Progress in Neuro-Psychopharmacology & Biological Psychiatry 56 (2015) 227–234

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

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

Ghrelin effects expression of several genes associated with depression-like behavior

María Belén Poretti [a], Mathias Rask-Andersen [b], Praveen Kumar [b], Susana Rubiales de Barioglio [c], Marta Fiol de Cuneo [a,1], Helgi B. Schiöth [b], Valeria Paola Carlini [a,*,1]

[a] Instituto de Fisiología, Instituto de Investigaciones en Ciencias de la Salud (INICSA, UNC-CONICET), Facultad de Ciencias Médicas, CONICET and Universidad Nacional de Córdoba, Argentina
[b] Department of Neuroscience, Functional Pharmacology, Uppsala University, BMC, Uppsala SE 75124, Sweden
[c] Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, 5016 Córdoba, Argentina

ARTICLE INFO

Article history:
Received 24 July 2014
Received in revised form 23 September 2014
Accepted 23 September 2014
Available online 5 October 2014

Keywords:
Corticotrophin-releasing hormone
Ghrelin
Olfactory bulbectomy
Opioid receptors
Vasopressin

ABSTRACT

Ghrelin (Ghr) is an orexigenic peptide that is being investigated for its potential role in development of anxiety-like behavior and modulation of depressive-like symptoms induced by bilateral olfactory bulbectomy (OB) in rodents. Olfactory bulbectomy is an animal model useful to study of depression and Ghr could be an alternative therapeutic tool in depression therapy. We studied the effects of intracerebroventricular (i.c.v.) Ghr administration on the expression of hypothalamic genes related to depression and mood (delta opioid receptor (DOR), mu opioid receptor (MOR) and kappa opioid receptor (KOR), lutropin-choriogonadotropic hormone receptor (LHCGR), serotonin transporter (SERT), interleukin 1 beta (IL-1b), vasopressin (AVP) and corticotrophin releasing hormone (CRH)) in OB animals, as well as changes in plasma levels of AVP, CRH and adenocorticotropic hormone (ACTH). We found that acute Ghr 0.3 nmol/μl administration increases gene expression of DOR, SERT and LHCGR in OB mice and decreased expression of IL-1b, suggesting that these genes could be involved in the antidepressant-like effects of Ghr. In addition, OB animals exhibit high AVP gene expression and elevated plasma concentrations of AVP and ACTH and acute Ghr 0.3 nmol/μl administration reduces AVP gene expression and the concentration of these hormones, suggesting that peptide-effects on depressive-like behavior could be mediated at least in part via AVP. In conclusion, this study provides new evidence about genes, receptors and hormones involved in the antidepressant mechanism/s induced by Ghr in OB animals.

1. Introduction

Ghrelin (Ghr) is a peptide hormone involved in energy metabolism through activation of the growth hormone secretagogue receptor (GHSR1a), which is expressed mainly in the pituitary and hypothalamus (Howard et al., 1996). Not surprisingly, in addition to its diverse actions in the brain such as regulation of the pituitary–gonadal axis, appetite, energy balance, sleep, and the immune system (Delporte, 2013), Ghr has been shown to participate in physiological responses to stress and mood (Patterson et al., 2010). Ghrelin has been reported to produce anxiolytic- and antidepressant-like responses in mice (Lutter et al., 2008), although it has also been demonstrated that chronic central Ghr administration increases both anxiety- and depression-like behavior in rats (Hansson et al., 2011). We have shown that acute central administration of this peptide increases anxiety-like behavior and reverses some symptoms of depression induced by olfactory bulbectomy (OB) in rodents (Carlini et al., 2002, 2004, 2008, 2012). Consistent with this, administration of antisense oligonucleotides for Ghr decreases anxiety-like behavior in rats (Kanehisa et al., 2006).

Major depressive disorder (MDD), one of the most prevalent and disabling mental disorders, is characterized by low mood, loss of motivation, feelings of despair, and an inability to feel pleasure, also known as anhedonia (Li et al., 2013). Numerous functional changes have been implicated in the etiology of the depression, including changes in a wide range of hypothalamic neurotransmitters (serotonin, norepinephrine, dopamine and their transporters); pro-inflammatory cytokines; neuropeptides of central nervous system, such as endogenous opioid peptides, neuropeptide Y (NPY) and hypothalamic hormones (corticotrophin-releasing hormone (CRH), vasopressin (AVP) and sexual hormones)

Abbreviations: ACT, β-actin; ACTH, adenocorticotropic hormone; ANOVA, analysis of variance; AVP, vasopressin; CRH, corticotrophin releasing hormone; CYCLO, cyclophilin; DOR, delta opioid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ghr, ghrelin; HO-1, heme oxygenase 1; HPA, hypothalamic–pituitary–adrenal axis; IFN-g, interferon gamma; IL-1b, interleukin 1 beta; IL-6, interleukin 6; KOR, kappa opioid receptor; LHCGR, lutropin-choriogonadotropic hormone receptor; MDD, major depressive disorder; MOR, mu opioid receptor; NPY, neuropeptide Y; OB, olfactory bulbectomy; RPL19, ribosomal protein 19; SERT, serotonin transporter; TNF-a, tumor necrosis factor alpha; TUB, β-tubulin.

* Corresponding author at: Instituto de Fisiología, Instituto de Investigaciones en Ciencias de la Salud (INICSA, UNC-CONICET) Facultad de Ciencias Médicas, CONICET and Universidad Nacional de Córdoba, Santa Rosa 1085, X5000ESU Córdoba, Argentina. Tel./fax: +54 351 4332019.
E-mail address: vcarlini@live.com.ar (V.P. Carlini).

[1] Established investigator from CONICET.

http://dx.doi.org/10.1016/j.pnpbp.2014.09.012
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(Bodnar and Klein, 2004; Heilig, 2004; Redrobe et al., 2002; Stogner and Holmes, 2000).

