

# Prodepressive effect induced by microinjections of MCH into the dorsal raphe: time course, dose dependence, effects on anxiety-related behaviors, and reversion by nortriptyline

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Melanin-concentrating hormone (MCH) administered within the rat dorsal raphe nucleus (DRN) has been shown to elicit prodepressive behaviors in the forced-swim test. The present study was designed to evaluate the time course (30 and 60 min) and dose dependence (25–100 ng) of this effect, and whether it would be antagonized by an intra-DRN microinjection of the MCH-1 receptor antagonist ATC0175 (ATC, 1 mmol/l) or intraperitoneal pretreatment with the noradrenergic antidepressant nortriptyline (20 mg/kg). The results showed that the behavioral effect of MCH was time and dose dependent as immobility was increased, and climbing decreased, only by the 50 ng MCH dose at  $T_{30}$ . The effect was mediated by MCH-1 receptors as a significant blockade of this behavioral response was observed in ATC-pretreated animals. ATC did not by itself modify animal behavior. Nortriptyline also prevented the prodepressive-like effect of MCH. Concomitantly, the effect of MCH (50 ng) at  $T_{30}$  on anxiety-related behaviors was assessed using the elevated plus-maze. Interestingly, these behaviors were

unchanged. In conclusion, MCH administration within the DRN elicits, through the MCH-1 receptor, a depression-related behavior that is not accompanied by changes in anxiety and that is prevented by a noradrenergic antidepressant. *Behavioural Pharmacology* 25:316–324 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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## Introduction

Previous anatomical and behavioral studies have shown that melanin-concentrating hormone (MCH) is involved in the modulation of emotional states, in addition to its classical functions such as food intake control, energy balance, and sleep behaviors (Chung *et al.*, 2009, 2011; Torterolo *et al.*, 2011; Blouin *et al.*, 2013). However, little is known about the brain regions, pathways, and mechanisms that relate the MCHergic system to depressive and anxiety states.

MCH is a cyclic neuropeptide of 19 amino acids. Its biological function in rodents is mediated by a G-protein-coupled receptor subtype known as MCH-1 (MCHR-1; Saito *et al.*, 2001). MCH is predominantly synthesized by neurons located in the lateral hypothalamus and the incertohypothalamic area (Bittencourt *et al.*, 1992; Saito *et al.*, 2001), which send projections to several brain regions (Bittencourt *et al.*, 1992; Bittencourt, 2011). There is evidence that MCHergic fibers reach the dorsal raphe nucleus (DRN) in rats (Bittencourt *et al.*, 1992; Lagos *et al.*, 2011a; Yoon and Lee, 2013). The DRN contains the vast majority of the serotonergic neurons of the brain (Azmitia and Segal, 1978; Jacobs and Azmitia, 1992; Monti, 2010; Calizo *et al.*, 2011) and it has been suggested that a dysfunction in the serotonergic neurons

of the DRN underlies major depression (Underwood *et al.*, 1999; Arango *et al.*, 2002).

We have reported previously that MCH induced a depressive-like behavior in the forced-swimming test (FST), that is a significant increase in immobility and decrease in climbing times, immediately following a microinjection into the DRN of the rat (time 0,  $T_0$ ). MCH immunoneutralization (i.e. anti-MCH antibodies microinjected into DRN) elicited an antidepressant-like behavioral response in the FST, defined by a significant decrease in immobility and increase in swimming behavior (Lagos *et al.*, 2011a). It has also been shown that MCH microinjected within the DRN increased, for at least 6 h, the time spent in REM sleep (Lagos *et al.*, 2009), a behavioral disturbance observed in depressive patients (Palagini *et al.*, 2013). Overall, these results support the idea that the MCHergic system plays a role in the induction of depressive-like behaviors through the modulation of neuronal activity within the DRN (Lagos *et al.*, 2009, 2011a).

The effect of MCH in the FST was partially prevented by systemic and subchronic treatment with fluoxetine, a selective 5-hydroxytryptamine (5-HT) reuptake inhibitor. This finding led us to propose that a serotonergic mechanism may be involved (Lagos *et al.*, 2011a).

However, it was observed that MCH induced a behavioral pattern that is in contrast to that described previously for a prototypical noradrenergic antidepressant, which decreases immobility by increasing climbing behavior, but not for a serotonergic antidepressant, which decreases immobility by increasing swimming behavior (Detke *et al.*, 1995; Cryan *et al.*, 2002). Therefore, a noradrenergic role in this prodepressive effect should also be considered.

In the present study, a detailed characterization (time course and dose dependence) of the behavioral effects elicited by MCH intra-DRN was performed in rats using the FST. The specificity of MCH action was studied using intra-DRN pretreatment with ATC0175 (ATC), a potent MCH antagonist with a high affinity for MCHR-1 (Chaki *et al.*, 2005). In addition, we evaluate whether the systemic pretreatment with nortriptyline (Nor), a tricyclic antidepressant and noradrenaline (NA) reuptake inhibitor (Roubert *et al.*, 2001; Dell'Osso *et al.*, 2011), would revert the prodepressive effects elicited by MCH. Considering that anxiety commonly co-occurs clinically with depression (Nutt and Stein, 2006), the effect of an MCH microinjection into the DRN on anxiety-related behaviors was also explored using the elevated plus-maze (EPM) test.

