cultures of fetal raphe nucleus neurons

Eugenia Saiz-Bianco[a], Jessika Urbanavicius[b], Giselle Prunell[c,*], Patricia Lagos[a,**]

[a] Departamento de Fisiología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay
[b] Departamento de Neurofarmacología Experimental, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay
[c] Departamento de Neuroquímica, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

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ABSTRACT

Melanin-concentrating hormone (MCH) is a neuropeptide present in neurons located in the hypothalamus that densely innervate serotonergic cells in the dorsal raphe nucleus (DRN). MCH administration into the DRN induces a depressive-like effect through a serotonergic mechanism. To further understand the interaction between MCH and serotonin, we used primary cultured serotonergic neurons to evaluate the effect of MCH on serotonergic release and metabolism by HPLC-ED measurement of serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) levels. We confirmed the presence of serotonergic neurons in the E14 rat rhombencephalon by immunohistochemistry and showed for the first time evidence of MCHergic fibers reaching the area. Cultures obtained from rhombencephalic tissue presented 2.2 ± 0.7% of serotonergic and 48.9 ± 5.4% of GABAergic neurons. Despite the low concentration of serotonergic neurons, we were able to measure basal cellular and extracellular levels of 5-HT and 5-HIAA without the addition of any serotonergic-enhancer drug. As expected, 5-HT release was calcium-dependent and induced by depolarization. 5-HT extracellular levels were significantly increased by incubation with serotonin reuptake inhibitors (citalopram and nortriptyline) and a monoamine-oxidase inhibitor (clorgyline), and were not significantly modified by a 5-HT1A autoreceptor agonist (8-OHDPAT). Even though serotonergic cells responded as expected to these pharmacological treatments, MCH did not induce significant modifications of 5-HT and 5-HIAA extracellular levels in the cultures. Despite this unexpected result, we consider that assessment of 5-HT and 5-HIAA levels in primary serotonergic cultures may be an adequate approach to study the effect of other drugs and modulators on serotonin release, uptake and turnover.

1. Introduction

Melanin-concentrating hormone (MCH) is a 19-amino acid inhibitory neuropeptide that activates two G-protein coupled specific receptors: MCHR-1 and MCHR-2, MCHR-1 being the only functional receptor in rodents (Shi, 2004; Saito et al., 1999, 2004). MCHergic neurons are located in the hypothalamus and extensively innervate several structures of the CNS, including the dorsal raphe nucleus (DRN), the area in the brain where the greater number of serotonergic neurons are concentrated (Bittencourt et al., 1992; Bittencourt and Diniz, 2018; Jacobs and Azmitia, 1992; Fu et al., 2010; Urbanavicius et al., 2016). A moderate expression of MCHR-1 mRNA in the rat DRN has been described in the literature (Hervieu et al., 2000; Saito et al., 2001). In our previous studies, we demonstrated that local MCH microinjection into the DRN of adult rats induced a depressive-like effect that was MCHR-1-dependent (Lagos et al., 2011b; Urbanavicius et al., 2014). This effect was partially prevented by the classical antidepressant fluoxetine, a selective serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibitor (SSRI), suggesting that MCH behavioral effect may be mediated by 5-HT (Lagos et al., 2011b). Moreover, local MCH microinjection into the DRN also induced an increase in the time the rats spend in the REM state of the sleep-wake cycle –an alteration observed in depressive patients, whereas immunoneutralization (microinjection of anti-MCH antibodies) induced the opposite effect (Lagos et al., 2009, 2011a). Furthermore, the infusion of a low concentration of MCH in the DRN induced a decrease in 5-HT release in this region as assessed by microdialysis (Urbanavicius et al., 2016). Our team also demonstrated that intracerebroventricular (i.c.v.) or local administration of MCH into

* Correspondence to: G. Prunell, Departamento de Neuroquímica, Instituto de Investigaciones Biológicas Clemente Estable, Avenida Italia 3318, 11600 Montevideo, Uruguay.
** Correspondence to: P. Lagos, Departamento de Fisiología, Facultad de Medicina, Universidad de la República, General Flores 2125, 11800 Montevideo, Uruguay.
E-mail addresses: gisellefprunell@gmail.com (G. Prunell), plagos@fmed.edu.uy (P. Lagos).

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the DRN inhibited the discharge of neurons present in this area, which were probably serotonergic given their typical electrophysiological characteristics (Devera et al., 2015). Finally, we showed that after i.c.v. administration of MCH conjugated with rhodamine (R-MCH), serotonergic neurons become rhodamine-positive, probably as a result of R-MCH interaction with MCHR-1 present in these neurons followed by the internalization of the complex R-MCH-MCHR-1 (Devera et al., 2015). Taken together, all the evidence suggests that MCH may modulate serotonergic neurons in the adult DRN and that a serotonergic mechanism may underlie its depressive-like effects.

Dysfunction of 5-HT neurotransmission is classically associated with the etiology of depression and is one of the main targets of conventional antidepressant therapies, including SSRIs or monoamino oxidase inhibitors (MAOI), MAO being the enzyme involved in the degradation process of 5-HT (WHO, 2013; Artigas et al., 2018). These drugs induce an increase in 5-HT extracellular levels (Belmaker and Agam, 2008; Yohn et al., 2017). Despite its high prevalence, current treatments for depression still have various shortcomings such as delayed onset of action and limited efficacy of the drugs (El-Hage et al., 2013; Outhred et al., 2013; Artigas et al., 2018). Understanding the modulatory influences of different neurotransmitter systems on the serotonergic system could help identify new molecular targets that could in turn be used to design more effective antidepressant therapies. A wealth of data points to neuropeptides as one of these alternative targets (Chaki et al., 2006; Mathé et al., 2007; Alldredge, 2010; Werner and Coveñas, 2013; Kormos and Gaszner, 2013). There is considerable evidence, including data from our group, indicating that the MCH system may be involved in depressive states (Borowsky et al., 2002; Chaki et al., 2005; Chaki, 2018; Lagos et al., 2011b; Urbanavicius et al., 2014, 2016). Moreover, MCHR-1 antagonists have been proposed as a possible effective antidepressant treatment (Borowsky et al., 2002; Georgescu et al., 2005; Chaki, 2018).

To further explore the proposed modulation of MCH on serotonergic neurons, we considered that an in vitro model of cultured serotonergic neurons would be an adequate and convenient approach. In particular, primary cell cultures from the raphe nuclei at E14 have been reported to contain serotonergic neurons with specific in vivo features: 5-HT synthesis from its precursor tryptophan, reuptake process mediated by SERT, vesicle storage and calcium-dependent release, as well as functional auto-receptors (5-HT1A) (Becquet et al., 1991, 1993a, 1993b; Héry et al., 1999; Lautenschlager et al., 2000). Published reports on the serotonergic metabolism in such neurons have measured [^{3}H]-5-HT extracellular levels under different conditions (Yamamoto et al., 1981; Becquet et al., 1991; Becquet et al., 1993a; Becquet et al., 1993b; Wichems et al., 1995; Héry et al., 1999; Lautenschlager et al., 2000; Birthelmer et al., 2007). Although this approach is highly sensitive, it involves the risks and challenges inherent to working with radioactive reagents. Besides they only evaluated 5-HT release after the addition of exogenous tryptophan, SSRI or MAOI, which affect the endogenous physiology of neurons (Yamamoto et al., 1981; Becquet et al., 1991; Héry et al., 1999). Another highly sensitive analytical method to measure 5-HT levels and its main metabolite −5-hydroxyindolacetic acid (5-HIAA)– is high-performance liquid chromatography with electrochemical detection (*HPLC-ED)*. This method avoids the potential hazards of working with radioactivity and has been successfully used to measure 5-HT and 5-HIAA intracellular content in primary cell cultures (Paolillo et al., 1993). HPLC-ED has also been successfully used to measure 5-HT and 5-HIAA extracellular levels under different pharmacological treatments in organotypic slices containing DRN (Higuchi et al., 2008; Nagayasu et al., 2010a; Nagayasu et al., 2010b). There are, however, no published reports on the use of HPLC-ED to assess endogenous and induced 5-HT and 5-HIAA release levels in primary cultures. We propose that this could be an adequate method to assess the mechanisms underlying MCH modulation on serotonergic neurons in vitro.