The available evidence suggests that genetic factors contribute to the depression therapy. Previous candidate gene studies were mainly focused on the monoaminergic system, since the monoaminergic theory of MDD held a pivotal position during the past years. Thus, monoamine transporters (particularly serotonin transporter (SERT) and norepinephrine transporter genes), serotonin receptors, the enzymes involved in monoamine breakdown, and monoamine synthesis were among the most studied candidates. In parallel to the discovery of other pathophysiological abnormalities in MDD, new candidate genes were object of pharmacogenetic studies. A dysregulation of the hypothalamic–pituitary–adrenal axis (HPA) plays a pivotal role in depression (Niitsu et al., 2013). Other than the monoaminergic theories of MDD, the inflammatory hypothesis was formulated, being found a higher pro-inflammatory cytokines expression in the peripheral blood of patients with MDD (Tsao et al., 2006).

Recently, much attention has been drawn to potential therapeutic actions of Ghr in the central nervous system. Specifically, genetic and pharmacological studies suggest that Ghr may be of clinical relevance for psychiatric conditions like mood disorders (Kurt et al., 2007; Nakashima et al., 2008; Schmid et al., 2006). However, the molecules and/or the mechanism involved in the antidepressant effects of Ghr in depression animal models are not yet elucidated.

Considering that gene expression analysis represents a powerful and sensitive tool for investigating molecules that are affected in psychiatric disorders (Molteni et al., 2013) and that acute Ghr administration reverses depressive-like behavior induced by OB surgery (Carlini et al, 2012), we studied the effects of central Ghr administration on the expression of some hypothalamic genes related to the depression and mood in animals with OB, as well as changes in plasma levels of AVP, CRH and adenocorticotropic hormone (ACTH). The genes analyzed were: opioid receptors: delta opioid receptor (DOR), mu opioid receptor (MOR) and kappa opioid receptor (KOR), lutropin-choriogonadotropic hormone receptor (LHCGR), SERT, interleucine 1-beta (IL-1b), AVP and CRH.

2. Materials and methods

2.1. Animals

Adult female mice (Albino Swiss-SWR/J (q)) with an initial body weight of ≅25 g were used in this study. The colony room was maintained under controlled temperature (20 ± 2 °C) and light (12 h light, 12 h dark), with access to water and food ad libitum. Before the surgery, animals were anesthetized using a combination of 55 mg/kg ketamine HCl (Vetanarcol König: Laboratorios König S.A., Argentina) and 11 mg/kg xylazine (Kensol König: Laboratorios König S.A., Argentina). After surgery, animals were housed in individual cages.

All procedures were performed in accordance to the guidelines of the Institutional Committee of Laboratory Animal Care and Use, School of Medical Sciences, National University of Córdoba.

2.2. Drugs and treatment

Ghrelin (Neosystem, France) was dissolved in saline, divided into aliquots and kept at −20 °C until the day of the experiment. Ghrelin was infused acutely by intracerebroventricular (i.c.v.) route.

2.3. Experimental procedure

The animals were divided into two groups:

a) Sham group (without OB): mice received the same surgery procedure that the bulbectomized animals, except that the olfactory bulbs were not removed.

b) Bulbectomized group: animals were subjected to the surgery procedure for ablation of the olfactory bulbs.

Thirty days after OB surgery, mice were i.c.v. cannulated and approximately seven days after, between 09:00 and 10:00 h, the animals were treated with sterile saline (sham-vehicle and OB-vehicle), Ghr 0.3 nmol/μl (sham-Ghr 0.3 and OB-Ghr 0.3) or Ghr 3.0 nmol/μl (sham-Ghr 3.0 and OB-Ghr 3.0). Thirty minutes later, the animals were killed by decapitation in a room separate from that in which the other animals were kept. Trunk blood was collected in heparinized Eppendorf tubes and immediately centrifuged (1700 rpm, 10 min, 4 °C). Individual plasma samples were frozen and stored (at −20 °C) for subsequent determination of AVP, CRH and ACTH concentrations. In addition, brains were removed and the hypothalamus was collected by dissection and preserved in RNAlater solution (Ambion, Austin, TX, USA), and stored at −20 °C.

The occurrence of estrous cycle was evaluated daily by vaginal smears to females of all the experimental groups, between day 16 after OB surgery and the final day of the treatment. Animals were administrated during the diestrus phase (approximately seven days after i.c.v. surgery).

2.4. Olfactory bulbectomy surgery

Midsagittal incision was given on the skull and the skin was retracted. The soft tissues overlying the skull were removed. The landmarks of the skull, bregma and lambda, were identified and the skull was oriented such that both points were positioned in horizontal level. After clearing the underlying fascia, a burr-hole 2 mm in diameter was drilled through the skull 8 mm anterior to the bregma and 2 mm to either side of the midline. While the olfactory bulbs were removed by suction, care was taken to avoid the damage to frontal cortex. Then the burr-holes were filled with haemostatic sponge in order to control the bleeding. Sham-operated rats were treated in the same way, but the olfactory bulbs were left undisturbed.

2.5. Intracerebroventricular surgery

After 30 days of OB, mice were placed in a stereotaxic apparatus and subjected to i.c.v. surgery using a steel guide cannula, according to the methods described by Franklin and Paxinos (2008). The coordinates relative to bregma were: anterior 0.2 mm, lateral 1.0 mm and vertical 2.8 mm. Cannulae were fixed to the skull surface with dental acrylic cement. Seven days after surgery, animals were infused with Ghr or saline using a 10 μl Hamilton syringe connected by Pe-10 polyethylene tubing to a 30-gauge needle extending it 0.75 mm beyond the guide cannula. Each infusion of 1 μl was delivered over a 1 min period.

