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Research report

Bicuculline, a $GABA_A$-receptor antagonist, blocked HPA axis activation induced by ghrelin under an acute stress

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HIGHLIGHTS

- Central administration of ghrelin in chicks induced anxiogenic like effect.
- Ghrelin significantly increased plasma ACTH and corticosterone level.
- Bicuculline methiodide blocks the behavioral and physiological effect of ghrelin.
- Ghrelin, $GABA_A$R and HPA axis interacts a complex way to regulate anxiogenic response.

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ABSTRACT

Ghrelin is a peptide of 28 amino acids with a homology between species, which acts on the central nervous system to regulate different actions, including the control of growth hormone secretion and metabolic regulation. It has been suggested that central ghrelin is a mediator of behavior linked to stress responses and induces anxiety in rodents and birds. Previously, we observed that the anxiogenic-like behavior induced by ghrelin injected into the intermediate medial *mesopallium* (IMM) of the forebrain was blocked by bicuculline (a $GABA_A$ receptor competitive antagonist) but not by diazepam (a $GABA_A$ receptor allosteric agonist) in neonatal meat-type chicks (Cobb). Numerous studies have indicated that hypothalamic–pituitary–adrenal (HPA) axis activation mediates the response to stress in mammals and birds. However, it is still unclear whether this effect of ghrelin is associated with HPA activation. Therefore, we investigated whether anxiety behavior induced by intra-IMM ghrelin and mediated through $GABA_A$ receptors could be associated with HPA axis activation in the neonatal chick. In the present study, in an Open Field test, intraperitoneal bicuculline methiodide blocked anxiogenic-like behavior as well as the increase in plasma ACTH and corticosterone levels induced by ghrelin (30 pmol) in neonatal chicks. Moreover, we showed for the first time that a competitive antagonist of $GABA_A$ receptor suppressed the HPA axis activation induced by an anxiogenic dose of ghrelin. These results show that the anxiogenic ghrelin action involves the activation of the HPA axis, with a complex functional interaction with the $GABA_A$ receptor.

Abbreviations: GHS-R, growth hormone secretagogue receptor; HPA axis, hypothalamic–pituitary–adrenal axis; CRH, corticotropin-releasing hormone; AVP, arginin-vasopresin hormone; ACTH, adrenocorticotropic hormone; PVN, paraventricular nucleus; IMM, intermediate medial *mesopallium*; $GABA_A$R, gamma aminobutyric acid type A receptor; OF, Open Field test; BB, breeding white wooden box; BIC, bicuculline methiodide; IPSP, inhibitory post-synaptic potentials; mIPSCs, miniature inhibitory postsynaptic currents; 5-HT, serotonin.

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1. Introduction

Ghrelin is a peptide of 26–28 amino acids having an *n*-octanoyl chain esterified to the serine at position 3 of the polypeptide chain, with a homology between species in the sequence of the first 8 amino acids which mediates its activity [1,2]. It is an endogenous ligand of the growth hormone secretagogue receptor 1a (GHS-R1a) and is mainly produced in the stomach [2,3]. Chicken ghrelin was originally isolated from the proventriculus, the glandular portion of the avian stomach, which indicated that this is the primary site of ghrelin production [4]. However, ghrelin-producing cells and GHS-R1a mRNA expression have also been detected in several parts of rodent and bird brain [3,5], with it having been observed that ghrelin acts on the central nervous system to regulate various actions, such as growth hormone secretion, food intake, energy expenditure and glucose homeostasis in several species [2,6]. Moreover, central ghrelin is a mediator of behavior linked to stress responses [7].

Numerous studies have indicated that hypothalamic-pituitary-adrenal (HPA) axis activation mediates the stress response in mammals and birds, and consequently, corticotropin-releasing hormone (CRH) and the arginin-vasopresin hormone (AVP) are released from the hypothalamus into the hypophysial portal vessels that access the anterior pituitary gland, and this induces the release of adrenocorticotropic hormone (ACTH) into the systemic circulation. The main target for circulating ACTH is the adrenal cortex, where it stimulates glucocorticoid synthesis and secretion. Glucocorticoids, such as cortisol and corticosterone, are the downstream effectors of the HPA axis and regulate physiological changes through ubiquitously distributed intracellular receptors. Although, the biological effects of glucocorticoids are usually adaptive, inadequate or excessive activation of the HPA axis may contribute to the development of pathologies [8].