## Methods

### Subjects

A total of 153 male Wistar rats (240–260 g), bred in the IIBCE animal facilities (Montevideo, Uruguay), were used in this study. Rats were housed in groups of five to six in plastic cages (50 × 37.5 × 21 cm), with food and water freely available, under controlled conditions (temperature 22 ± 2°C, 12 h light–dark cycle, lights on at 07:00 h). All procedures were carried out in accordance with the IIBCE Bioethics Committee's requirements under the current ethical regulations of the National Law on Animal Experimentation No. 18.611, taking into account the 'Guidelines for the care and use of laboratory animals' (8th ed., National Academy Press, Washington DC, 2011). Adequate measures were adopted to minimize discomfort or stress of the animals, and all efforts were made to use the minimal number of animals necessary to produce reliable scientific data.

In all behavioral experiments, rats were used only once. Experiments were conducted between 11:00 and 16:00 h, and behavior was rated simultaneously by two trained researchers.

### Surgical procedures

Animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (5 mg/kg) from Konsol Köning S.A Laboratory and mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A small hole was drilled in the skull at coordinates that were determined

relative to the Bregma according to the atlas of Paxinos and Watson (2005): anterior, –7.8 mm and lateral to the midline, +3.2 mm. A stainless-steel guide cannula (21 G, length 20 mm) was slowly inserted at a 30° angle and left 1 mm above the target (–5 mm vertical from the dura mater) to minimize cellular damage at the injection site. Three small stainless-steel screws serving as anchors were cemented to the skull with dental acrylic. After recovering from anesthesia, the rats were returned into their home cages in groups of three to four until the day of the experiment (5–6 days after guide cannula implantation).

### Drugs and microinjection procedure

MCH (Phoenix Pharmaceuticals Inc., Belmont, CA, USA; #070-47) was diluted in saline to obtain a final concentration of 0.125, 0.25, and 0.5 µg/µl (25, 50, and 100 ng, respectively).

Aliquots were prepared, frozen at –20°C, and thawed immediately before use. The MCHR-1 antagonist N-[cis-4-[[4-(dimethylamino)-2-quinazolinyl] amino] cyclohexyl]-3,4-difluorobenzamide hydrochloride, ATC (Tocris Bioscience, Bristol, UK), was diluted in a 1.5% solution of Tween 80 and distilled water to obtain a final concentration of 1 mmol/l. Nor hydrochloride was generously donated by Urufarma Laboratories (Montevideo, Uruguay).

One day before the behavioral experiments, rats were brought in their home cages to the experimental room, where they remained undisturbed until the beginning of the experimental sessions. On the test day, MCH, ATC, or their respective vehicle solutions were microinjected into the DRN through an administration cannula (27 G, length 21 mm) connected to an infusion pump (Harvard Apparatus; Insteck, Plymouth Meeting, PA, USA) and introduced slowly through the guide cannula toward a final position in the DRN (–6 mm vertical). Then, the animals were allowed to move freely in a cage (26 × 20 × 13 cm) while MCH, ATC, or vehicle was perfused at a flow rate of 0.1 µl/min for 2 min (final volume of 0.2 µl); thereafter, the cannula was left in position for one additional minute to allow drug diffusion.

### Forced-swim test

The FST was performed as described previously (Detke *et al.*, 1995; Lucki, 1997; Lagos *et al.*, 2011a). Briefly, rats were placed in a plexiglas cylinder (30 cm internal diameter, 50 cm height) containing 23–24°C water at a depth of 30 cm, preventing the rats from supporting themselves touching the bottom with their paws. Animals were exposed to a swim session for 15 min (pretest session) and they were then dried and returned to their home cages. Twenty-four hours later, a test session (5 min) was carried out, during which immobility (passive behavior) as well as swimming and climbing time (active behaviors) were recorded (Detke *et al.*, 1995). The total duration of

each behavior was scored in seconds (s). Immobility was assigned when no additional activity was observed other than that required to keep the head and nose above the water level. Swimming behavior was defined as movements (usually horizontal) throughout the cylinder, and climbing behavior consisted of vigorous and upward-directed movements of the forepaws along the cylinder wall (Porsolt *et al.*, 1978; Detke *et al.*, 1995; Cryan *et al.*, 2002).

For the time-course study, MCH (50 ng) or saline was microinjected into the DRN in different groups of animals, and behavioral effects in the FST were evaluated independently 30 and 60 min ( $T_{30}$  and  $T_{60}$ , respectively) after the end of each microinjection procedure. Two other doses of MCH (25 and 100 ng) were tested in other groups of animals at  $T_{30}$  only to evaluate the dose dependence of the behavioral effects.

To study the specificity of MCH action, a second group of implanted animals was pretreated with intra-DRN ATC 1 mmol/l or vehicle, followed 10 min later by an intra-DRN injection of MCH or saline (vehicle + saline, vehicle + MCH, ATC + saline, and ATC + MCH groups).

A third group of implanted animals was pretreated with three doses of Nor (20 mg/kg, intraperitoneally) administered 1 h after the end of the pretest session and 5 and 1 h before the test session (Detke *et al.*, 1995; Cryan *et al.*, 2002). A control group received vehicle (saline) injections under the same schedule. Animals pretreated with either vehicle or Nor were then microinjected with intra-DRN MCH or saline and 30 min later were tested in the FST (vehicle + saline, vehicle + MCH, Nor + saline, and Nor + MCH groups).