In the present study, we investigated the interaction of MCH with primary serotonergic neurons obtained from the rhombencephalon of E14 embryos using a morphological and neurochemical approach. To this end, we first determined the presence of serotonergic and MCHergic neurons and fibers in the embryos. Thereafter, we performed a phenotypic characterization of the primary cultures obtained from this area, identifying and quantifying serotonergic and GABAergic neurons. We also evaluated whether HPLC measurement of 5-HT and 5-HIAA levels was an adequate method to assess modulation of the system through different pharmacological treatments known to affect serotonergic transmission. Finally, we studied MCH effect on serotonergic metabolism in our in vitro system.

2. Materials and methods

2.1. Animals

The experiments were performed using Sprague-Dawley rat embryos on gestation day 14 (E14) obtained from the IIBCE's animal facility. All experimental procedures involving animals were in accordance with IIBCE Bioethics Committee's requirements and Uruguayan law on animal experimentation (Law N° 18.611).

2.2. Immunohistochemistry of E14 entire embryos and rhombencephalon whole mounts

E14 entire embryos were fixed by immersion in 4% paraformaldehyde (PFA, overnight, 4 °C) and then cryoprotected in 30% sucrose solution (in PBS 0.1 M) for 48 h (4 °C). Afterward, they were cut along the sagittal plane (30 μm) by a cryostat (Leica CM 1900, Nussloch, Germany) and selected sections were mounted on Pro-plus slides to perform immunohistochemistry (Paxinos et al., 1990). After blocking endogenous peroxidase activity with H_2O_2 1% (60 min) and non-specific binding with normal donkey serum (NDS) 3% (60 min), the sections were incubated overnight at room temperature (RT) with the primary antibodies rabbit anti-5-HT (1:5000, ImmunoStar, Hudson, WI, USA; # 20080) or rabbit anti-5-HT transporter (SERT) (1:5000, ImmunoStar, # 24330) or rabbit anti-MCH (1:1000, Phoenix Pharmaceuticals Inc., # H-070-47) in a humidified chamber. Following several washes in PBS 0.1 M, the sections were incubated for 90 min with a biotinylated secondary antibody (1:600, donkey anti-rabbit, Jackson ImmunoResearch, West Grove, USA, # 711-065-152) and then with ABC kit (1:300, Vector Laboratories Inc., Burlingame, CA, USA, # SK-6100) for 60 min. A positive reaction was obtained by incubation with DAB kit (Vector Laboratories Inc., # SK-4100) for 10 min. Omission of primary antibodies was used as a negative control of the reaction. Images were obtained with an Olympus IX81 microscope and a DP72 camera, and processed with Photoshop and Acrobat Illustrator softwares.

Whole mounts from E14 rhombencephalon as obtained for primary cultures (see below) were processed by fixation on 4% PFA (60 min, 4 °C) followed by several washes in PBS 0.1 M, blocking with NDS 3% (60 min) and incubation for 48 hs at 4 °C with goat anti-5-HT antibodies (1:5000, Immunostar, # 20079). They were then incubated for 90 min (RT) with Cy3-conjugated donkey anti-goat antibody (1:800, Jackson ImmunoResearch, # 705-165-147), washed in PBS and mounted on slides with 50% glycerol. Omission of primary antibodies was used as a negative control of the reaction. The tissues were observed using an epifluorescence microscope (Olympus IX81) equipped witha DP72 camera, and images were processed with Photoshop and Acrobat Illustrator software.

2.3. Primary ventral rhombencephalic cultures

Primary rhombencephalon cultures were prepared from E14 embryos according to Lautenschlager et al. (2000), with modifications. Briefly, the embryos were removed from pregnant rats at gestation day

14 and the rhombencephalon was dissected out. The neural tube was opened along the dorsal suture and a strip of tissue of approximately 1 mm in width was dissected from the ventral midline (König et al., 1988). The dissected tissue was then mechanically dissociated in culture medium containing Neurobasal medium (Gibco, Thermo Fischer Scientific, Whaltham, MA, USA) supplemented with 2% B27 (Gibco, Thermo Fischer Scientific) and 2 mM L-Glutamine (Sigma-Aldrich, San Luis, MI, USA). After centrifugation (1000 *g*, 5 min), the pellet containing the cells was re-suspended in culture medium and plated on culture plates (Cellstar, Greiner Bio-One, Frickenhausen Germany) or cover glasses previously coated with poly-L-lysine (0,1 mg/ml, Sigma-Aldrich). A density of 1,000,000 cells/well on 24-wells plates containing cover glasses was used for immunohistochemical experiments that were analyzed by confocal microscopy. A density of 600,000 cells/well on 48-wells plates was used for immunohistochemical studies analyzed by epifluorescence microscopy and for analytical studies analyzed by HPLC-ED. Cultures were kept at 37 °C and 5% CO_2 in a humidified incubator (Revco Ultima, Thermo Fischer Scientific) and were fed by replacing half of the medium at day in vitro 7 (DIV 7). Immunohistochemical and analytical experiments were performed at DIV 14.

2.4. Immunohistochemistry of primary cultures

Cells were fixed with 4% PFA/5% sucrose in PBS at 37 °C for 20 min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (15 min). After blocking nonspecific binding with 5% bovine serum albumin in PBS (30 min), the cultures were incubated with primary antibodies diluted in the blocking solution at 4 °C overnight. 5-HT positive neurons were identified using goat or rabbit anti-5-HT antibodies (1:500, ImmunoStar; # 20079 and # 20080). ϒ-aminobutyric acid (GABA) neurons were identified with rabbit anti-GABA antibodies (1:500, Sigma-Aldrich; #A-2052), while mouse anti-tyrosine hydroxylase (TH) antibodies were used to identify catecholaminergic neurons (1:1000, Millipore, Temecula, CA, USA, # MAB318). To detect astrocytes, we used mouse anti-glial fibrillary acidic protein (GFAP) antibodies (1:400, Sigma-Aldrich, # MS-1407). Culture cells were then incubated with secondary antibodies (donkey anti-mouse, donkey anti-rabbit or donkey anti-goat Alexa Fluor 488 or 594 at 1:1000 dilution, Jackson ImmunoResearch) at RT for 60 min, followed by staining with DNA intercalating dye Hoechst 33258 (1 μg/ml, Sigma-Aldrich) for 10 min. Omission of primary antibodies was used as a negative control of the reaction.

To quantify the number of serotonergic and GABAergic neurons in primary cultures, we analyzed two wells per experiment (in 48-well plates) and photographed five 200× pre-determined fields per well with an Olympus IX81 fluorescent microscope equipped with a DP71 Olympus camera. Images were processed with Image J software to quantify the number of immunolabeled cells and the total viable cells (identified as Hoechst-positive stained large and opaque nuclei). Data are presented as the percentage of immunolabeled cells (serotonergic or GABAergic) in relation to the total number of viable cells. Confocal representative images of 5-HT and GABA- positive neurons were obtained using an Olympus BX6 microscope equipped with an FV300 confocal module and a DP70 Olympus camera.

2.5. Determination of cellular and extracellular 5-HT and 5-HIAA levels by HPLC-ED

5-HT and 5-HIAA levels were measured at the IIBCE Analytic Chemistry Platform according to Higuchi et al. (2008) and Nagayasu et al. (2010a, 2010b) procedures for organotypic slices, with modifications. Briefly, the culture medium of DIV 14 primary cultures was replaced with Krebs–Ringer–Henseleit (KRH) buffer (146 mM NaCl, 27 mM KCl, 10 mM $MgCl_2$, 12 mM $CaCl_2$, 100 mM D-glucose, 150 mM HEPES, 50 mM HEPES-Na, 2 mM ascorbic acid; pH 7.4) and incubated for 15 min at 37 °C for stabilization. The cultures were then incubated for 30 min at 37 °C with 120 μl of fresh KRH buffer (control group corresponding to basal endogenous levels) or KRH buffer supplemented with the different compounds to be evaluated.