It has been shown that ghrelin and growth hormone secretagogues (GHS) have an important role on the activation of the HPA axis. In mammals, it has been observed that the acute administration of ghrelin and GHS increased the levels of ACTH and glucocorticoids, independently of gender, by acting at the hypothalamic level through an increase in the release of CRH and AVP [3,6,9,10]. Recently, Cabral et al. [11] observed that the peripheral and central administration of ghrelin indirectly activates the hypophysiotropic CRH neurons, and consequently, the HPA axis, since this cell type does not express the GHS-R. In addition, it was demonstrated that ghrelin activates paraventricular nucleus (PVN) CRH neurons via inhibition of local GABAergic tone [12]. In neonatal chicks, Saito et al. [13] demonstrated that astressin (CRH_2 receptor antagonist) attenuated the rise in plasma corticosterone induced by central ghrelin.

The intracerebroventricular (i.c.v.) administration of ghrelin into the amygdala, hippocampus, hypothalamus or raphe nucleus of rodents induced an anxiogenic response measured as reduced activity in the open arm of an elevated plus maze [14–16]. Similarly, in chicks, we showed for the first time that i.c.v. administration of ghrelin also induced an anxiogenic-like behavior [17], which was also observed when ghrelin was injected into the intermediate medial *mesopallium* (IMM) of the chick forebrain. Although, this effect was blocked by bicuculline (a $GABA_A$ receptor ($GABA_AR$) competitive antagonist), diazepam (a $GABA_AR$ allosteric agonist) did not block the anxiogenic-like behavior, suggesting that ghrelin plays a significant role in the acute stress response pattern via the GABAergic system [18]. However, it is still unclear whether anxiety induced by ghrelin is associated with HPA axis activation.

It is noteworthy that there is a substantial degree of homology in fundamental neural systems and mechanisms between mammals and birds. In particular, the IMM area could be considered to be a homologous area to the mammalian neocortex, and thereby constitutes an important center of integration that relates the sensory and motor system and receives afferents from different brain regions related to motivational aspects of behavior [19–21].

In the present study, we investigated whether the anxiety behavior induced by intra-IMM ghrelin and mediated through $GABA_AR$ could be associated with HPA axis activation in neonatal chicks.

2. Materials and methods

2.1. Animals

Day-old meat-type chicks (Cobb) of both sexes were obtained after hatching from the commercial hatchery INDACOR (Argentina) when they were only a few hours old. They were then housed in a breeding white wooden box (BB, 90 × 40 × 60 cm) before performing an Open Field test (OF), which was illuminated from above with a hanging incandescent lamp kept in a small room (3 × 3 m) at a controlled temperature (30–32 °C) in a 12–12 h dark-light cycle (lights on at 7 a.m.). Tap water and food were freely available, with daily food replenishment (Cargill, broiler BB, and 20% minimum crude protein 12.34 MJ/kg) and maintenance chores being carried out at 9 a.m.

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and following the Arrive guidelines as approved by the Animal Care and Use Committee of the Universidad Nacional de Córdoba. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs and injections

Bicuculline methiodide (Sigma Chemical Co), a $GABA_AR$ antagonist, was dissolved in 0.85% saline and intraperitoneally (*i.p.*) injected at doses of 0.0036, 0.036, 0.36 and 36 mg/kg body weight at a final volume of 100 μl. The final dose of bicuculline methiodide used for co-administration with ghrelin produced no behavioral effects *per se*. The ghrelin peptide (rat acyl-ghrelin, Innovagen, Sweden) was dissolved in 0.85% saline containing 0.1% Evans Blue solution and was bilaterally injected into the IMM (intra-IMM) at the anxiogenic dose of 30 pmol, as indicated by Gastón et al. [18]. Briefly, intra-IMM injections were made 2–3 mm to the left and the right of the midline and 3–4 mm from the suture between the forebrain and the cerebellum, using a Hamilton syringe of 10 μl volumes at a volume 3 μl/hemispheres, according to the method of Davis et al. [22]. The depth of the brain injection was controlled by plastic tubing on the 27 gauge needle, which limited the depth of injection to 2.5 mm [23]. An acrylic device was used to hold the heads of chicks, which had bilateral holes in the acrylic head-plate to accommodate the needle of the microsyringe. The stress suffered by this method is minimal, as this system does not require implantation of cannulae, and also avoids problems associated with other methods such as that of ear bars [24].