### Open-field test

To control for the nonspecific effects of MCH on the behaviors evaluated in the FST, an additional group of animals was treated with MCH or saline and locomotor activity was measured. The open-field test (OFT) was conducted as described previously (Lagos *et al.*, 2011a). The apparatus consisted of a square box (60 × 60 cm) with red acrylic walls (40 cm high), homogeneously illuminated (150 lux). Locomotor activity was recorded automatically by a camera connected to a computer equipped with Ethovision software 7.0 (Noldus, Wageningen, the Netherlands). We measured the horizontal locomotor activity, defined as the total distance moved in meters (m). Time (s) spent in the center of the field was also recorded. Before each session, the floor was cleaned with alcohol (30%). Thirty minutes ( $T_{30}$ ) after intra-DRN MCH (25, 5.0 and 100 ng) or saline microinjections, the animals, naive to the OFT, were individually placed in the center of the field and locomotion was recorded during 5 min.

### Elevated plus-maze

The EPM was made of wood and consisted of two opposed open arms (50 × 10 cm) crossed at a 90° angle

with two opposed closed arms (50 × 10 × 40 cm), which extended from a 10 × 10 cm central platform. The maze was elevated 50 cm above the floor (Pellow and File, 1986) and homogeneously illuminated (190–200 lux). Thirty minutes ( $T_{30}$ ) after intra-DRN MCH (50 ng) or saline microinjections, the animal was individually placed on the central platform of the EPM facing a closed arm and allowed to freely explore the EPM for 5 min. The frequency of entries and time spent in open and closed arms, frequency of risk assessment, and head dipping were recorded. EPM performance was also videotaped and the distance moved (m) by the animal was recorded automatically using the Ethovision software. The EPM was cleaned with alcohol (30%) between animals.

### Histology

To confirm the microinjection site, rats were deeply anesthetized with urethane (1.2 g/kg, intraperitoneally; Sigma, St. Louis, MO, USA) and perfused transcardially with a heparinized saline solution, followed by a fixative solution containing 4% paraformaldehyde. Brains were dissected out and processed for histological procedures. Brains were coronally sectioned at 30 μm using a cryostat (Leica Microsystem CM 1900, Wetzlar, Germany). Then, sections were mounted on gelatin-coated slides and stained by the Nissl method. Brain sections were analyzed to determine the correct cannula placement and the microinjection sites following the atlas of Paxinos and Watson (2005). Animals with cannula placement and microinjections outside the DRN were discarded. Microphotographs indicating localizations of microinjections within the DRN have been shown in previous studies from our group (Lagos *et al.*, 2009, 2011a, 2011b).

### Statistical analysis

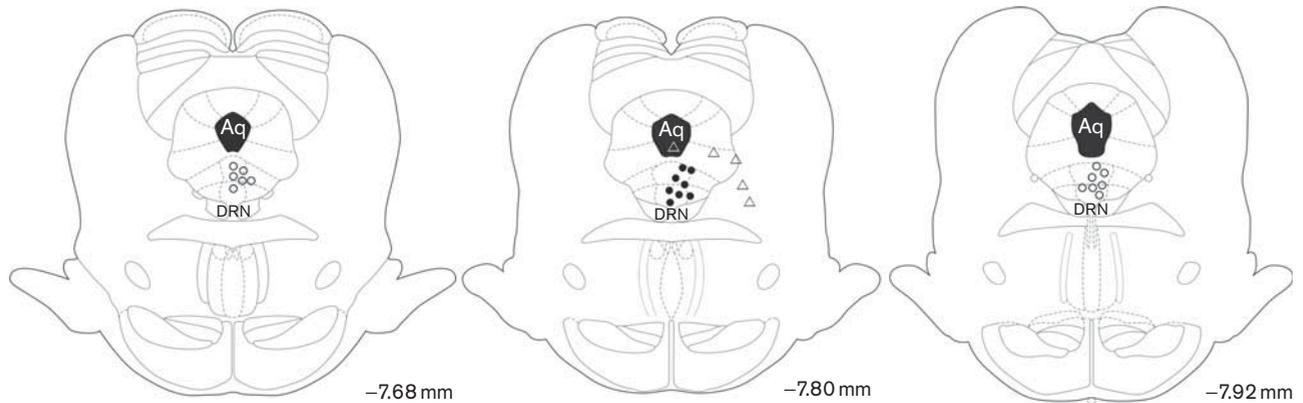
Data are reported as mean ± SEM and were analyzed by one-way analysis of variance for independent measures, followed by a post-hoc Newman–Keuls test. The two-tailed unpaired Student's *t*-test was also utilized. Statistical significance was set at a *P* value of less than 0.05.

## Results

### Evaluation of cannula localization

In Fig. 1 the DRN sites where MCH, ATC, or vehicle were microinjected are shown in composite diagrams. The microinjection sites of 137 rats are marked as dark spots (at −7.80 mm from the bregma) and in open circles (at −7.68 and −7.92 mm) for 16. Each dark spot represents 9–17 microinjection sites. Open triangles represent the microinjection sites of 25 animals that were discarded (each represents 5–10 injection sites) because the cannula was localized in the periaqueductal gray, aqueduct, or lateral to the DRN. Variable or no behavioral effects were observed when cannula placement was outside the DRN. Only those animals with a correct anatomical localization were included in the data analysis.

Fig. 1



Representative localization of microinjection sites in the dorsal raphe nucleus. The figure shows a schematic diagram of coronal sections at three different levels ( $-7.68$ ,  $-7.80$ , and  $-7.92$  mm from Bregma) according to the atlas of Paxinos and Watson (2005), showing the MCH or saline microinjection sites as dark spots (each representing 9–17 animals) and 16 open circles (each representing an individual animal). Open triangles represent 5–10 sites of injection outside the DRN. DRN, dorsal raphe nucleus; MCH, melanin-concentrating hormone.