After the incubation period, the KRH buffer (extracellular sample) was collected from the wells in perchloric acid (PCA, final concentration 0.1 M). In some experiments, cellular samples were collected by scraping the cells in 200 μl of 0.1 M PCA and then sonicated. Samples were then centrifuged at 15.000 rpm for 10 min at 4 °C, and 100 μl of the supernatants were analyzed by HPLC-ED to measure 5-HT and 5-HIAA levels. The HPLC system (Waters 2465, Milford, MA, USA) was equipped with a C-18 column (Luna® 3 μm C18(2) 100 Å, LC 100 × 2 mm, Phenomenex, Torrance, CA, USA) and a security guard cartridge (3.2 to 0.8 mm internal diameter, Phenomenex) maintained at 37 °C. The electrochemical detection system (Epsilon e5P, BASi, West Lafayette, IN, USA) was set to an oxidation potential of + 0,65 V (glassy carbon working electrode vs. Ag/AgCl reference electrode). The mobile phase was composed of 0.15 M citric acid, 0.6 mM sodium octyl sulphate, 4% acetonitrile and 1.6% tetrahydrofuran at pH 3.0, and was delivered at a flow rate of 0.3 ml/min using an HPLC pump (Waters 1525, Milford, MA, USA). The concentration of 5-HT and 5-HIAA in the samples was calculated by comparison with the signal obtained with standard solutions of known concentrations. The detection limit in our system was 19 ± 1 pg for 5-HT and 32 ± 15 pg for 5-HIAA.

2.6. Pharmacological treatments

We studied the response of rhombencephalic primary cultures under 30 min treatments with potassium chloride (KCl; 50 mM), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA; 2 mM), citalopram (0.1, 1, 10 and 100 μM), nortriptyline (1, 10 and 100 μM), clorgyline (0.1, 1, 10 and 100 μM), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT; 1, 10 and 100 nM), WAY 100635 (10, 100 and 1000 nM), and MCH (10, 100 and 1000 nM). All substances were dissolved in KBH and obtained from Sigma-Aldrich, except for MCH that was obtained from Phoenix Pharmaceuticals Inc.

2.7. Data analysis

Data on 5-HT and 5-HIAA levels were analyzed with GraphPad Prism 5 software, and are presented in pg as mean ± SEM of three to five independent experiments performed in triplicate. Significant differences were determined using *t*-test or one-way ANOVA test followed by post hoc Tukey multiple comparison test. Statistically significant difference was considered at a $P < 0.05$.

3. Results

3.1. Identification of serotonergic and MCHergic neurons and fibers in E14 embryos

The location of serotonergic neurons in E14 rat embryos was determined by immunohistochemistry using anti-5-HT and anti-SERT antibodies. In sagittal sections, serotonergic neurons were densely distributed near the rostral border of the rhombencephalon (presumed anlage of the B4-9 complex of the adult raphe nuclei) in two discrete groups of cells: a rostral cluster (RC) and a caudal cluster (CC) (Fig. 1). Their cell bodies were intensely stained and were small and spindle-shaped as is characteristic of immature neurons (Fig. 1B2 and C2). The serotonergic fibers were displayed in an extensive network of bundles projecting from both groups of serotonergic neurons in rostral and caudal direction. SERT-immunoreactivity was less intense than serotonergic immunoreactivity, but displayed a similar distribution pattern of immunostained somas and fibers. Fig. 1D shows a representative bundle of SERT-positive fibers and neurons located at the pontine flexure.

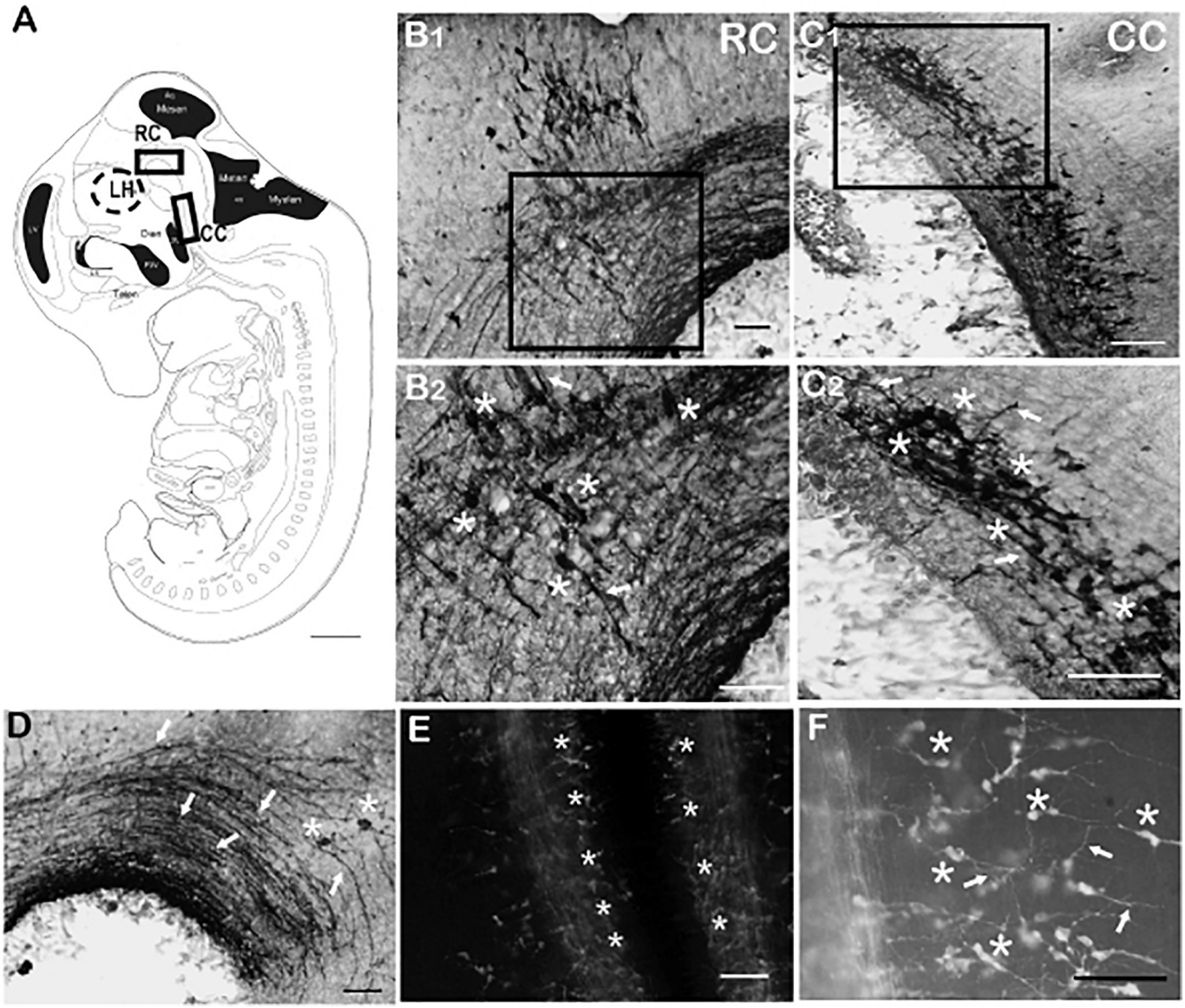

Fig. 1. Localization of serotonergic neurons and fibers in E14 rat embryos and whole mounts obtained from the rhombencephalon. A) Drawing of the sagittal plane of an E14 rat embryo (modified from Paxinos et al., 1990). The two black boxes indicate the position of the rostral cluster (RC) and the caudal cluster (CC) of serotonergic neurons, and the dashed oval indicates the area corresponding to the lateral hypothalamus (LH). B1) Representative microphotograph showing RC serotonergic neurons present at the pontine flexure. B2) Magnification of the area inside the black box in B1. C1) Representative photomicrograph of CC serotonergic neurons. C2) Magnification of the area inside the black box in C1. D) SERT-positive fibers located in the RC area where few serotonergic somatas are present. E) Serotonergic neurons immunostained in an open whole mount obtained from the rhombencephalon of an E14 embryo. F) Magnified photomicrograph of another whole mount with serotonergic neurons. Asterisks indicate serotonergic somata, and arrows indicate immunostained neurites. Scale bars: A = 1 mm; B1 to D = 50 μm; E to F = 100 μm.