2.3. Experimental design

Chicks of 4–6 days old weighing approximately 100 g were used in the experiments. To obtain a bicuculline methiodide dose-response curve, chicks were carefully individually captured and placed in a cardboard box before being taken to a separate room where they were injected *i.p.* with saline or different doses of bicuculline methiodide and maintained for 20 min in the BB, after which, they were exposed to OF for 10 min. In order to evaluate the ghrelin effects, 4–6 day-old chicks were gently individually captured and placed in a cardboard box, before being taken to a separate room where they were injected *i.p.* with saline or 0.036 mg/kg

of bicuculline methiodide and 20 min later injected intra-IMM with saline or ghrelin. Immediately following this, they were exposed to OF for 10 min. Thus, the four experimental groups were as follows: saline *i.p.* plus saline intra-IMM (saline); bicuculline methiodide *i.p.* plus saline intra-IMM (BIC); saline *i.p.* plus ghrelin intra-IMM (Ghrelin) and bicuculline methiodide *i.p.* plus ghrelin intra-IMM (BIC + Ghrelin).

2.4. Open Field test

Immediately after the treatments, chicks were placed in the center of a 60 × 60 cm OF apparatus with 30 cm high sides, which was made of white wood and had the floor marked off into 25 squares of 12 × 12 cm each, illuminated by a 100 W overhead bulb [25]. The following types of behaviors were analyzed for 10 min: latency to ambulate, locomotor activity (number of squares crossed), latency to defecate, number of defecations and attempts to escape. Spontaneous activity was recorded by a digital camera suspended 1.5 m above the center of the apparatus, with the monitoring system being set up in a separate room to avoid disturbing the birds [18]. The number of vocalizations (distress calls) was simultaneously recorded for 10 min and counted using a computer with Audacity software (Audacity, San Antonio, TX) [26]. After testing, the floor of the OF apparatus was cleaned with towels wet with 70% ethanol, and immediately after, all birds were decapitated and the trunk blood was collected. In addition, chick brains were removed and fixed in 4% formaldehyde solution to verify the site of the injection by optical microscope. Chicks that had not been injected correctly into the IMM were discarded from the analysis.

2.5. Hormone assays

In order to assess possible differences in basal ACTH and corticosterone levels, some chicks from each group (Saline, BIC, Ghrelin and BIC + Ghrelin) were not exposed to the OF test and remained in the BB and before being decapitated 10 min after injection. Trunk blood was collected in 50 ml chilled dry tubes with ethylenediaminetetraacetic acid. Then, blood samples were centrifuged (1,000 × *g* for 20 min at 4 °C), and plasma samples were collected in 1.5 ml dry tubes and stored at −20 °C until hormone determination was performed. Hormonal measurements were only obtained in animals in which the plasma minimum volume required was collected (300 μl to ACTH and 20 μl to corticosterone assays).

The plasma ACTH concentration was determined by a two-site sequential chemiluminescent immunometric assay (IMMULITE 2000 SIEMENS), with the detection limit of the assay being 5 pg/ml and the intra-assay and inter-assay coefficients of variation being <10% respectively. The plasma corticosterone concentration was determined using a corticosterone enzyme immunoassay kit (MP Biomedicals), with the detection limit of the assay being 5 ng/ml and the intra- and inter-assay coefficients of variation being <10% respectively. Both procedures were carried out following the manufacturer's instructions. Finally, blood collections were taken between 09:00 a.m. and 12:00 p.m., in order to avoid unspecific variability linked to diurnal fluctuations in circulating hormone levels. All measurements were conducted in duplicate.

2.6. Statistical analysis

Data from the OF experiments had a non-normal distribution and were analyzed using the Kruskal–Wallis non-parametric tests. Whenever the test indicated significant effects ($p < 0.05$), a Dunn *post-hoc* test was carried out. ACTH and corticosterone data were analyzed by a one-way ANOVA or by a two-way ANOVA (condition × treatments), and when the analysis indicated significant effects, a Newman Keuls *post-hoc* test was carried out. When necessary, normality and homoscedasticity were achieved by performing a $\log_{10}$ transformation. A p value < 0.05 was considered to represent a significant difference in all cases.

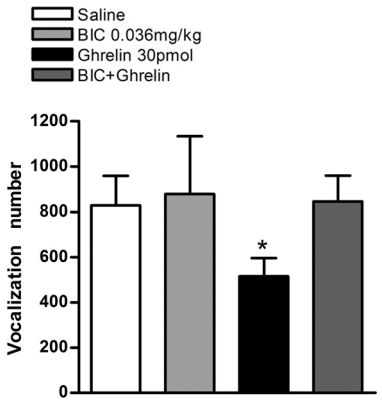

Fig. 1. Effect of *i.p.* bicuculline methiodide administration on the number of vocalizations induced by an intra-IMM anxiogenic dose of ghrelin, in 4–6 day-old chicks exposed to an Open Field. Bars represent median and interquartile range. n = 12–13. *p < 0.05 compared to other groups (Dunn's *post-hoc* test).