2.6. Gene expression

2.6.1. RNA isolation and cDNA synthesis

Thirty minutes after i.c.v. administration, the animals were killed by decapitation and its brains were removed, collected the hypothalamus by dissection. Removal of the hypothalamus was conducted from the ventral side of the brain. The optic chiasm was dissected away from the anterior portion of the hypothalamus, followed by dissection of the mammillary nuclei from the posterior of the hypothalamus. The entire hypothalamus was dissected including the arcuate, ventromedial, dorsomedial, and paraventricular nuclei. Tissue samples were preserved in RNAlater solution and then homogenized by sonication in TRIzol (Invitrogen, Sweden) using a sonicator. Chloroform was added to the homogenate, which was then centrifuged at 10,000 ×g at 4 °C for 15 min. The aqueous phase was then transferred into a new tube and the RNA was precipitated with isopropanol. Pellets were washed with 75% ethanol, air-dried at room temperature and dissolved in RNAse-free water. DNA was removed by treatment with DNAse I (Roche

Diagnostics, Sweden) at 37 °C for 4 h and the enzyme was thereafter inactivated by heating the samples at 75 °C for 15 min. The absence of genomic DNA was determined by PCR analysis with primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_017008; forward TCCCTCAAGATTGTCAGCAA and reverse CACCACCTTC TTGATGTCATC). RNA concentrations were measured using a NanoDrop1 ND-1000 spectrophotometer (NanoDrop1 Technologies, Delaware, USA). cDNA was synthesized with M-MLV reverse transcriptase using random hexamers as primers, according to the manufacturer's recommendations (GE Healthcare, Sweden).

2.6.2. Real-time PCR

The cDNA was analyzed with a MyIQ thermal cycler (Bio-Rad Laboratories, Sweden). Each real-time PCR reaction with a total volume of 20 μl contained cDNA synthesized from 25 ng total RNA, 0.25 M of each primer, 20 mM Tris/HCl (pH 8.4), 50 nM KCl, 4 mM Mg Cl2, 0.2 mM dNTP, SYBR Green (1:50,000). A real-time PCR reaction was performed with 0.02 U/l Taq DNA polymerase (Invitrogen, Sweden) under the following conditions: initial denaturation for 4 min at 95 °C followed by 50 cycles of 15 s each at 95 °C, 30 s at 55–62 °C (i.e. at the optimal annealing temperature for each primer pair), and 30 s at 72 °C. This step was followed by 1 min at 55–62 °C (optimal annealing temperature) and a melting curve with 84 cycles of 10 s at 55 °C, increased by 0.5 °C per cycle. All experiments were performed in duplicate. Measurements in which the threshold cycle (Ct) values between the duplicates had a difference equal to or greater than 0.9 were repeated. A negative control for a given primer pair and a positive control with 25 ng of genomic DNA was included in each plate. The following housekeeping genes were used to define expression normalization factors: GAPDH, β-tubulin (TUB), ribosomal protein 19 (RPL19), cyclophilin (CYCLO) and β-actin (ACT). Normalization factors were calculated using the GeNorm method described by Vandesompele et al. (2002). The primers RPL19, Cyclo, GAPDH and the primers studied (DOR, MOR, KOR, LHCGR, SERT, IL-1b, AVP and CRH) were designed using Beacon Primer Design 4.0 software (Premier Biosoft, USA) (Appendix 1).

2.7. Hormone assays

Plasma concentrations of AVP, CRH and ACTH were assayed using commercial mouse I^{125} RIA kits following the manufacturer's instructions (Phoenix Pharmaceutical Inc., USA). The range of detection for the radioimmunoassays was 10–1280 pg/ml. The lowest detection limit for each hormone was: ACTH 28.4 pg/ml, CRH 26.5, pg/ml AVP 31.1 pg/ml. The inter-, and intra-assay coefficients of variation were 9% and 1% respectively.

2.8. Statistical methods

Data were analyzed using a STATISTICA — StatSoft (Version 10) statistical package. Comparisons between groups were performed by a two-way analysis of variance (ANOVA), with two blocks: condition (sham and OB) and treatment (saline, Ghr 0.3 nmol/μl or Ghr 3.0 nmol/μl). When required, pairwise contrasts of main effects or simple main effects were conducted using Bonferroni post-test. Data are expressed as mean ± standard error mean (SEM) and p values lower than 0.05 were considered statistically significant.

3. Results

3.1. Molecular analysis of genes following olfactory bulbectomy and central ghrelin administration

The effects of acute i.c.v. Ghr administration on gene expression of opioid receptors into the hypothalamus are shown in Fig. 1. As can be seen in the panel A, OB animals treated with Ghr 0.3 nmol/μl showed an increase in DOR expression, compared to OB-vehicle animals ($p = 0.0216$). On the contrary, no changes were seen in sham animals treated with Ghr ($p > 0.05$). The OB mice that received Ghr 0.3 nmol/μl also exhibited a higher expression of DOR gene in comparison to sham mice that received the same dose of peptide ($p = 0.0059$). The two-way ANOVA test revealed a significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 5.48$, $p = 0.0090$); a significant effect of the animals condition ($F(1, 24) = 8.24$, $p = 0.0072$) and a no significant effect of Ghr administration ($F(2, 24) = 2.58$, $p = 0.0911$).