Anti-5-HT immunofluorescence performed on whole mount preparations showed a bilateral distribution of embryonic serotonergic neurons (Fig. 1E). As shown in Fig. 1F, these cells have characteristics common to immature neurons, with short and tapering processes with varicosities. Omission of primary antibodies did not display any positive reaction in any of the assays.

The immunohistochemistry against MCH in sagittal sections of E14 embryos revealed the presence of MCHergic neurons (Fig. 2A) and fibers in the lateral hypothalamus. MCHergic neurons were mildly stained and presented the typical morphology of immature neurons with a non-homogeneous distribution of the positive signal in their somata. The MCHergic fibers were intensively stained and were present in different lengths and diameters. Some MCHergic fibers were present in the area of the rhombencephalon where serotonergic neurons are found (Fig. 2B1, B2 and B3). Such fibers displayed varicosities, a characteristic of peptidergic fibers. The sections assayed for MCH immunohistochemistry were located at similar sagittal planes as those assayed for 5-HT and SERT.

3.2. Morphological and phenotypical characterization of primary cultures at DIV 14

The cultures presented 2.2 ± 0.7% of serotonergic neurons (n = 22 cultures quantified) as identified by immunofluorescence for 5-HT. These neurons were round-shaped, multipolar and presented long processes with varicosities (Fig. 3A). Some processes extended several micrometers away from the cell body and displayed diverse and tortuous ramifications with a very strong staining pattern that formed an extensive network of fibers (Fig. 3A).

As expected, the percentage of GABAergic neurons in the cultures

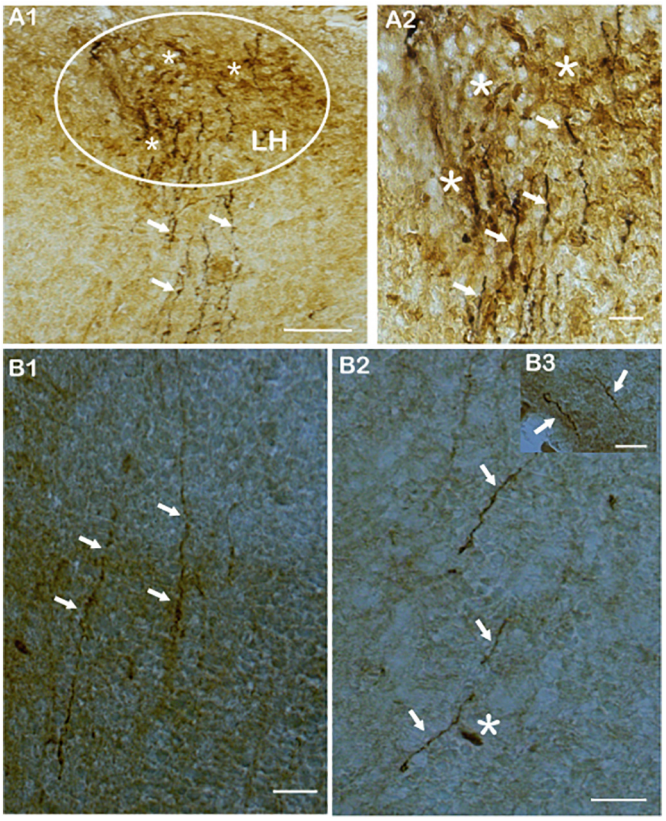

Fig. 2. Localization of MCHergic neurons and fibers in E14 embryos by immunohistochemistry. A1) Representative photomicrograph of lateral hypothalamus where somatas of MCHergic neurons were immunostained with anti-MCH antibodies. The area enclosed by an oval corresponds to the dotted oval in Fig. 1A and includes the lateral hypothalamus. A2) Magnified area from A1 showing the immature feature of MCHergic neurons and positive immunostained fibers. B1–B3) Representative positive immunostained MCHergic fibers of different length and thickness located in the rhombencephalon where serotonergic neurons are present (B1 and B2 correspond to the CC, and B3 to the RC area in Fig. 1A). Asterisks indicate MCHergic somata, and arrows indicate immunostained fibers. Scale bars: A1 = 50 μm; A2 = 25 μm; B1 to B3 = 50 μm.

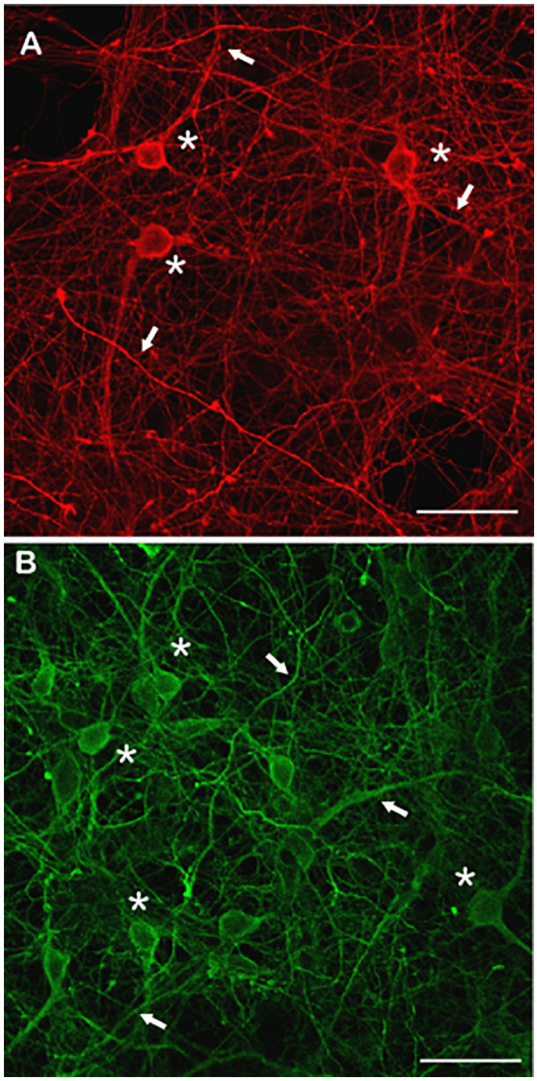

Fig. 3. Morphological characterization of cultured neurons from rhombencephalon after DIV 14: serotonergic and GABAergic neurons. Representative photomicrographs showing 5-HT-positive neurons (A, red) and GABA-positive neurons (B, green). Asterisks indicate immunopositive neurons and arrows indicate immunostained neurites. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was much higher than that of serotonergic neurons. GABA-positive cells represented 48.9 ± 5.4% of total alive cells (n = 4 cultures quantified). GABAergic neurons in culture were multipolar, round or spindle-shaped (Fig. 3B), and displayed a dense network of neurites.

We did not detect catecholaminergic neurons (TH-positive cells) or astrocytes (GFAP-positive cells) in our cultures.

3.3. Functional and pharmacological characterization of rhombencephalic primary cultures: quantification of 5-HT and 5-HIAA levels by HPLC-ED

3.3.1. Quantification of basal levels

Under our chromatographic conditions, we were able to measure basal endogenous levels of 5-HT and 5-HIAA in cellular and extracellular samples after 30 min of incubation with KRH buffer (Table 1). As expected, cellular 5-HT levels were higher than extracellular ones (about 50 times higher), whereas 5-HIAA levels showed the opposite relation, with higher levels in the extracellular samples compared with the intracellular ones. The extracellular/intracellular ratio for 5-HT was about 200 times higher than for 5-HIAA. Since extracellular 5-HT levels in the cultures represent the amount of neurotransmitter available to interact with *serotonergic* receptors, and are thus a more sensitive indicator of serotonergic transmission than intracellular levels, we chose the extracellular samples to analyze and evaluate modifications of 5-HT and 5-HIAA levels under different pharmacological treatments.

Table 1
5-HT and 5-HIAA basal levels quantified by HPLC-ED.

	Intracellular	Extracellular	Extra/intracellular
5-HT	2294 ± 1727	42 ± 27	0.02 ± 0.11
5-HIAA	450 ± 309	1896 ± 1257	4.51 ± 0.96
5-HIAA/5-HT	0.20 ± 0.07	40.88 ± 21.01	–

Serotonin and 5-HIAA basal levels and 5-HIAA/5-HT ratio quantified in cellular samples (intracellular) and released into the medium (extracellular) after 30 min-incubation with KBH. Values are expressed in pg per well as mean ± SEM, n = 7.