### Time course of the effect of MCH in the FST

Figure 2 shows the effect of MCH (50 ng) in the FST, evaluated 30 (Fig. 2a–c) and 60 min (Fig. 2d–f) after microinjection into the DRN. MCH significantly increased the immobility time compared with the respective control group at  $T_{30}$  [ $t_{(12)} = 3.18$ ,  $P < 0.01$ ]; this parameter was not significantly altered at  $T_{60}$  [ $t_{(12)} = 2.09$ ,  $P = 0.06$ ; Fig. 2d]. For swimming behavior, no significant differences were found between saline-treated and MCH-treated groups at either evaluation time [ $t_{(12)} = 1.88$ ,  $P = 0.08$  and  $t_{(12)} = 1.54$ , NS for  $T_{30}$  and  $T_{60}$ , respectively; Fig. 2b and e]. However, MCH led to a significant decrease in climbing time at  $T_{30}$  [ $t_{(12)} = 2.23$ ,  $P < 0.05$ ] and  $T_{60}$  [ $t_{(12)} = 2.42$ ,  $P < 0.05$ ] compared with the respective control group (Fig. 2c and f). Overall, MCH induced a clear depressive-like response at  $T_{30}$ , but this effect was less evident at  $T_{60}$ .

### Dose dependence of the effect of MCH in the FST

As significant differences in immobility and climbing time were observed 30 min after MCH (50 ng) microinjection, we selected this time to test the effect of MCH at two more doses (25 and 100 ng). Data from the previous test with MCH 50 ng at  $T_{30}$  were included in the analysis. Figure 3 shows that MCH elicits different behavioral profiles depending on the dose. A significant reduction in immobility time was observed when MCH was microinjected at the lowest concentration (25 ng) [ $t_{(10)} = 2.33$ ,  $P < 0.05$ ; Fig. 3a], without modifying swimming (Fig. 3b) or climbing (Fig. 3c) time [ $t_{(10)} = 0.78$ , 0.70, respectively, NS]. As reported above, MCH (50 ng) significantly increased immobility time ( $P < 0.01$ ; Fig. 3a), with a concomitant decrease in climbing time ( $P < 0.05$ ; Fig. 3c), but no effect on swimming time (Fig. 3b). Neither immobility [ $t_{(16)} = 1.27$ ,  $P = 0.22$ ] nor swimming [ $t_{(16)} = 1.31$ ,  $P = 0.22$ ] behavior was altered significantly by

MCH 100 ng; however, a significant decrease in climbing behavior was observed [ $t_{(16)} = 2.61$ ,  $P < 0.05$ ; Fig. 3c].

Locomotor activity was unchanged at any of the doses of MCH tested [ $F_{(3,24)} = 0.51$ , NS; Table 1]; thus, nonspecific effects can be excluded as an explanation for the FST results. However, the time spent in the center of the field was significantly increased by MCH 50 ng [ $F_{(3,24)} = 15.63$ ,  $P < 0.001$ ; post-hoc  $P < 0.001$ ], but not by MCH 25 and 100 ng.

### Effects of MCH in the FST in ATC pretreated animals

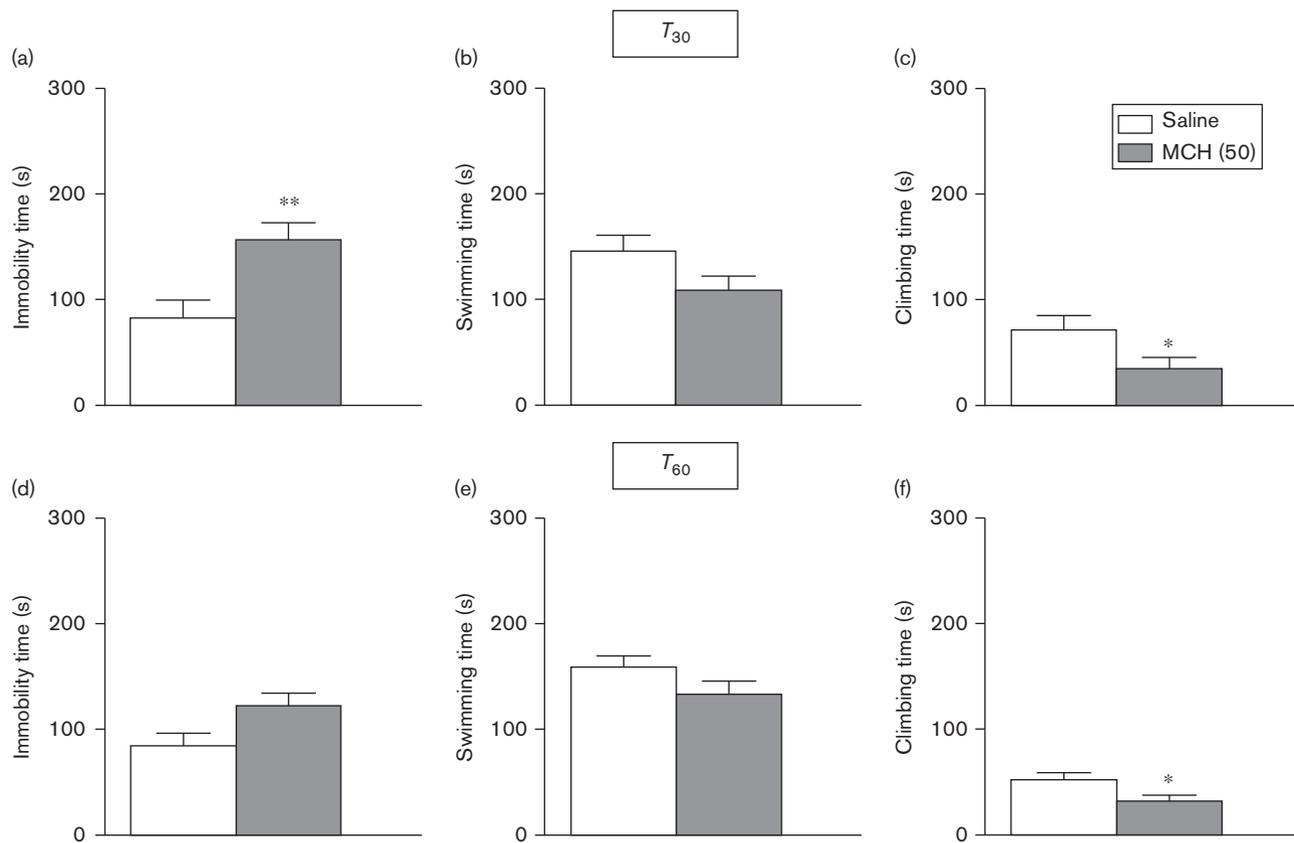
Figure 4 shows the effect on behavior in the FST of intra-DRN MCH (50 ng), evaluated at  $T_{30}$ , in ATC-pretreated or vehicle-pretreated animals. For immobility time, a significant effect of treatment was observed [ $F_{(3,24)} = 4.33$ ,  $P < 0.05$ ]. MCH significantly increased immobility time in vehicle-pretreated animals (vehicle + saline vs. vehicle + MCH,  $P < 0.05$ ; Fig. 4a), but this effect was absent in ATC-pretreated animals (vehicle + MCH vs. ATC + MCH,  $P < 0.05$ ; Fig. 4a). Intra-DRN ATC did not alter immobility time compared with the control group.