3. Results

3.1. Effect of i.p. bicuculline methiodide administration on anxiety-like behavior

The Kruskal-Wallis test revealed a significant effect of treatments on ambulation latency ($H = 16.09$, $p = 0.0029$), with Dunn's *post-hoc* test showing that at a dose of 36 mg/kg of bicuculline induced a significant increase in ambulation latency respect to the saline, 0.036 and 0.36 mg/kg groups ($p < 0.05$). The Kruskal–Wallis test did not indicate any significant effect of treatments on the number of ambulations ($H = 5.132$, $p = 0.2741$), defecation latency ($H = 4.080$, $p = 0.3953$), number of defecations ($H = 3.714$, $p = 0.4461$), attempts to escape ($H = 5.011$, $p = 0.2862$). However, it could be observed that these behavioral parameters varied with respect to saline control (Table 1). The final dose of bicuculline used in the studies described below produced no behavioral effects *per se*.

3.2. Effect of i.p. bicuculline administration on anxiety-like behavior induced by intra-IMM ghrelin

The Kruskal–Wallis test revealed a significant effect of treatments on ambulation latency ($H = 28.91$, $p < 0.0001$), number of ambulations ($H = 18.73$, $p = 0.0003$), defecation latency ($H = 19.81$, $p = 0.0002$), number of defecations ($H = 22.57$, $p < 0.0001$), attempts to escape ($H = 8.132$, $p = 0.0434$) and vocalizations ($H = 10.98$, $p = 0.0118$). Dunn's *post-hoc* test demonstrated that intra-IMM ghrelin (30 pmol) induced a significant increase in ambulation ($p < 0.05$) and defecation ($p < 0.05$) latencies respect to the saline, BIC and BIC + Ghrelin groups. However, the *post-hoc* test revealed that intra-IMM ghrelin induced a significant decrease in the number of ambulations ($p < 0.05$), number of defecations ($p < 0.05$), attempt to escape ($p < 0.05$) and vocalizations ($p < 0.05$) respect to the other groups (Table 2 and Fig. 1). Overall, this suggests that bicuculline was able to block the anxiogenic-like behavior induced by ghrelin.

Table 1
Effect of bicuculline *i.p.* administration on anxiety-like behavior in an Open-Field test.

BIC (mg/kg)	Saline	0.0036	0.036	0.36	36
Ambulation latency (s)	15 (7–28)	22 (16–32)	15 (9–33)	16 (12–67)	175 (69–462)*
Number of ambulation	112 (58–316)	102.5 (73.5–188.5)	104 (30.5–286.5)	62 (40–157)	43 (5–98)
Defecation latency (s)	63 (45–110)	77.5 (49.5–352.5)	137.5 (51–338.5)	97 (9–457)	178 (94–600)
Number of defecation	2 (1–3)	1.5 (0.5–2.5)	1.5 (1–2)	1 (0–2)	1 (0–2)
Attempts to escape	2 (0–10)	3.5 (1–17.5)	2 (0–12.5)	7 (0–11)	0 (0–1)

Each value is expressed as median and interquartile range. n = 6–7.
* $p < 0.05$ compared to saline group (Dunn's *post-hoc* test).

Table 2
Effect of bicuculline administration on anxiety-like behavior induced by ghrelin in an Open-Field test.

	Saline	BIC (0.036 mg/kg)	Ghrelin (30 pmol)	BIC + Ghrelin
Ambulation latency (s)	23 (11–63)	19 (11.5–35.5)	523 (321–600)*	36 (12–62.5)
Number of ambulation	58 (33.5–113)	89.5 (44.5–110)	2 (0–26)*	55.5 (19–112.5)
Defecation latency (s)	18 (8–417)	107.5 (28.5–278)	600 (379–600)*	85.5 (6–173.5)
Number of defecation	2 (1.5–2.5)	1 (1–2)	0 (0–1)*	1 (1–2)
Attempts to escape	2 (0–5.5)	1 (0–3.5)	0 (0–0)#	0 (0–4.5)

Each value is expressed as median and interquartile range. n = 12–13.
* $p < 0.05$ compared to other groups.
# $p < 0.05$ compared to saline group only. (Dunn's *post-hoc* test).