3.3.2. Effects of KCl and EGTA

When primary cultures were incubated for 30 min with KCl 50 mM, 5-HT extracellular levels significantly increased compared to controls (Fig. 4A). 5-HIAA levels showed no significant changes, whereas 5-HIAA/5-HT ratio –an indirect indicator of neurotransmitter turnover–

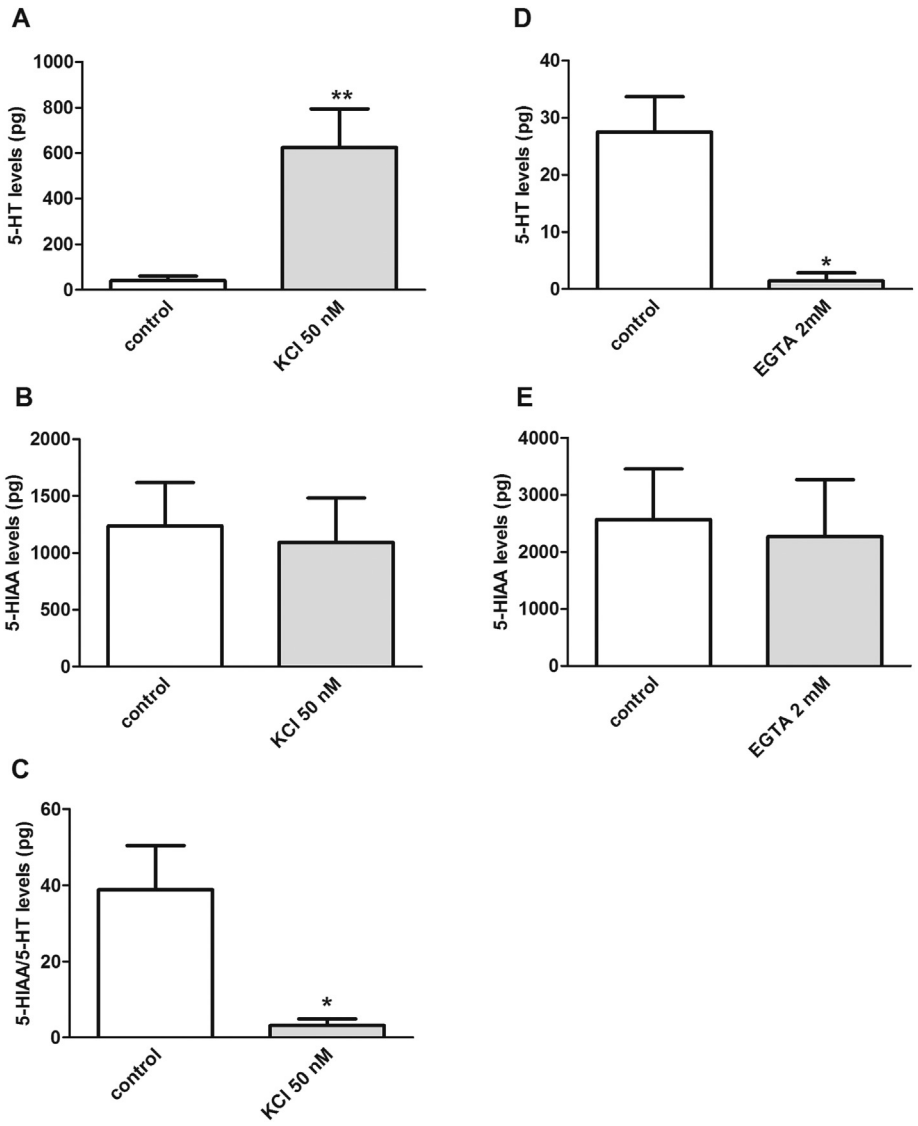

Fig. 4. Effects of KCl and EGTA on 5-HT and 5-HIAA extracellular levels. Cultures were incubated for 30 min with KCl (50 mM) (A, B, C) or EGTA (2 mM) (D, E). Extracellular levels of 5-HT (A, D), 5-HIAA (B, E) and 5-HIAA/5-HT ratio (C) were determined. Data are expressed in pg as mean ± SEM $*p < .05$ and $**p < .01$ versus control (0, KBH) (*t*-test). $n = 4$ experiments.

was significantly reduced (Fig. 4B and C).

Removal of Ca^{2+} from the medium by incubation with EGTA induced a decrease in 5-HT levels in the extracellular space (non-detectable levels with our analytical method in two of the three experiments done) (Fig. 4D). 5-HIAA levels were not modified after EGTA treatment (Fig. 4E).

3.3.3. Effects of 5-HT reuptake inhibitors

In order to assess the normal function of the 5-HT reuptake process in cultured serotonergic neurons, we first studied the effect of a 30-min incubation with SSRI citalopram (0.1–100 µM). As Fig. 5 shows, citalopram induced the expected increase in extracellular 5-HT levels, which followed a typical inverted U-shaped curve and was statistically significant at 1 and 10 µM concentrations compared with basal conditions (Fig. 5A). 5-HIAA levels showed no significant modification in the concentrations assessed (Fig. 5B). The 5-HIAA/5-HT ratio showed a significant decrease in all citalopram concentrations tested (Fig. 5C).

Nortriptyline (1–100 µM), a non-selective reuptake inhibitor, also induced a dose-dependent increase in 5-HT extracellular levels which became significant at 10 and 100 µM (Fig. 5D). 5-HIAA levels did not change under the effect of any of the concentrations of nortriptyline assayed (Fig. 5E). However, 1 and 10 µM of nortriptyline induced a decrease in the 5-HIAA/5-HT ratio (Fig. 5F).

3.3.4. Effects of a monoamine oxidase-A inhibitor (MAOI)

The 30-min incubation with clorgyline (0.1–100 µM) –a well-known MAOI– induced an expected dose-dependent increase in 5-HT extracellular levels that was statistically significant at 10 µM (Fig. 6A). 5-HIAA levels showed a tendency to decrease that did not reach statistical significance at any of the concentrations assessed (Fig. 6B). On the other hand, 5-HIAA/5-HT ratio decreased at every clorgyline concentration tested (Fig. 6C).

3.3.5. Effects of a 5-HT1A receptor agonist and antagonist

In order to assess the presence and functionality of 5-HT1A autoreceptors in cultured 5-HT neurons, we performed a 30-min incubation with a 5-HT1A agonist and a 5-HT1A antagonist. The incubation with 8-OH-DPAT (1–100 nM) –a 5-HT1A agonist– induced a decreasing trend in 5-HT levels only at 1 nM concentration, which had no statistical significance (Fig. 7A). No significant changes were observed in 5-HIAA levels or 5-HIAA/5-HT ratio after 8-OH-DPAT treatment at any of the concentrations tested (Fig. 7 B and C). WAY 100635 (1–1000 nM) –a 5-