In relation to gene expression of KOR and MOR (Fig. 1 panels B and C, respectively), OB mice that received Ghr 0.3 nmol/μl exhibited a higher expression of MOR gene in comparison to sham mice that received the same dose of peptide ($p = 0.0216$). In KOR analysis, the two-way

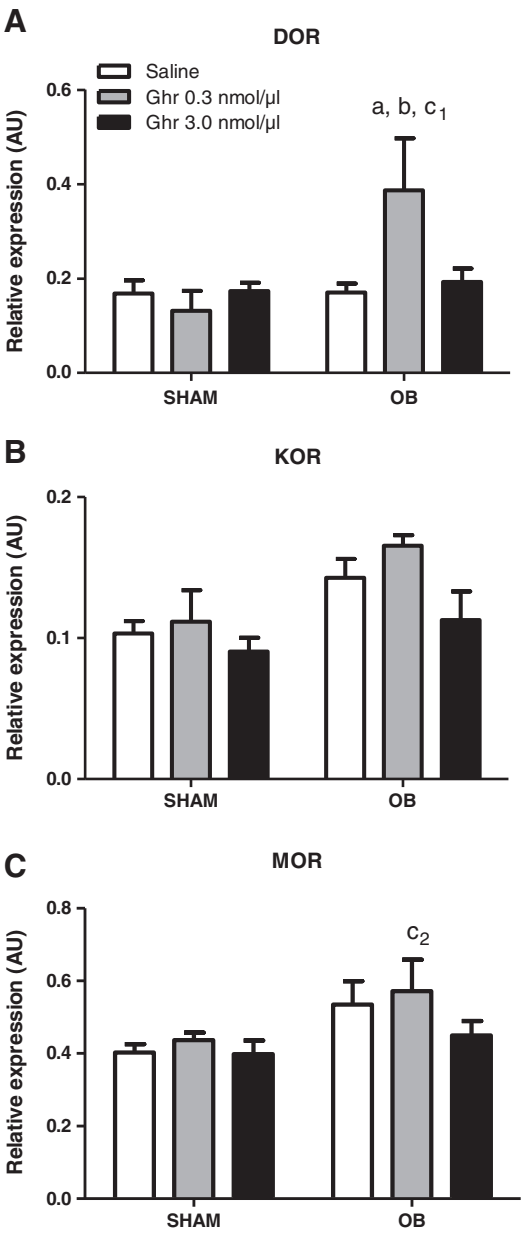

Fig. 1. Effects of acute i.c.v. administration of Ghr on opioid receptors gene expression in the hypothalamus of sham and OB mice. Panel A: delta opioid receptor (DOR). Panel B: kappa opioid receptor (KOR). Panel C: mu opioid receptor (MOR). Sham (without OB). Bulbectomized group (OB). Animals were i.c.v. cannulated and treated with saline (vehicle), Ghr 0.3 nmol/μl or Ghr 3.0 nmol/μl. Data are expressed as Mean ± SEM. n = 5 animals in each group. a: significant differences vs. sham-vehicle ($p = 0.0116$) b: significant differences vs. OB-vehicle ($p = 0.0216$); c: significant differences vs. sham-Ghr 0.3 nmol/μl (c_1: $p = 0.0059$, c_2: $p = 0.0216$).

ANOVA test revealed a no significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 0.41$, $p = 0.6652$); a no significant effect of the animals condition ($F(1, 24) = 2.32$, $p = 0.1168$) and a no significant effect of Ghr administration ($F(2, 24) = 7.26$, $p = 0.0118$).

In MOR analysis, the two-way ANOVA test revealed a no significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 0.60$, $df = 2$, $p = 0.5569$); a significant effect of the animals condition ($F(1, 24) = 7.99$, $p = 0.0081$) and a no significant effect of Ghr administration ($F(2, 24) = 1.53$, $p = 0.2328$).

Fig. 2 shows the effects of acute i.c.v. Ghr administration on LHCGR expression in the hypothalamus. The two-way ANOVA test revealed a no significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 2.13$, $p = 0.1357$); a significant effect of the animals condition ($F(1, 24) = 5.62$, $p = 0.0241$) and a significant effect of Ghr administration ($F(2, 24) = 7.17$, $p = 0.0028$).

A lower expression of LHCGR was observed in sham-Ghr 3.0 nmol/μl compared to sham-vehicle ($p = 0.0032$). The expression was significantly up-regulated in the OB-Ghr 0.3 nmol/μl compared to OB-vehicle ($p = 0.0219$) and sham-vehicle ($p = 0.0411$).

The effects of acute i.c.v. Ghr administration on SERT gene expression in the hypothalamus are shown in Fig. 3. As can be seen, sham animals treated with Ghr 3.0 nmol/μl showed a lower SERT expression compared to sham-vehicle ($p = 0.0209$). The OB-vehicle mice presented a lower level of SERT expression in relation to sham-vehicle ($p = 0.0034$), that was attenuated when the animals were treated with Ghr 0.3 nmol/μl ($p = 0.0418$). The two-way ANOVA test revealed a significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 7.75$, $p = 0.0018$); a significant effect of the animals condition ($F(1, 24) = 4.40$, $p = 0.0416$) and a significant effect of Ghr administration ($F(2, 24) = 5.58$, $p = 0.0022$).

Fig. 4 shows the Ghr effects on IL-1b expression. The two-way ANOVA test revealed a significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 3.77$, $p = 0.0347$); a significant effect of the animals condition ($F(1, 24) = 6.46$, $p = 0.0039$) and a no significant effect of Ghr administration ($F(2, 24) = 2.76$, $p = 0.0793$). A higher expression of IL-1b was observed in OB mice that received saline compared to sham-vehicle ($p = 0.0015$). The Ghr 3.0 nmol/μl administration reduced IL-1b expression in OB animals ($p = 0.0049$). No significant differences were observed in sham animals treated with Ghr.

Acute Ghr effects on gene expression of CRH and AVP are shown in Fig. 5. As can be seen in panel A, we did not observe differences in CRH gene expression. The two-way ANOVA test revealed a no significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 5.09$, $p = 0.9714$); a no significant effect of the animals condition ($F(1, 24) = 3.64$, $p = 0.0653$) and a no significant effect of Ghr administration ($F(2, 24) = 1.62$, $p = 0.2145$).

The OB-vehicle animals present a higher level of AVP expression in relation to sham-vehicle ($p = 0.0103$) (panel B), that was attenuated in mice treated with Ghr 0.3 and 3.0 nmol/μl ($p = 0.0009$ and $p = 0.0001$, respectively). It was observed an increase on AVP expression ($p = 0.0036$) in sham animals i.c.v. infused with Ghr 3.0 nmol/μl.