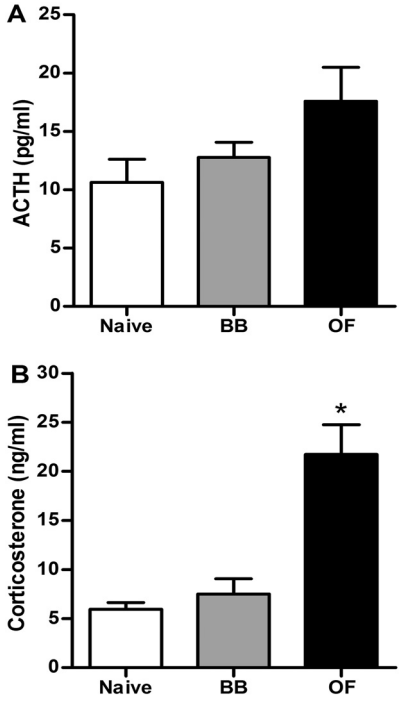

Fig. 2. Effect of the Open Field (OF) exposure on plasma ACTH (A, n = 6–11) and corticosterone (B, n = 5–8) concentrations in 4–6 day-old chicks, compared to those not exposed to an acute stressor (naive and breeding box (BB) groups). Chicks of BB and OF groups were injected intra-IMM with saline. Naive group were undisturbed chicks, housed in BB but not injected and unexposed to OF. Bars represent means ± SEM. *$p < 0.05$ compared to the other groups (Newman Keuls *post-hoc* test).

3.3. Effect of the Open Field test on plasma ACTH and corticosterone levels

A one-way ANOVA revealed no significant effect of OF exposure on plasma ACTH levels ($F_{2,24} = 1.85$, $p = 0.1797$) (Fig. 2A). However, this test showed a significant effect of OF exposure on plasma corticosterone levels ($F_{2,19} = 13.86$; $p = 0.0002$). In addition, the Newman Keuls *post-hoc* test revealed a significant increase in the plasma corticosterone concentration in chicks exposed to OF compared to those not exposed to the stressor (naive and BB groups) ($p < 0.05$) (Fig. 2B). This indicates that the handling and the procedure of the stereotaxic injection were not stressful factors, but that the OF test acted as an acute stressor for neonatal chicks.

3.4. Effect of intra-IMM administration of ghrelin on plasma ACTH and corticosterone levels after the Open Field test

Regarding plasma ACTH levels, a two-way ANOVA (condition × treatment) revealed an independent significant main effect of treatment ($F_{3,70} = 9.99$; $p < 0.0001$), but no significant effect of condition ($F_{1,70} = 0.36$; $p = 0.5489$) and no interaction between the two variables ($F_{3,70} = 0.47$; $p = 0.7038$). For the Breeding Box condition (unstressed), the Newman Keuls *post-hoc* test showed a significant increase in plasma ACTH levels in chicks injected with intra-IMM ghrelin (30 pmol) compared to saline ($p < 0.05$) or BIC (0.036 mg/kg) ones ($p < 0.05$). Similar results were observed between the same groups ($p < 0.05$) exposed to the Open Field (stress condition) ($p < 0.05$). In addition, it was observed that *i.p.* pre-treatment with BIC significantly blocked the rise in plasma ACTH levels induced by intra-IMM ghrelin, but only in the group exposed to the Open Field ($p < 0.05$) (Fig. 3A).

With regard to corticosterone levels, a two-way ANOVA (condition × treatment) revealed an independent significant main effect of treatment ($F_{3,52} = 8.88$; $p = 0.0001$) and condition ($F_{1,52} = 23.82$; $p < 0.0001$), but showed no interaction between the two ($F_{3,52} = 1.64$; $p = 0.1906$). For the Breeding Box condition, a Newman Keuls *post-hoc* test revealed a significant increase in the plasma corticosterone levels in the group injected with intra-IMM ghrelin (30 pmol) compared to the saline ($p < 0.05$) or BIC (0.036 mg/kg) groups ($p < 0.05$). Similar results were obtained for plasma corticosterone levels in the groups exposed to the Open Field ($p < 0.05$). The *i.p.* pre-treatment with BIC significantly blocked the increase in the plasma corticosterone levels induced by intra-IMM ghrelin, but only in the group exposed to the Open Field ($p < 0.05$). Furthermore, exposure to the stressor induced a significant increase in the plasma corticosterone levels in the saline, BIC, and Ghrelin groups ($p < 0.05$) compared to the same groups without OF exposure (Fig. 3B). Overall, these data indicate that ghrelin induced HPA activation, which was blocked by *i.p.* BIC pre-treatment in chicks exposed to the OF test.