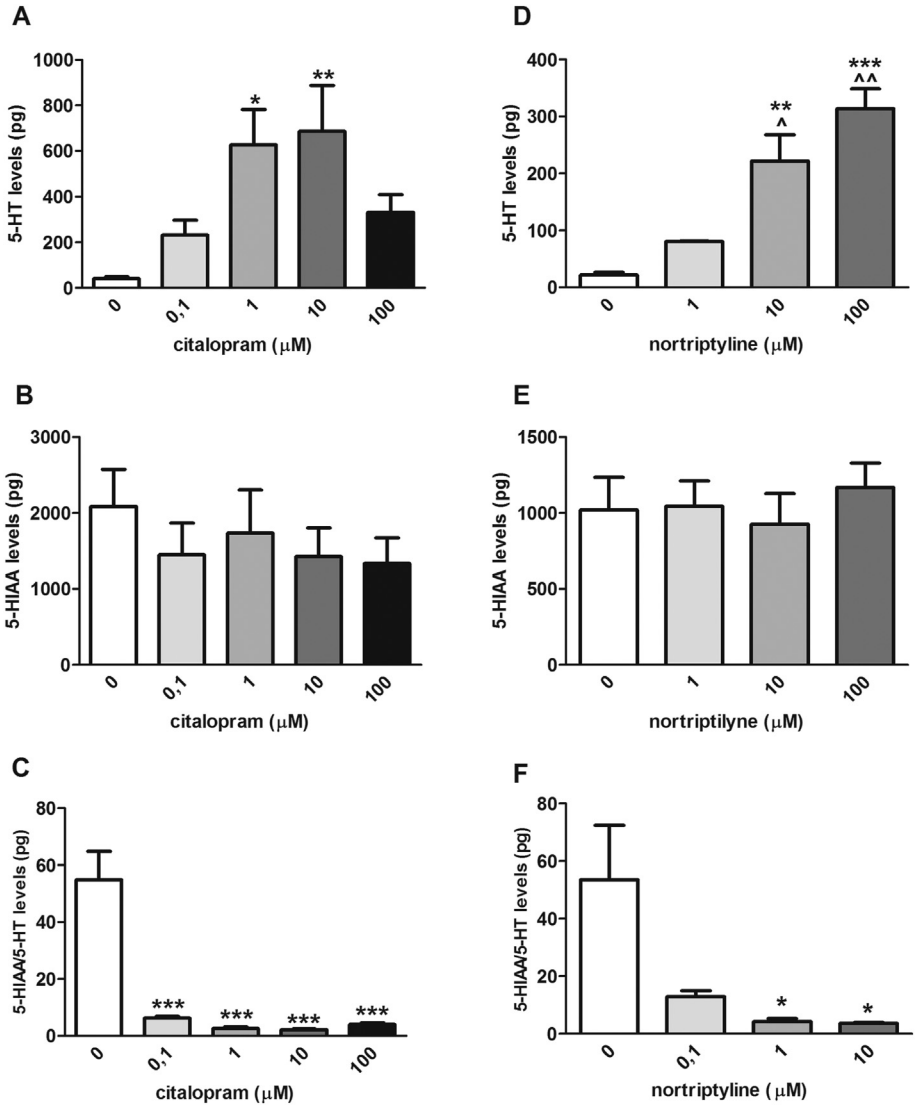

Fig. 5. Effects of citalopram and nortriptyline on 5-HT and 5-HIAA extracellular levels. Cultures were incubated for 30 min with citalopram (0.1–100 μM) (A, B, C) or nortriptyline (1–100 μM) (D, E, F). Extracellular levels of 5-HT (A, D), 5-HIAA (B, E) and 5-HIAA/5-HT ratio (C, F) were determined. Data are expressed in pg as mean ± SEM * $p < .05$, ** $p < .01$ and *** $p < .01$ versus control (0, KBH); ^$p < .05$, ^^$p < .01$ versus nortriptyline 1 μM (One-way ANOVA test followed by post hoc Tukey multiple comparison test). $n = 3–5$ experiments.

HT1A antagonist– did not induce significant modifications in 5-HT or 5-HIAA levels or in the 5-HT/5-HIAA ratio (Fig. 7 D–F).

3.4. Effects of MCH on 5-HT metabolism in primary cultures

After validation of our preparation and analytical method to evaluate serotonergic transmission, we assessed the effect of MCH on 5-HT and 5-HIAA extracellular levels in primary cultures. A 30-min treatment with MCH at 10, 100 and 1000 nM did not induce significant changes in 5-HT or 5-HIAA extracellular levels or in 5-HIAA/5-HT ratio (Fig. 8 A-C).

4. Discussion

The results of the present study provide morphological evidence of the presence of MCHergic fibers reaching the area where serotonergic neurons are present in E14 embryos, but we were unable to show neurochemical modifications of serotonergic metabolism under the effect of MCH in primary cultures. In spite of these results, we propose that analysis by HPLC-ED of 5-HT and 5-HIAA levels in primary rhombencephalic cultures may be used as an appropriate and reliable model to evaluate the effects of neuromodulators on the serotonergic system, including the possible development of novel antidepressants.

4.1. Identification of serotonergic and MCHergic neurons and fibers in E14 embryos

Our data on the location and morphology of serotonergic neurons in E14 rat embryos are consistent with previous publications (Yamamoto et al., 1981; Lautenschlager et al., 2000; Czesak et al., 2007). Immunostained sagittal embryonic sections indicated that 5-HT-positive neurons were distributed in two clusters of cells in the rhombencephalon (RC and CC), whereas whole mount immunostaining showed that they were bilaterally disposed. We also confirmed previous reports by Schroeter and Bakely (1996) and Zhou et al. (2000) on the presence of SERT-immunopositive neurons and fibers in the rhombencephalon.

Besides, we evidenced the presence of positive immunostained MCHergic fibers in the regions where serotonergic neurons are located in E14 rhombencephalon, indicating that MCH could already be modulating the serotonergic system at this embryonic stage. These data gave us an appropriate morphological frame to dissect out the ventral part of the rhombencephalon to perform our primary cultures.

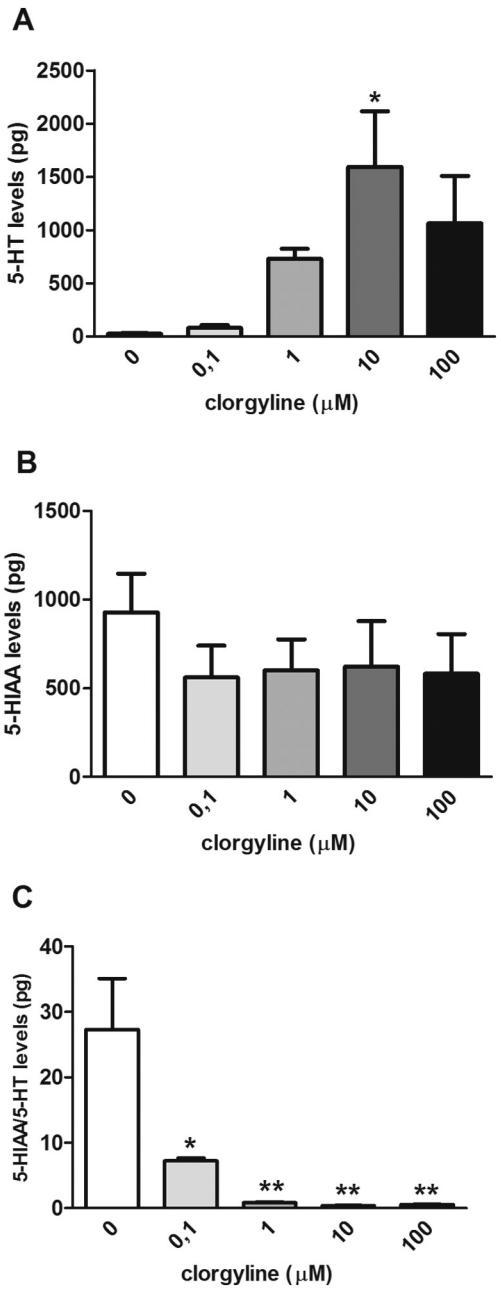

Fig. 6. Effects of clorgyline on 5-HT and 5-HIAA extracellular levels. Cultures were incubated with clorgyline (0.1–100 μM) for 30 min, and extracellular levels of 5-HT (A), 5-HIAA (B) and 5-HIAA/5-HT ratio (C) were determined. Data are expressed in pg as mean ± SEM * $p < .05$ and ** $p < .01$ versus control (0, KBH) (One-way ANOVA test followed by post hoc Tukey multiple comparison test). $n = 3$ experiments.

In addition, we demonstrated the presence of MCHergic neurons in the lateral hypothalamus at E14, confirming data from previous studies showing that MCHergic neurons ontogenesis occurred between E10 and E16 in rat embryos (Brischoux et al., 2001). Specifically, two groups of MCHergic neurons have been described: one of them is generated at E11 and innervates structures like the brainstem and the spinal cord. The other group of neurons is generated between E12 and E13 and sends projections to the cerebral cortex (Brischoux et al., 2002). Previous in situ hybridization studies against prepro-MCH (the MCH peptide precursor) and studies using MCH-GFP mice have already suggested that MCHergic neurons are present at E14 in mice and that they innervate the area where serotonergic neurons are located (Croizier et al., 2011; Croizier et al., 2015; Chometton et al., 2016). Data presented here show for the first time positive MCHergic immunostaining of hypothalamic neurons and fibers that project to the rhombencephalon in E14 rat embryos.