No significant treatment effects were observed on swimming behavior [ $F_{(3,24)} = 1.35$ , NS; Fig. 4b], but there was a significant treatment effect on climbing behavior [ $F_{(3,24)} = 4.42$ ,  $P < 0.05$ ]. A significant reduction in climbing behavior was elicited by MCH (vehicle + saline vs. vehicle + MCH,  $P < 0.05$ ; Fig. 4c) and this effect was significantly prevented by pretreatment with ATC (vehicle + MCH vs. ATC + MCH,  $P < 0.05$ ). ATC *per se* did not significantly alter climbing behavior compared with the control group.

### Effects of MCH in the FST in nortriptyline-pretreated animals

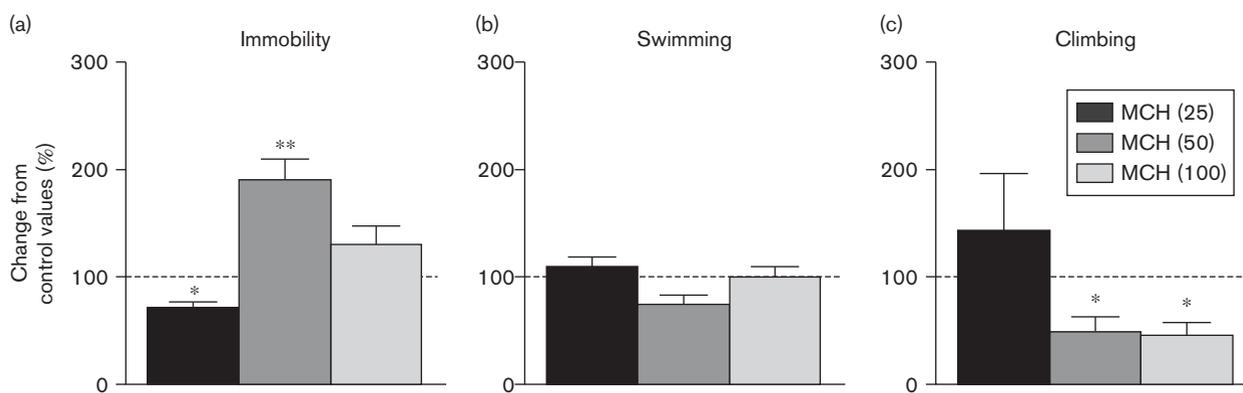
Figure 5 shows the effects of MCH (50 ng), evaluated 30 min after microinjection into the DRN in

Fig. 2



Effects of MCH 50 ng or saline on behavior in the FST 30 ( $T_{30}$ ; a–c) and 60 min ( $T_{60}$ ; d–f) after microinjection into the DRN. Bars represent mean  $\pm$  SEM of time spent in immobility, swimming, or climbing behavior. \* $P < 0.05$ ; \*\* $P < 0.01$ , relative to the respective control group; Student  $t$ -test;  $n = 7$  per group. DRN, dorsal raphe nucleus; FST, forced-swim test; MCH, melanin-concentrating hormone.

Fig. 3



Effects of MCH 25, 50, and 100 ng or saline on behavior in the FST 30 min after microinjection into the DRN. Data are shown as percent change in relation to the respective control group  $\pm$  SEM. The absolute values (mean  $\pm$  SEM) for control and treated animals, respectively, were as follows: Immobility time (a) 25 ng,  $99.5 \pm 11.0$  versus  $71.1 \pm 5.1$ ; 50 ng,  $82.4 \pm 16.7$  versus  $156.7 \pm 16.2$ ; 100 ng,  $86.2 \pm 13.1$  versus  $112.1 \pm 15.4$ . Swimming time (b) 25 ng,  $169.0 \pm 11.3$  versus  $184.0 \pm 15.3$ ; 50 ng,  $145.9 \pm 14.5$  versus  $108.6 \pm 13.4$ ; 100 ng,  $165.7 \pm 11.0$  versus  $165.9 \pm 14.6$ . Climbing time (c) 25 ng,  $31.5 \pm 8.4$  versus  $44.8 \pm 16.9$ ; 50 ng,  $71.7 \pm 13.1$  versus  $35.2 \pm 8.7$ ; 100 ng,  $48.1 \pm 6.4$  versus  $22.0 \pm 5.7$ . \* $P < 0.05$ ; \*\* $P < 0.01$ , for difference from the respective control group, two-tailed unpaired Student  $t$ -test;  $n = 6-9$  per group. DRN, dorsal raphe nucleus; FST, forced-swim test; MCH, melanin-concentrating hormone.