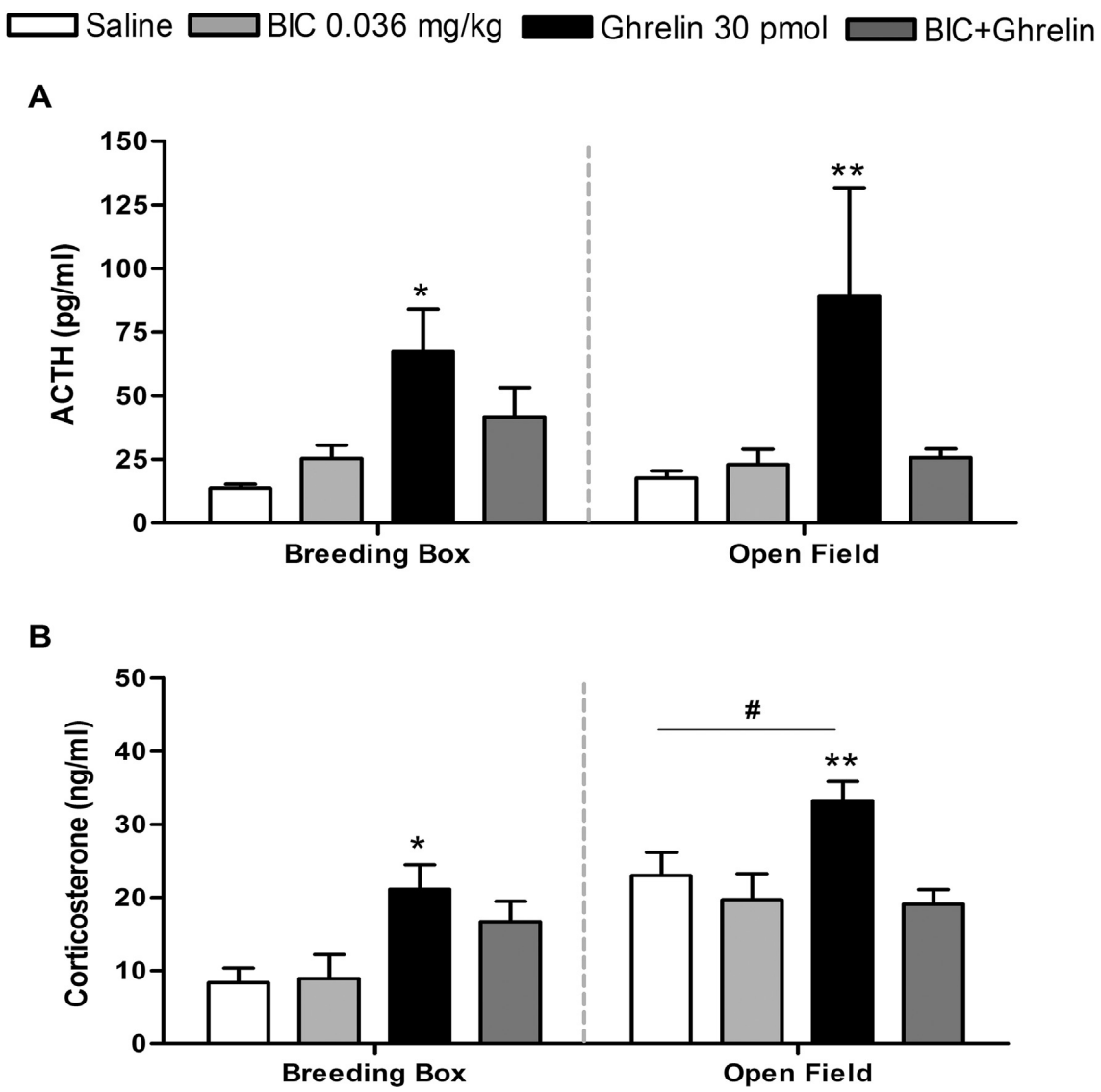

Fig. 3. Effect of intra-IMM ghrelin and *i.p.* bicuculline administration on plasma ACTH (A, n = 8–11) and corticosterone (B, n = 6–9) concentrations in 4–6 day-old chicks. Bars represent means ± SEM. *p < 0.05 compared to saline and BIC groups in the unstressed breeding box condition. **p < 0.05 compared to other groups under the stressed Open Field condition. #p < 0.05 compared to corresponding groups in the unstressed breeding box condition. In all cases, a Newman Keuls *post-hoc* test was carried out.

4. Discussion

The present results show for the first time that bicuculline, an antagonist of $GABA_AR$, blocks the HPA axis activation induced by an anxiogenic ghrelin dose administered intra-IMM.