4.2. Morphological and phenotypical characterization of primary cultures

Our cultures presented 2.2% of serotonergic neurons, 48.9% of GABAergic neurons, and no catecholaminergic cells or astrocytes. These results are consistent with previous reports: Lautenschlager et al. (2000) obtained 4% of serotonergic neurons at DIV 14, while Yamamoto et al. (1981) and Czesak et al. (2007) reported 40% and 54% of GABAergic neurons, respectively, in similar preparations (Yamamoto et al., 1981; Lautenschlager et al., 2000; Czesak et al., 2007). The presence of GABA neurons at E14 is not surprising since GABAergic fibers have been detected as early as E13 in the brainstem, mesencephalon and diencephalon of rodents, and GABAergic cell bodies were found in the lateral cortical anlage at E14 (Henschel et al., 2008). Previous reports have also described the presence of catecholaminergic neurons and astrocytes in primary rhombencephalic cultures (Lautenschlager et al., 2000; Czesak et al., 2007). However, we did not detect TH or GFAP-positive cells in our preparations. Slight differences in dissection procedures or culture protocols may account for this discrepancy. We did not perform further studies to address the cellular phenotype of the other cells present in our cultures, but based on previous publications they were probably progenitors and other types of neurons (Czesak et al., 2007).

4.3. Functional and pharmacological characterization of rhombencephalic primary cultures: quantification of 5-HT and 5-HIAA levels by HPLC-ED

Although serotonergic neurons represented a low proportion of the culture cells, they developed an extensive neurite network. These neurons synthesized and released enough 5-HT and 5-HIAA for us to quantify their basal endogenous levels with our method with high reproducibility and reliability. As expected, the great majority of the 5-HT content was present inside the serotonergic neurons (probably in the vesicular pool), with intracellular neurotransmitter levels being > 50 times higher than extracellular ones. The opposite relation was found for 5-HIAA since extracellular 5-HIAA levels were 4 times higher than intracellular ones. To our knowledge, this is the first study to report basal endogenous and released 5-HT and 5-HIAA levels in primary cultures without the addition of a radioactive serotonergic precursor or 5-HT. Paolillo et al. (1993) described a method for the simultaneous determination of 5-HT and 5-HIAA in primary cultures using HPLC-ED, but they only detected intracellular levels and were unable to measure released 5-HT and 5-HIAA, a key parameter for a reliable estimation of serotonergic transmission and metabolism (Paolillo et al., 1993). In another study, Gu and Azmitia also combined rhombencephalon tissue culture and HPLC-ED to investigate drug toxicity on serotonergic neurons, but they were unable to detect released 5-HT in basal conditions (Gu and Azmitia, 1993). Other reports assessing released 5-HT in primary cultures pre-loaded the cells with [^{3}H]-tryptophan or [^{3}H]-5-HT (Yamamoto et al., 1981; Becquet et al., 1991; Becquet et al., 1993a; Becquet et al., 1993b; Wichems et al., 1995; Héry et al., 1999; Lautenschlager et al., 2000; Birthelmer et al., 2007). The detection of radiolabeled molecules is highly sensitive and overcomes the low neurotransmitter levels, but is associated with intrinsic health and environmental risks (Holtzhauer, 2006). In this regard, we consider that our experimental approach is more suitable and safer.

In order to study the physiology of the serotonergic neurons in culture, we challenged the preparation with diverse pharmacological treatments known to modify serotonergic transmission. On the whole, cultured serotonergic cells responded as expected to the different pharmacological treatments, indicating that this in vitro system is a suitable model to evaluate serotonergic transmission. For example,

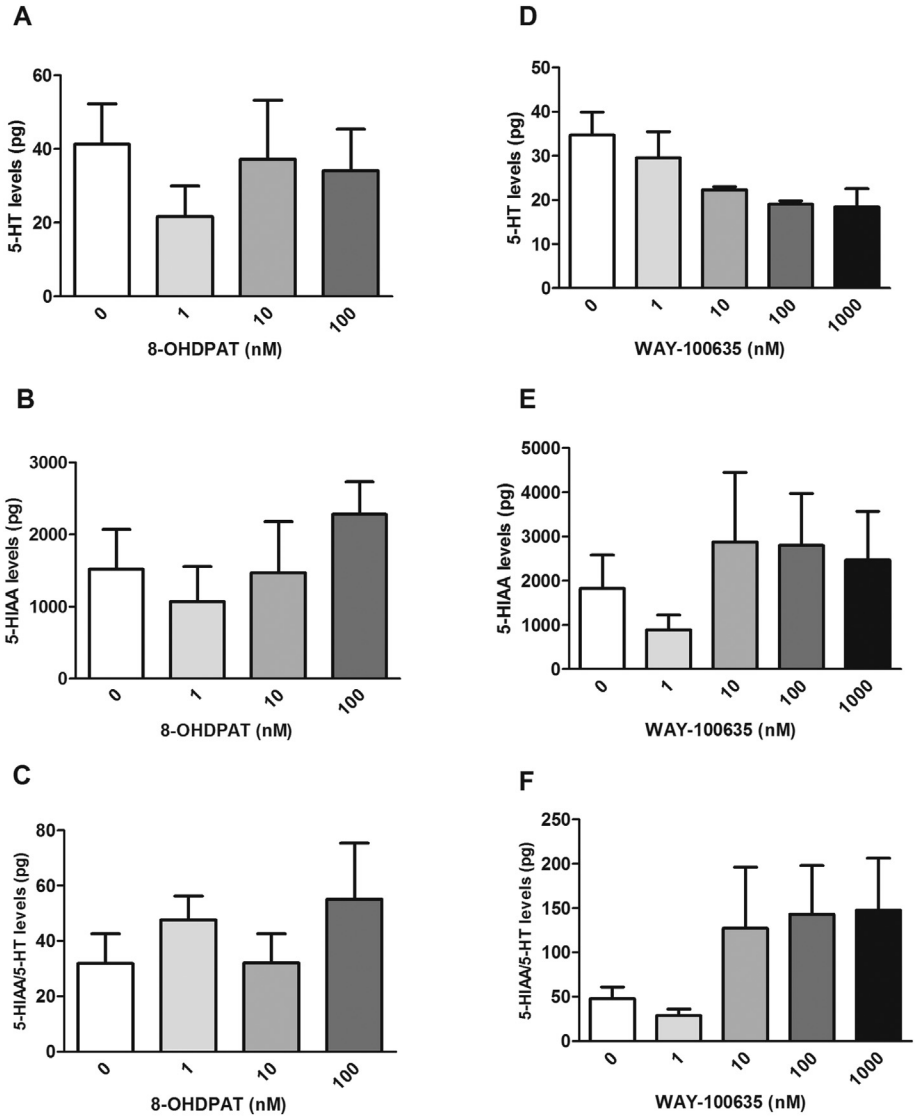

Fig. 7. Effects of 8-OH-DPAT and WAY-100635 on 5-HT and 5-HIAA extracellular levels. Cultures were incubated for 30 min with 8-OH-DPAT (1–100 nM) (A, B, C) and WAY-100635 (1–1000 nM) (D, E, F). Extracellular levels of 5-HT (A, D), 5-HIAA (B, E) and 5-HIAA/5-HT ratio (C, F) were determined. Data are expressed in pg as mean ± SEM. n = 3–5 experiments.

depolarization with KCl (50 mM) resulted in an 8-fold increase of 5-HT extracellular levels. Similar results were obtained by Yamamoto et al. (1981) after incubation with KCl 44 mM (Yamamoto et al., 1981). Lautenschlager et al. (2000) also reported an increase in [3H]-5-HT secretion after a 5 min incubation with KCl 50 mM, which was blocked by Tetanus toxin, indicating that neurotransmitter release is due to synaptic vesicles exocytosis (Lautenschlager et al., 2000). In line with this observation, the drastic reduction of 5-HT extracellular levels after treatment with EGTA indicated that 5-HT release from serotonergic neurons in our cultures was indeed calcium-dependent. Other published studies have reported similar results in primary cultures and organotypic slices (Becquet et al., 1991; Birthelmer et al., 2007; Nagayasu et al., 2010a; Nagayasu et al., 2010b).