The two-way ANOVA test revealed a significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 21.21$, $p = 0.0001$); a significant effect of the animals condition ($F(1, 24) = 8.19$, $p = 0.0052$) and a significant effect of Ghr administration ($F(2, 24) = 6.45$, $p = 0.0048$).

3.2. Effects of ghrelin on plasma levels of AVP, CRH and ACTH in sham and OB mice

As can be seen in Fig. 6, panel A, plasma CRH levels were similar in sham and OB mice treated with vehicle. The i.c.v. Ghr 3.0 nmol/μl infusion increased plasma CRH levels in the sham group in compared to saline treated animals ($p = 0.0001$). The two-way ANOVA test

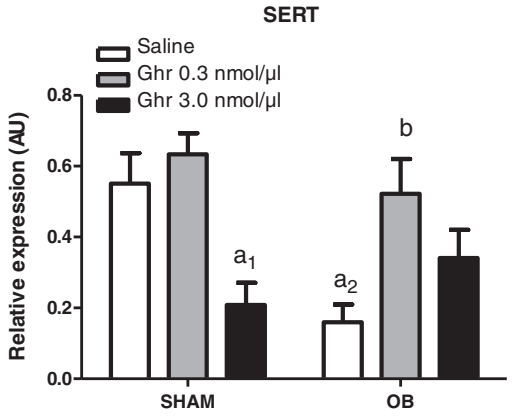

Fig. 3. Effects of acute i.c.v. administration of Ghr on serotonin transporter (SERT) gene expression into the hypothalamus in sham and OB mice. Sham (without OB). Bulbectomized group (OB). Animals were i.c.v. cannulated and treated with saline (vehicle), Ghr 0.3 nmol/μl or Ghr 3.0 nmol/μl. Data are expressed as Mean ± SEM. n = 5 animals in each group. a: significant differences vs. sham-vehicle (a_1: $p = 0.0209$, a_2: $p = 0.0034$); b: significant differences vs. OB-vehicle ($p = 0.0418$).

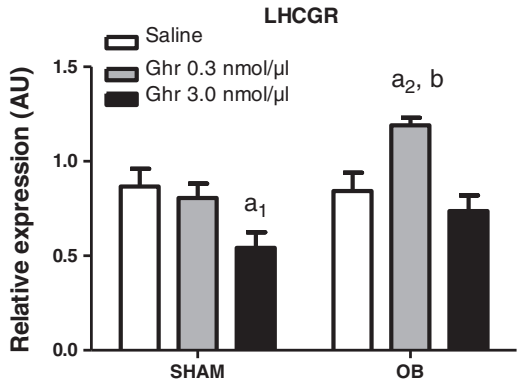

Fig. 2. Effects of acute i.c.v. administration of Ghr on luteinizing hormone receptor (LHCGR) gene expression in the hypothalamus of sham and OB mice. Sham (without OB). Bulbectomized group (OB). Animals were i.c.v. cannulated and treated with saline (vehicle), Ghr 0.3 nmol/μl or Ghr 3.0 nmol/μl. Data are expressed as Mean ± SEM. n = 5 animals in each group. a: significant differences vs. sham-vehicle (a_1: $p = 0.0032$, a_2: $p = 0.0411$); b: significant differences vs. OB-vehicle ($p = 0.0219$).

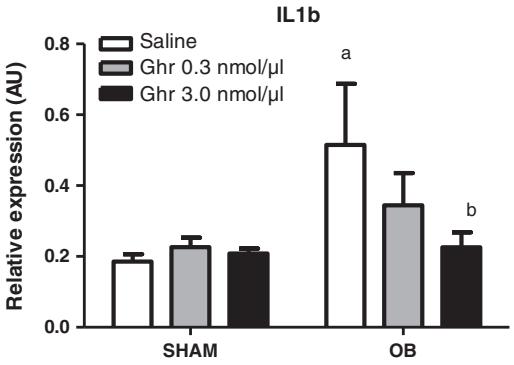

Fig. 4. Effects of acute i.c.v. administration of Ghr on interleukin 1 beta (IL-1b) gene expression into the hypothalamus in sham and OB mice. Sham (without OB). Bulbectomized group (OB). Animals were i.c.v. cannulated and treated with saline (vehicle), Ghr 0.3 nmol/μl or Ghr 3.0 nmol/μl. Data are expressed as Mean ± SEM. n = 5 animals in each group. a: significant differences vs. sham-vehicle ($p = 0.0015$); b: significant differences vs. OB-vehicle ($p = 0.0049$).