In chicks, an OF response is primarily a fear of novelty and isolation, in addition to a tendency to reinstate contact with conspecifics [27], and represents a compromise between opposing tendencies to reinstate contact and to avoid detection by potential predators [25]. Thus, changes in the latencies to ambulate and defecate may be established as an indication of fear in the task, while the number of crossed squares, escape attempts and distress calls can be interpreted as socially motivated behavior patterns in order to reinstall contact for isolated chicks [17,25]. However, in the presence of a potential predator, these responses would serve to increase detectability and as such might be maladaptive; vocal behavior in particular might seem self-defeating because of its potential to attract predators [28]. Here, we confirm the behavioral pattern of response after an acute stressor induced by intra-IMM ghrelin (30 pmol) administration (Table 2), which was previously reported by Gastón et al. [18]. Also, we observed a significant decrease of the number of vocalizations induced by an anxiogenic dose of ghrelin in the OF test, which was blocked by bicuculline (Fig. 1). This distress call decrease may be associated with an increase in arousal, alertness, and heightened attention of the chick in response to an acute stressor, at least in this case.

It has been reported that bicuculline methiodide passes the blood brain barrier (BBB) poorly [29,30]. Here, we observed that the highest dose of bicuculline methiodide (36 mg/kg) increased the latency to ambulate in neonatal chicks exposed to an OF test (Table 1) whereas an ineffective dose (0.036 mg/kg) blocked the central anxiogenic action of ghrelin (Table 2 and Fig. 1). These behavioral observations may have been due to the fact that neonatal chicks have a different permeability of BBB compared to that of the adult bird. Indeed, several studies have demonstrated that substances injected peripherally in neonatal chicks enter the brain more rapidly and attain higher concentrations than in the adult bird [31], including electrolytes such as chloride [32,33] and also biologically active compounds such as noradrenaline [34], serotonin [35,36] and GABA [37]. Adult BBB characteristics for the chicken are not present until about the fourth week of life [32]. Moreover, the BBB exist in the choroid plexi and in essentially all areas of the brain

except the hypothalamus, where blood-borne substances diffuse with ease into the extracellular spaces [31]. In mammals, changes has been described in the BBB related to age [38], with Mares et al. [39] also observing that *i.p.* administered bicuculline methiodide was able to cross the BBB in immature but not in adult rats. Thus, we do not rule out that bicuculline methiodide may be able to cross the BBB in neonatal chicks, and act at different pallial brain levels or on the HPA axis.

Successful adaptation to frightening or stressful stimuli requires not only the ability to perceive and respond to a stimulus, but also the capacity to be able to control the stress responses appropriately [40]. One physiological characteristic of the stress response is HPA axis activation, with the plasma corticosterone level being the main indicator used to assess this [40,41]. Jones and Merry [42] observed elevated plasma corticosterone levels in chickens, which was associated with stress induced by exposure in pairs or individually to an OF respect to undisturbed group. In agreement, in our study, neonatal chicks exposed to OF revealed a significant plasma corticosterone level increase compared to unexposed birds, indicating HPA activation in response to the acute stressor (Fig. 2B). However, the plasma ACTH levels did not show any significant changes between the groups of birds exposed and unexposed to OF (Fig. 2A), perhaps due to a negative feedback on the hypothalamus and pituitary induced by a higher release of corticosterone from the adrenal cortex. In this regard, several studies have indicated that a fast negative feedback may occur within seconds to minutes (<30 min) and involves the inhibition of ACTH release by glucocorticoids, a non-genomic effect without transcriptional regulation or protein synthesis [43–46].

In the present study, a significant increase in plasma ACTH and corticosterone levels induced by ghrelin (30 pmol) was observed in chicks kept in the Breeding Box (unstressed) or exposed to the Open Field (Fig. 3). Similarly, Saito et al. [13] found that i.c.v. ghrelin led to increased plasma corticosterone levels in a dose-dependent and time-dependent manner in neonatal chicks that had been previously isolated in individual cages for 24 h. In other investigations, increased plasma ACTH and corticosterone levels after ghrelin administration were observed in humans and rodents [47–50] demonstrating that ghrelin might be mediating HPA axis activation.

It has been reported that the activation of $GABA_AR$ from limbic and cortical neurons that project toward hypothalamic PVN leads to a GABA-mediated inhibition of the CRH neurons and consequently the HPA axis, associated with an anxiolytic-like effect [51]. Thus, the elevated plasma ACTH and corticosterone levels after ghrelin intra-IMM administration suggests a reduction in GABAergic inhibition in the hypothalamus, which may explain the anxiogenic effect observed in the OF test. However, we observed that previous administration of 0.036 mg/kg of bicuculline blocked the increase of both the plasma ACTH and corticosterone concentrations induced by 30 pmol of ghrelin in the OF test (Fig. 3). Furthermore, Roberto et al. [52] reported that CRH enhances GABAergic transmission, and more recently, Cruz et al. [53] found that ghrelin administered in the rat central amygdala induced an increase in GABAergic activity in both the amplitude of the inhibitory post-synaptic potentials (IPSP) and the frequency of miniature inhibitory postsynaptic currents (mIPSCs). This suggests a potential ghrelin role in regulating GABAergic neurotransmission. Therefore, the anxiogenic action induced by ghrelin, under an acute stressor, may have been modulated by the GABAergic system through $GABA_AR$, implying HPA axis activation. It will be necessary in future studies to use other specific $GABA_AR$ antagonists in order to determine the extent of $GABA_AR$ involvement in the ghrelin effects.