**Table 1** Effect of MCH microinjection into the DRN in the open-field test

Parameters	Treatment			
	Control	MCH (25)	MCH (50)	MCH (100)
Distance moved (m)	22.2±1.1	18.9±3.1	21.9±1.6	20.8±2.2
Center time (s)	3.1±1.4	3.2±1.2	11.2±1.2***	1.2±0.4

Values are represented as mean±SEM.

The control group was microinjected with saline.

One-way ANOVA, followed by Newman-Keuls test,  $n=6-7$  for each group.

The effects were evaluated 30 min after the microinjection.

ANOVA, analysis of variance; DRN, dorsal raphe nucleus; MCH, melanin-concentrating hormone.

\*Statistical differences versus the control group.

\*\*\* $P<0.001$ .

Nor-pretreated or vehicle-pretreated animals. A significant effect of treatment on immobility time was observed [ $F_{(3,30)}=8.09$ ,  $P<0.001$ ]. Post-hoc analysis showed a significant increase in the immobility time following MCH relative to vehicle-pretreated animals (vehicle + saline vs. vehicle + MCH,  $P<0.01$ ; Fig. 5a), but Nor pretreatment significantly prevented this effect (vehicle + MCH vs. Nor + MCH,  $P<0.001$ ; Fig. 5a). Administration of Nor plus intra-DRN saline induced a nonsignificant reduction in immobility time compared with the control group.

No treatment effects were observed on swimming behavior [ $F_{(3,30)}=0.66$ , NS; Fig. 5b]. However, in the case of climbing behavior, there was a significant treatment effect [ $F_{(3,30)}=16.11$ ,  $P<0.001$ ]. Post-hoc tests showed that, compared with the control group, MCH significantly reduced climbing behavior (vehicle + saline vs. vehicle + MCH,  $P<0.01$ ; Fig. 5c), and this effect was significantly prevented by Nor pretreatment (vehicle + MCH vs. Nor + MCH,  $P<0.001$ ). Consistent with the literature (Cryan *et al.*, 2002; Consoni *et al.*,

2006), systemic administration of Nor significantly increased climbing time compared with the control group (Nor + saline vs. vehicle + saline,  $P<0.01$ ).

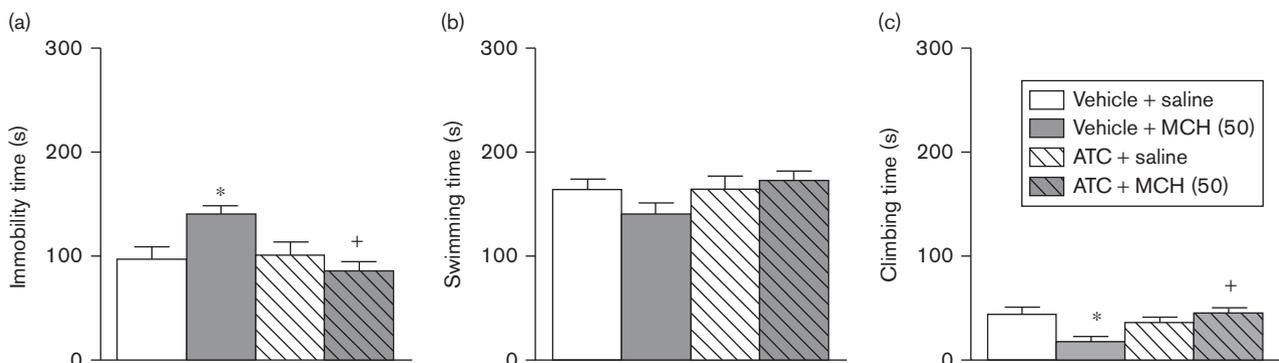
### Evaluation of the effect of MCH in the elevated plus-maze

Table 2 shows that the microinjection of MCH (50 ng) into the DRN at  $T_{30}$  did not modify anxiety-related behaviors or the distance moved in the EPM.