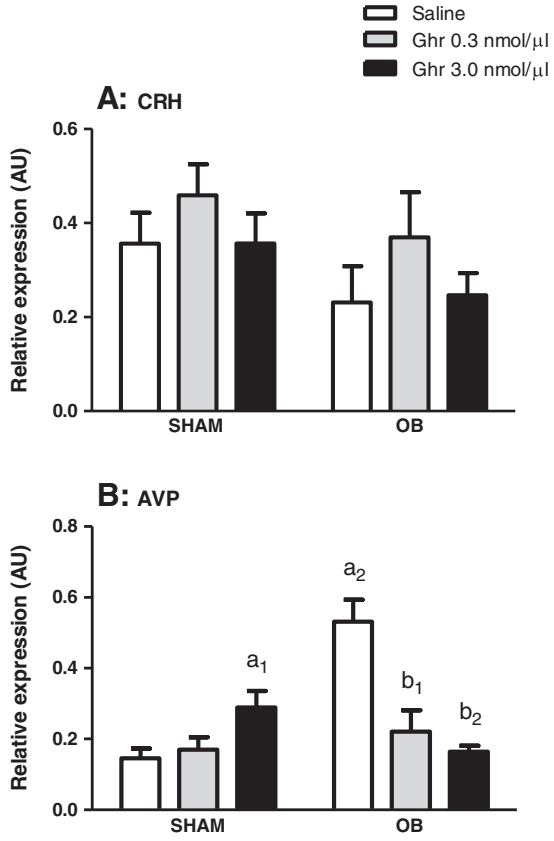

Fig. 5. Effects of acute i.c.v. Ghr administration on plasma levels of AVP, CRH and ACTH in sham and OB mice. Corticotrophin releasing hormone (CRH); vasopressin (AVP), adrenocorticotropic hormone (ACTH). Sham (without OB). Bulbectomized group (OB). Animals were i.c.v. cannulated and treated with saline (vehicle), Ghr 0.3 nmol/μl or Ghr 3.0 nmol/μl. Data are expressed as Mean ± SEM. n = 5 animals in each group. a: significant differences vs. sham-vehicle (a_1: p = 0.0036, a_2: p = 0.0103); b: significant differences vs. OB-vehicle (b_1: p = 0.0009, b_2 p = 0.0001).

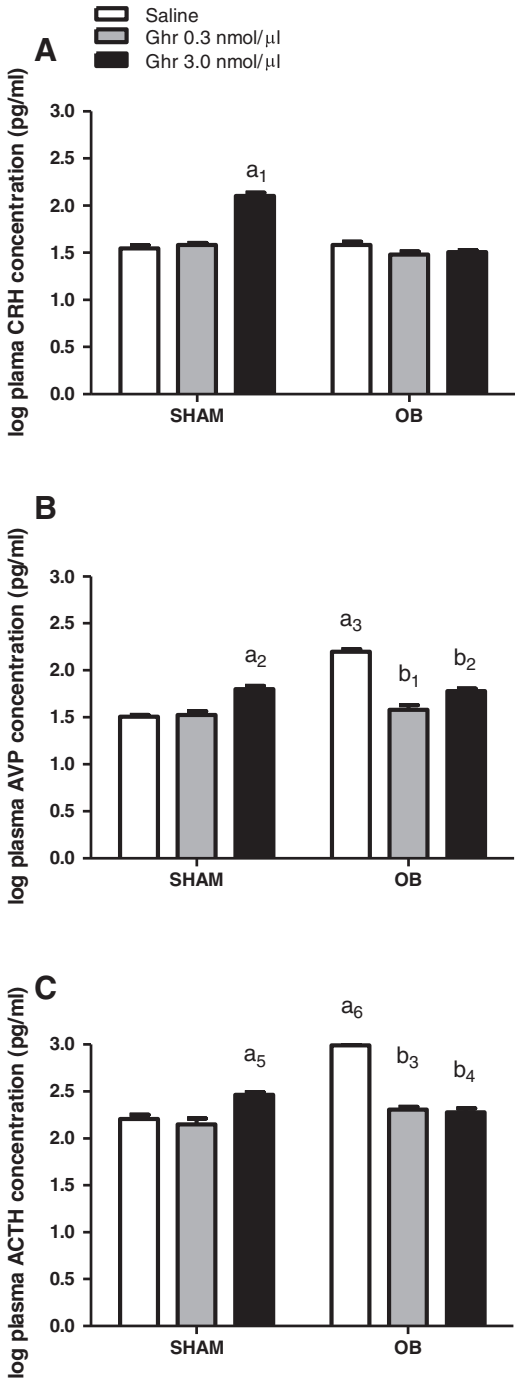

Fig. 6. Effects of Ghr on plasma levels of AVP, CRH and ACTH in sham and OB mice. Corticotrophin releasing hormone (CRH); vasopressin (AVP), adrenocorticotropic hormone (ACTH). Sham (without OB). Bulbectomized group (OB). Animals were i.c.v. cannulated and treated with saline (vehicle), Ghr 0.3 nmol/μl or Ghr 3.0 nmol/μl. Data are expressed as Mean ± SEM. n = 5 animals in each group. a: significant differences vs. sham-vehicle (a_1: p = 0.0001, a_2: p = 0.0001, a_3: p = 0.0002, a_4: p = 0.0001, a_5: p = 0.0001, a_6: p = 0.0001); b: significant differences vs. OB-vehicle (b1: p = 0.0001; b2: p = 0.0001; b3: p = 0.0001; b4: p = 0.0001).

revealed a significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 84.58$, $p = 0.0001$); a significant effect of the animals condition ($F(1, 24) = 103.70$, $p = 0.0001$) and a significant effect of Ghr administration ($F(2, 24) = 65.52$, $p = 0.0001$).

Plasma levels of AVP and ACTH were observed to be increased in OB-vehicle treated mice compared to sham-vehicle treated ($p = 0.0001$ (AVP); $p = 0.0001$ (ACTH)). In the sham group, Ghr 3.0 nmol/μl infusion was observed to increase AVP ($p = 0.0002$) and ACTH ($p = 0.0001$), while in the OB group, both infusion of Ghr (0.3 and 3.0 nmol/μl) reduced plasma concentration of these hormones ($p = 0.0001$ (AVP) and $p = 0.0001$ (ACTH)).

The two-way ANOVA test revealed a significant interaction between the Ghr treatment and condition (sham and OB) ($F(2, 24) = 61.99$, $p = 0.0001$ (AVP); $F(2, 24) = 127.50$, $p = 0.0001$ (ACTH); a significant effect of the animals condition ($F(1, 24) = 77.44$, $p = 0.0001$ (AVP); $F(1, 24) = 100.06$, $p = 0.0001$ (ACTH)) and a significant effect of Ghr administration) ($F(2, 24) = 42.74$, $p = 0.0001$ (AVP); $F(2, 24) = 68.83$, $p = 0.0002$ (ACTH)).