Immunohistochemistry studies showed in this work confirmed that SERT is expressed at E14. In addition, functional studies indicated that the transporter was working properly in the serotonergic cultured cells. Indeed citalopram and nortriptyline induced a significant dose-dependent increase in extracellular 5-HT levels associated with a reduction in neurotransmitter turnover. These results are consistent with SERT inhibition inducing accumulation of released 5-HT in the extracellular space. Besides, the potency of both drugs on rising 5-HT extracellular levels reflected the different inhibition constant reported for rat SERT, which is higher for nortriptyline (Owens et al., 1997). A citalopram concentration of 100 μM (the highest concentration tested) did not induce significant changes, probably because of internalization or down-regulation of SERT molecules inside the neurons as a result of the high concentration of the inhibitor (Lau et al., 2008). Our results are consistent with those of Nagayasu et al. (2013) in organotypic slices containing DRN from P0 to P2 rats: they show a similar dose-response curve of citalopram concentrations on 5-HT extracellular levels (Nagayasu et al., 2013).

MAO catalyzes the oxidative deamination of different biogenic amines, with MAO A being the isoform that preferentially metabolizes 5-HT (Shih, 1991; Bortolato et al., 2010). Our results suggested that the enzyme was functional in our cultures, as indicated by MAO A inhibition with clorgyline inducing an increase in 5-HT extracellular levels and a nonsignificant decreasing trend in its main metabolite, which resulted in a decreased 5-HT/5-HIAA ratio. Similar results were obtained in a previous study where clorgyline produced an increase in 5-HT released levels in primary rhombencephalic cultures, although at a lower concentration (Gu and Azmitia, 1993).

Among the 14 known mammalian serotonergic receptors the most

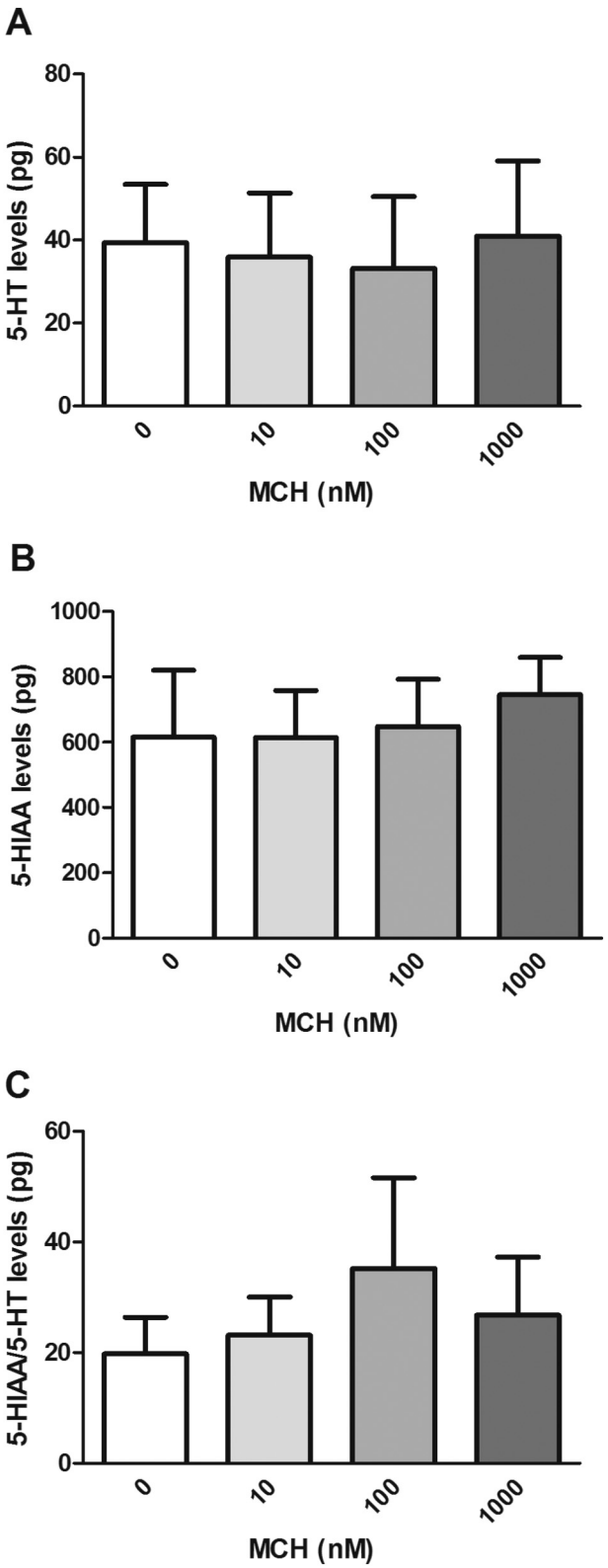

Fig. 8. Effects of MCH on 5-HT and 5-HIAA extracellular levels. Cultures were incubated for 30 min with MCH (10–1000 nM), and extracellular levels of 5-HT (A), 5-HIAA (B) and 5-HIAA/5-HT ratio (C) were determined. Data are expressed in pg as mean ± SEM. n = 3–5 experiments.

abundant subtype expressed in serotonergic adult neurons is the somatodendritic 5-HT1A autoreceptor (Zhou et al., 1999; Riad et al., 2000). This receptor mediates negative feedback inhibition of serotonergic neurons, and its mRNA and protein are expressed at E14.5 in rodents (Hillion et al., 1994; Bonnin et al., 2006). As described and demonstrated through binding and functional studies (Héry et al., 1999), serotonergic cultured neurons obtained from E14 embryos present functional 5-HT1A receptors. These studies also showed that stimulation of this subtype of receptor by different agonists induces a decrease in [^{3}H]-5-HT release, with a reduction of about 30% after 8-OH-DPAT at 10^{-7} to 10^{-9} M. In line with these results, 8-OH-DPAT at 10^{-9} M concentration caused approximately a 50% reduction in extracellular 5-HT levels in our cultures, but this was not significantly different from control conditions.

In accordance with previous publications, the 5-HT1A antagonist WAY 100635 did not induce any modification in released 5-HT levels (Héry et al., 1999; Birthelmer et al., 2007). This result is in line with electrophysiological studies done on neonatal DRN slices indicating that the basal release of 5-HT during physiological discharge of serotonergic neurons does not reach the levels required to activate 5-HT1A autoreceptors (Johnson, 1994).

4.4. Assessment of the effects of MCH on 5-HT metabolism in cultures

In this work we evaluated for the first time the effect of MCH on serotonergic metabolism in a primary culture of embryonic neurons. Our results indicate that MCH had no effect on serotonergic metabolism in our in vitro preparation, since incubation with MCH did not significantly change 5-HT or 5-HIAA extracellular levels compared to basal conditions. This observation did not support our initial hypothesis about MCH modulating serotonergic neurons. A possible explanation for these negative results would be that, although MCH fibers are already present at this early embryonic stage, MCH is not yet able to modulate serotonergic neurons. In fact, to date, no published reports have described the presence of MCHR-1 protein or mRNA in neurons present in the CNS of E14 rats. Further studies under other experimental conditions (chronic incubation or longer incubation times with MCH, different MCH concentrations, etc.) and determination of the presence of MCHR-1 protein will be necessary to complement our results and establish if MCH induces an effect on embryonic serotonergic cultured neurons. Eventually, postnatal serotonergic cultures could be an alternative and adequate preparation to answer our question.

5. Conclusion

Our findings confirmed that MCHergic fibers were present on the location of serotonergic neurons in the E14 rat embryo. However, we were not able to evidence a modulatory effect of MCH on 5-HT metabolism in the rhombencephalic primary cultures obtained at this embryonic stage. Despite this limitation, our results suggest that determining 5-HT and its main metabolite by HPLC-ED in rhombencephalic primary cultures is a useful and sensitive approach to study modulation of serotonergic neurotransmission through compounds affecting 5-HT release, uptake and turnover under carefully controlled conditions.

Acknowledgements

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