### Discussion

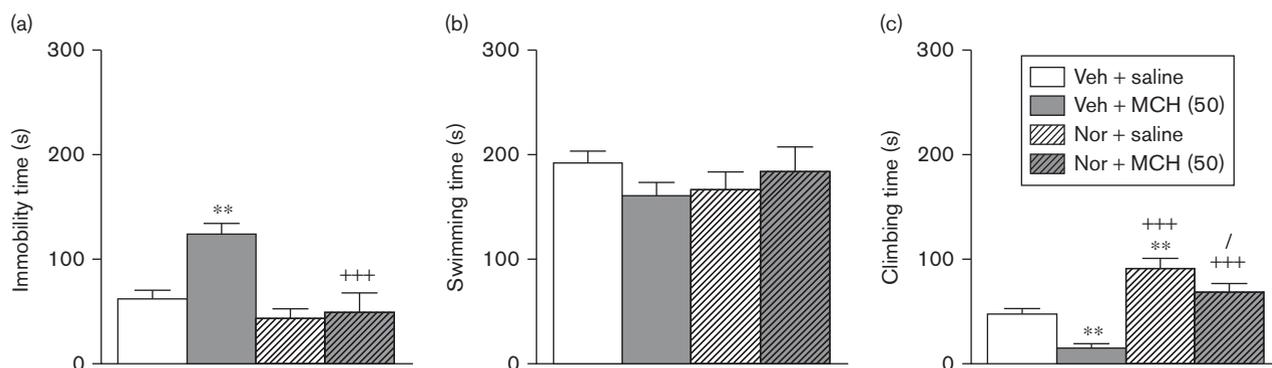
In the present study, we characterized the time course and dose-dependent behavioral effects induced by MCH microinjection into the DRN. This evaluation showed that the effect of MCH was time and dose dependent. Although MCH induced a clear depressive-like effect at 30 min after microinjection ( $T_{30}$ ) using a 50 ng dose, this behavioral response disappeared 60 min after MCH microinjection. Also, the lower dose of MCH (25 ng) seemed to elicit an antidepressive effect at  $T_{30}$ , whereas the higher dose assayed (100 ng) only decreased the climbing behavior. On the basis of these results and according to previous data (Lagos *et al.*, 2011a) collected at  $T_0$ , we considered that pretreatment times of 0–30 min and the 50 ng dose are the optimal conditions under which to continue the study of the mechanisms underlying the induction of depressive-related behaviors by intra-DRN MCH. Furthermore, the induction of a depressive-like response involves the activation of MCH-1 receptors, as ATC, the specific MCHR-1 antagonist, significantly reversed the effects of MCH.

Although a putative mechanism was not investigated directly, we have previously suggested that a link between the depressive-like effect of MCH and a decreased serotonergic neuronal activity within the DRN could be considered (Lagos *et al.*, 2011a). Several lines of

**Fig. 4**

Effect of MCH (50 ng) or saline on behavior in the FST 30 min after microinjection into the DRN in vehicle-pretreated or ATC-pretreated animals. The MCHR-1 antagonist ATC and vehicle were microinjected into the DRN 10 min before MCH microinjection. The bars represent mean±SEM of immobility time (a), swimming time (b), and climbing time (c). \* $P<0.05$  versus vehicle + saline; +  $P<0.05$  versus vehicle + MCH, one-way ANOVA, followed by the Newman-Keuls test;  $n=6-7$  per group. ANOVA, analysis of variance; DRN, dorsal raphe nucleus; FST, forced-swim test; MCH, melanin-concentrating hormone.

Fig. 5



Effects of MCH (50 ng) or saline on behavior in the FST 30 min after the microinjection into the DRN in vehicle-pretreated or nortriptyline (Nor)-pretreated animals. Nor and vehicle were administered intraperitoneally 24, 5, and 1 h before the FST test session. The bars represent mean  $\pm$  SEM of immobility time (a), swimming time (b), and climbing time (c). \*versus vehicle+saline; <sup>+</sup>versus vehicle+MCH; <sup>/</sup>versus Nor +saline; <sup>/</sup> $P < 0.05$ ; <sup>\*\*</sup> $P < 0.01$ ; <sup>+++</sup> $P < 0.001$ ; one-way ANOVA, followed by the Newman-Keuls test;  $n = 7-8$  per group. ANOVA, analysis of variance; DRN, dorsal raphe nucleus; FST, forced-swim test; MCH, melanin-concentrating hormone.

**Table 2** Effect of MCH microinjection into the DRN in the elevated plus-maze

Parameters	Treatment	
	Saline	MCH (50)
Entries OA (%)	19.8 $\pm$ 8.6	20.3 $\pm$ 5.6
Time OA (%)	8.2 $\pm$ 3.5	10.1 $\pm$ 3.5
Entries CA (%)	80.2 $\pm$ 8.6	79.6 $\pm$ 5.6
Time CA (%)	83.0 $\pm$ 4.1	82.9 $\pm$ 4.8
Risk assessment	1.8 $\pm$ 0.4	3.4 $\pm$ 0.6
Head dipping	4.5 $\pm$ 1.9	4.2 $\pm$ 1.7
Distance moved (m)	13.1 $\pm$ 1.1	14.4 $\pm$ 1.7

Values are represented as mean  $\pm$  SEM.

Student *t*-test,  $N = 7$  for each group.

No statistical differences were found between the control and the MCH-treated groups.

The effects were evaluated 30 min after the microinjection.

CA, closed arms; DRN, dorsal raphe nucleus; MCH, melanin-concentrating hormone; OA, open arms.

evidence support this hypothesis. It is well known that the DRN receives a dense network of MCHergic fibers (Bittencourt *et al.*, 1992; Torterolo *et al.*, 2008; Lagos *et al.*, 2009), which probably modulates DRN serotonergic neuronal activity. In preliminary studies, we observed that the intracerebroventricular or juxtacellular application of MCH into the DRN mainly decreased the firing rate of recorded units, which, from their basal electrophysiological characteristics, resembled the serotonergic neuronal phenotype (Pascovich *et al.*, 2011). In addition, perfusion of MCH (30  $\mu$ mol/l) intra-DRN decreased extracellular 5-HT levels measured by microdialysis (Urbanavicius *et al.*, 2013). In behavioral experiments, we had shown previously that the depressive-like profile induced by MCH introduction into the DRN was partially prevented by the systemic and subchronic administration of fluoxetine, an antidepressant drug, which increases the serotonergic neurotransmission by the inhibition of 5-HT reuptake (Lagos *et al.*, 2011a).