4. Discussion

It has been demonstrated that Ghr signaling contributes to neural control of anxiety and emotional states as acute i.c.v. administration of Ghr 0.3 nmol/μl produced antidepressant-like effects in tail suspension test, and reversed depressive-like behavior induced by OB (Carlini et al., 2012). Nevertheless, the neural mechanisms underlying these Ghr effects remain obscure. The present work studies the effects of acute i.c.v. Ghr administration on the expression of hypothalamic genes related to depressive disorders in OB mice, such as DOR, MOR, KOR, LHCGR, SERT, IL-1b, AVP and CRH. We found that acute Ghr 0.3 nmol/μl administration increases gene expression of DOR, SERT and LHCGR in OB mice and decreased IL-1b, suggesting that these genes could be involved in the antidepressant-like effect of the peptide reported (Carlini et al., 2012; Lutter et al., 2008).

In recent years, the opioid system has been employed as a target for treatment of depression and mood disorders. Delta-opioid receptor activation has been shown to produce antidepressant-like effects in a number of preclinical assays and in depression animal models (Broom et al., 2002; Perrine et al., 2006; Tejedor-Real et al., 1998; Torregrossa et al., 2006). Changes observed in the DOR expression in our experimental model are in accordance with evidence proving that Ghr reverses depressive-like symptoms in the OB animals (DOR up-regulation was seen in OB animals infused with Ghr 0.3 nmol/μl) this effect was not found with the higher dose of Ghr employed (Ghr 3.0 nmol/μl). In contrast to these findings, Skibicka et al. (2012) were unable to observe altered DOR expression in the ventral tegmental area after Ghr infusion for seven days, but were able to observe increased MOR expression in this structure. Differences in experimental models, state of the subjects (normal or depressive), administration protocols (acute, sub-chronic or chronic), and/or the doses employed, could be responsible for some discrepancies in the reports and, in consequence, this topic needs further investigation.

The serotonergic system plays a fundamental role in a wide variety of physiological and behavioral processes and the maintenance of serotonin neurotransmission largely depends of its transporter activity. Serotonin transporter has been also the focus of investigation in MDD (Haddley et al., 2008). It has been demonstrated that mice with constitutive reductions in SERT expression show elevated anxiety-related behavior (Holmes et al., 2003), enhanced stress reactivity (Bartolomucci et al., 2010; Li et al., 1999; Tjurmina et al., 2002), and in some background strains, increased depressive-like behavior (Holmes et al., 2002; Nonkes et al., 2010; Olivier et al., 2008). In addition, it has been shown that Ghr promotes a direct regulatory effect on serotonin release (Brunetti et al., 2002; Ghersi et al., 2011) and that mRNA expression of serotonin receptor subtypes in the dorsal raphe and amygdala are affected by acute central infusion of Ghr (Hansson et al., 2014).

In the gene expression analysis, there was also evidence that Ghr influences the central serotonin system reflected by SERT down-regulation in sham-Ghr 3.0 nmol/μl mice. Coincidently, animals treated with Ghr 3.0 nmol/μl exhibited anxiogenic responses in the elevated plus maze (Carlini et al., 2008). The fact that treatment with Ghr 0.3 nmol/μl induces SERT up-regulation could partly explain the antidepressant effects of the peptide in OB mice. Moreover, SERT down-regulation induced by Ghr 3.0 nmol/μl administration in sham animals could explain the anxiety-like responses reported in a previous study (Carlini et al., 2008).

The inflammatory theory of MDD suggests that alterations in cytokines and other autoimmune factors result in neuronal damage, neurodegenerative diseases, and clinical depression (Dantzer et al., 2008; Hayley et al., 2005). Patients with MDD present altered levels of pro- and antiinflammatory cytokines in circulation (Dowlati et al., 2010; Howren et al., 2009; Leonard and Maes, 2012; Maes, 1994; Miller et al., 2009). Tsao et al. (2006) found that the expression of IL-1b, IL-6, TNF-a and IFN-g were higher in the peripheral blood mononuclear cells of MDD patients. In addition, postmortem studies have also described gene expression alterations in a variety of these cytokines in the frontal cortex of patients with MDD (Shelton et al., 2011). Moreover, IL-b and TNF-a have been described as predictors of antidepressant treatment response (Hepgul et al., 2013).

Our results shows that IL-1b expression was increased in OB-vehicle compared to sham mice treated with vehicle and that the acute Ghr 3.0 nmol/μl administration in OB animals reduces IL-1b up-regulation. In addition, we did not observe changes in gene expression of other pro-inflammatory cytokines, such as IL-6 and heme oxygenase 1 (HO-1) (not shown). Although, some studies have demonstrated that Ghr modulates the pro-inflammatory and anti-inflammatory cytokines inhibiting the production of TNF-a, IL-6 and IL-8 in human endothelial cells (Waseem et al., 2008).

It has been demonstrated that central actions of estrogens can profoundly modify mood and depressive episodes (Douma et al., 2005). The fact that LHCGR expression was increased in OB mice infused with Ghr 0.3 nmol/μl, provides evidence that the peptide antidepressant-like activity could be associated with a variation in LHCGR hypothalamic expression. Interestingly, it has been observed that LHCGR is down-regulated by stress-related hormones such as CRH and proopiomelanocortin during an acute stress condition thereby altering sexual function (Rivier and Rivest, 1991).

It is argued that an HPA overstimulation has been frequently associated with MDD (Barden, 2004; Holsboer and Barden, 1996; Nemeroff et al., 1984; Raadsheer et al., 1994; Scott and Dinan, 1998) and HPA activation is a potential mechanism by which Ghr could be regulating depression-related behavior (Smith and Vale, 2006; Spencer et al., 2012). We were not able to observe a change on the hypothalamic CRH gene expression and on plasma CRH levels in OB mice, suggesting that antidepressant-like effect of Ghr could be independent of CRH. In relation to AVP, OB animals exhibit high AVP gene expression and elevated plasma concentrations of AVP and ACTH, which was reduced by acute Ghr 0.3 nmol/μl administration. Our findings are in line with previous studies showing that bulbectomy leads to an increase in AVP in the pituitary, but do not induce any effect on CRH expression into the hypothalamus in mice (Marcilhac et al., 1999).