Moreover, microinjections of MCH into the DRN promoted the generation of REM sleep (Lagos *et al.*, 2009), a behavioral disturbance distinctive of depressive patients (Palagini *et al.*, 2013), whereas the immunoneutralization of this neuropeptide within the DRN inhibited REM sleep (Lagos *et al.*, 2011b). These results suggested that MCH inhibits the 'permissive' REM-off serotonergic neurons of the DRN (Monti, 2010; Palagini *et al.*, 2013). In accordance with all these results, it was shown that inhibition of DRN serotonergic neurons by electrical stimulation of the subthalamic nucleus elicited a depression-related behavior in the FST, which was reversed by citalopram, another 5-HT-enhancing antidepressant (Temel *et al.*, 2007). Taken together, this evidence strongly supports a serotonergic mechanism underlying the physiological effects of intra-DRN MCH. However, the participation of other neurotransmitters cannot be ruled out as the three doses of MCH tested induced different patterns of behavior in the FST. It is possible that depending on the dose, MCH modulates the serotonergic system directly and other neurotransmission systems indirectly. In fact, the activity of serotonergic neurons is highly controlled by local GABAergic interneurons, intrinsic serotonergic mechanisms, and afferent pathways of other neurotransmission systems to the DRN, such as GABAergic, glutamatergic, serotonergic, or noradrenergic (Adell *et al.*, 2002). In the present study, we showed that systemic pretreatment with Nor prevented the behavioral effect of intra-DRN MCH. Moreover, Nor could completely restore to control levels the behavioral changes induced by MCH (i.e. increased immobility time and decreased climbing behavior), which were only partially blocked by fluoxetine pretreatment (the increase in immobility time was significantly blocked, but the decrease in climbing behavior was not affected; Lagos *et al.*, 2011a). It is

possible that the systemic administration of Nor compensates the behavioral deficit by an antidepressant effect that is independent of the mechanism by which MCH elicits a prodepressive response. However, other interpretations could also be considered.

DRN 5-HT neurons receive ascending noradrenergic afferents that originate from the locus coeruleus (LC) (Anderson *et al.*, 1977; Baraban and Aghajanian, 1981) and it is well established that NA neurons modulate the serotonergic system (Clement *et al.*, 1992; Yoshioka *et al.*, 1992). Therefore, it is possible to consider a local effect of MCH on noradrenergic neurotransmission within the DRN. The intra-DRN microinjection of MCH could directly inhibit the noradrenergic transmission (probably through MCHergic receptors located on noradrenergic terminals), leading to disruption of a local 5-HT/NA balance. This could explain the behavioral pattern observed after MCH and its restoration by Nor treatment. Conversely, the serotonergic innervation of the LC derives partly from the DRN (Morgane and Jacobs, 1979; Vertes and Kocsis, 1994), and there is considerable evidence indicating that the serotonergic system functionally influences brain NA neurons (Haddjeri *et al.*, 1997; Kaehler *et al.*, 1999; Szabo and Blier, 2001). Therefore, a distal (or indirect) effect of intra-DRN MCH on LC NA neurons cannot be excluded.

Taken together, our data suggest that changes in the noradrenergic system are also directly or indirectly involved in the action of MCH. In this context, we have recently reported a prodepressive effect of MCH microinjected into the median raphe nucleus (MnR; López Hill *et al.*, 2013), although some differences were detected. A lower efficacy of MCH was observed in comparison with DRN microinjection (100 ng of MCH was needed to induce a depressive-like response in the MnR), and a different behavioral pattern was elicited by MCH (i.e. an increase in immobility time and decrease in swimming behavior, with no change in climbing behavior). We have proposed that MCH acts differently on the activity of MnR and DRN neurons altering the 5-HT/NA balance in both regions (López Hill *et al.*, 2013). Additional experiments should be conducted to clarify the anatomical and neurochemical bases of the action of MCH in the raphe nuclei.

In the present paper, we also showed that the depressive-like effect of MCH 50 ng at  $T_{30}$  was not accompanied by changes in anxiety-related behaviors, evaluated in the EPM. A similar lack of effect on anxiety-related behaviors in the EPM was observed previously following an intra-DRN microinjection of MCH at doses of 50 and 100 ng ( $T_0$ ) (Urbanavicius, 2013, unpublished data). The increase observed in the time spent in the center of the OFT suggests an anxiolytic effect of MCH. However, although it is unclear why MCH induced this effect in the OFT, the EPM is considered a more specific and

widely used paradigm to assess behaviors related to experimental anxiety (Pellow and File, 1986; Razafsha *et al.*, 2013).

These findings suggest that MCHergic projections to DRN neurons do not have functional consequences for anxiety, at least at the same dose at which the prodepressive effect is observed. The effect of central administration of MCH on anxiety-related behavior is unclear (Basso *et al.*, 2006): the intracerebroventricular injection of MCH or infusion directly into different brain regions has been reported to induce both anxiogenic (Gonzalez *et al.*, 1996; Smith *et al.*, 2006) and anxiolytic effects (Monzon and De Barioglio, 1999; Kela *et al.*, 2003; Carlini *et al.*, 2006). Here, we provide evidence that the DRN is not involved in the induction of anxiety-related behaviors by MCH. On the contrary, our data support a role for an MCHergic modulation on DRN neurons in the regulation of depression-related behaviors.

## Conclusion

We showed that MCH is functionally related to depressive-like, but not anxiety-related behaviors. In addition, the mechanism of action of MCH was shown to be mediated by MCHR-1 and probably involves a modulation of serotonergic and noradrenergic neurotransmission in the DRN.

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## Conflicts of interest

There are no conflicts of interest.

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