Considering that OB animals treated with Ghr 0.3 nmol/μl: 1) exhibited similar CRH gene expression and plasma CRH levels and 2) plasma levels of AVP and ACTH were lower compared to OB-vehicle, it is hypothesized that Ghr-effects on depressive-like behavior could be mediated at least in part via AVP.

On the other hand, our results also shows that i.c.v. administration of Ghr 3.0 nmol/μl increases plasma levels of CRH and AVP in sham mice, which could explain the anxiogenic effect reported by Carlini et al. (2008). In this context, it has been reported that Ghr facilitates ACTH release via activation of CRH (Korbonits and Grossman, 2004; Mozid et al., 2003) and that intraperitoneal Ghr injection increases hypothalamic CRH gene expression in mice (Asakawa et al., 2001).

It has been shown that following olfactory bulbectomy appears anatomical, behavioral and biochemical changes, such as a hyperactivity of cholinergic and noradrenergic system at the central level (van Riezen and Leonard, 1990; Jancsár and Leonard, 1984; Song and Leonard, 1995; Broekkamp et al., 1986), and/or a reduction in the serotonin concentration, which is compensated by a increase in tryptophan hydroxylase activity, serotonin transporter sites and 5HT2A receptor number (Huether et al., 1997; Zhou et al., 1998; Grecksch et al., 1997). These changes as well as other yet unknown could probably be responsible of opposite Ghr effects on gene expression of SERT, LHCGR and AVP and plasma concentration of AVP and ACTH in sham and OB mice, further suggesting that Ghr effect could be dependent of the basal condition of the animal. However, it is important take into account that the present work has some limitations to affirm this, in consequence future approaches on the bulbectomized animal model of depression may assist in our deeper understanding of the mechanisms of action of Ghr effect and possibly a greater insight into the psychopathology of depression.

In conclusion, this study provides new evidence about genes, receptors and hormones involved in the antidepressant mechanisms induced by Ghr in OB animals. Ghrelin has potential for use as treatment of depression and undoubtedly, knowledge about the molecular mechanisms of action of the antidepressant-like effects of Ghr is valuable for development of novel therapies in this field.

Policy and ethics

All authors certify that the experiments were carried out in accordance with: the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and the guidelines of the Institutional Committee of Laboratory Animal Care and Use, School of Medical Sciences, National University of Córdoba.

In addition, every attempt to minimize the number of animals used and their suffering was made.

Submission declaration

The information provided in this paperwork has not been previously published, except in the form of an abstract; the manuscript has been read and approved by all authors in addition to the institutional authorities. If the manuscript is accepted, it will not be published elsewhere, including electronically, in the same form, in English or in any other language, without the written consent of the copyright-holder.

Contributors

Valeria P. Carlini designed the study and wrote the first draft of this paper. Valeria P. Carlini, María Belén Poretti, Mathias Rask-Andersen and Praveen Kumar carried out experiment and performed the analysis of this data. Helgi B. Schiöth, Susana Rubiales de Barioglio and Marta Fiol de Cuneo contributed to the design of the study, data analysis and reviewed the manuscript. All the above mentioned authors contributed equally and have approved the final version of the manuscript.

Disclosure of financial relationships

The manuscript does not present any possible conflict of interest in the conduction and/or reporting of the research presented in this work.

Acknowledgments

This work was supported by grants from CONICET (Consejo Nacional de Investigación Científica y Técnica), SECyT-UNC (Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba), the Swedish Research Foundation (VR) and the Swedish Brain Research Foundation. We thank Grupo Pilar — GEPSA for the donation of the animals' pelleted food. We also would like to thank Dr. Paul Hobson, native speaker, for revision of the manuscript.

Appendix 1. Real-time PCR primers. β-tubulin: TUB; ribosomal protein 19: RPL19; histone H3: H3; cyclophilin: CYCLO; β-actin: ACT; succinate dehydrogenase complex, subunit B: SUCB; glyceraldehyde-3-phosphate dehydrogenase: GAPDH; delta opioid receptor: DOR; mu opioid receptor: MOR; kappa opioid receptor: KOR; lutheinizante hormone receptor: LHCGR; serotonin transporter: SERT; interleukin 1 beta: IL-1β; corticotrophin-releasing hormone: CRH; vasopressin: AVP

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)	Annealing temperature
RPL19	AATCGCCAATGCCAACTC	GGAATGGACAGTCACAGG	55 °C
β-TUB	AGTGCTCCTCTTCTACAG	TATCTCCGTGGTAAGTGC	55 °C
β-ACT	CCTTCTTGGGTATGGAATCC TGTG	CAGCACTGTGTTGGCATA GAGG	55 °C
GAPDH	GCCTTCCGTGTTCCTACC	GCCTGCTTCACCACCTTC	55 °C
Cyclo	TTTGGGAAGGTGAAAGAAGG	ACAGAAGGAATGGTTTGA TGG	55 °C
DOR	GCTGGTGGACATCAATCG	AGGAAGGCGTAGAGAACC	60 °C
MOR	CCTGCCGCTCTTCTCTGG	CGGACTCGGTAGGCTGTA AC	60 °C
KOR	CACCTTGCTGATCCCAAAC	TTCCCAAGTCACCGTCAG	60 °C
LHCGR	ACTTATACATAACCACCATA CCA	AACTCCAGCGAGATTAGC	61 °C
SERT	ATCTTCACCATTATCTACTTCA	CAGGACAGAAAGGACAAT	57 °C
IL-1β	TACCCAAAGAAGAAGATG	GAAGTCAATTATGTCCTG	55 °C
AVP	GCTCAACACTACGCTCTC	CTTGGGCAGTTCTGGAAG	58 °C
CRH	TACCAAGGGAGGAGAAGA GAG	GGACGACAGAGCCACCAG	60 °C

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