Peripherally (after crossing the blood brain barrier) as well as centrally synthesized ghrelin (in the hypothalamus) regulates diverse functions of the central nervous system, including stress-associated behavioral functions [54], with circulating ghrelin levels having been found to rise following stress and some studies suggesting that increasing ghrelin contributes to the mechanisms responsible for the development of stress-induced anxiety [7]. Related to this, rats given either a drug to stimulate the ghrelin receptor, or gene therapy to overexpress growth hormone over a prolonged period, became much more susceptible to fear than normal rats [55]. Thus, the elevation of ghrelin levels following stress or trauma exposure could be one of the factors for maintaining an anxiogenic profile. In our experiments, chicks with exogenous ghrelin exhibited a higher fearfulness, as observed by the inhibitory behavior and/or the increase in ACTH and corticosterone levels. In addition, under stress conditions, blocking of the $GABA_AR$ by bicuculline in chicks injected with ghrelin reduced the fear to saline group values. These results reveal that ghrelin action in the stress response involves activation of the HPA axis, with an intricate functional interaction with $GABA_AR$. Thus, ghrelin delivery into the brain can mimic vulnerability to stress in individuals with a high production of ghrelin.

It is known that the $GABA_A$ergic neurotransmission efficiency can be readily modulated by altering the aggregation of these receptors at the synapses, thereby regulating the number of available postsynaptic receptors and consequently the synaptic strength [56]. Inhibitory synaptic plasticity that involves the rearrangement of the GABAergic synapse components leads to modulation of intracellular trafficking of $GABA_AR$ to the membrane surface and its surface lateral diffusion, providing a faster control in the number of receptors at the synapse [57]. Thus, ghrelin and stress may have had an additive effect, which induced a greater recruitment of $GABA_AR$ to the synaptic sites. This could explain the differential modulation of the GABAergic system on the effects of ghrelin under both unstressed (Breeding Box) and stressed (Open Field) conditions. Further studies on elucidating this point are now necessary.

Ghrelin and $GABA_ARs$ have a complex interaction, perhaps due to the participation of serotonergic pathways. Several studies have shown that a decrease in the release of 5-HT may be associated with an anxiolytic response [58,59], whereas a higher release enhances anxiety [60]. In chicks, 5-HT had a stimulatory role on the HPA axis, which may have been mainly mediated by $5HT_{2C}$ receptors [61]. In rodents, a hyperactivity of corticolimbic 5-HTergic projections is implicated in the induction of anxious states. Moreover, $5\text{-}HT_{1A}$ receptor agonists elicit a pronounced increase in the activity of the HPA axis in response to stress, and GABA may exert an inhibitory effect on the 5-HT release at the level of cell body and terminal corticolimbic 5-HTergic projections [62].

Hansson et al. [63] found that acute central administration of ghrelin increased the serotonergic turnover and the mRNA expression of a number of serotonin receptors, in the amygdala and dorsal raphe of mice. In addition, GHS-R1a knock-out mice showed a decreased mRNA expression of serotonergic receptors. These authors suggested that hypothalamic neurons sensitive to ghrelin, which project toward the amygdala may act on presynaptic terminals to affect the release of 5-HT or the gene expression, although it is not known whether ghrelin exerts a direct or indirect effect on serotonergic pathways. We hypothesize that ghrelin might act directly on GHS-R of 5-HT neurons to stimulate 5-HT synthesis and secretion of 5-HT. However, ghrelin might also act indirectly through GABAergic interneurons that project toward the GABAergic neurons responsible for inhibiting 5-HT neuronal cells. Thus, it is possible that ghrelin could increase anxiety by activating the 5-HTergic pathways (Fig. 4A).

As in the case of the GABAergic system, the organization and functionality of the 5-HTergic system are similar in birds and mammals [64], with a dense expression of their receptors in areas such as *arcopallium*, *mesopallium*, hypothalamus and dorsal raphe nucleus [65–69]. Although, cells that synthesize and secrete ghrelin and

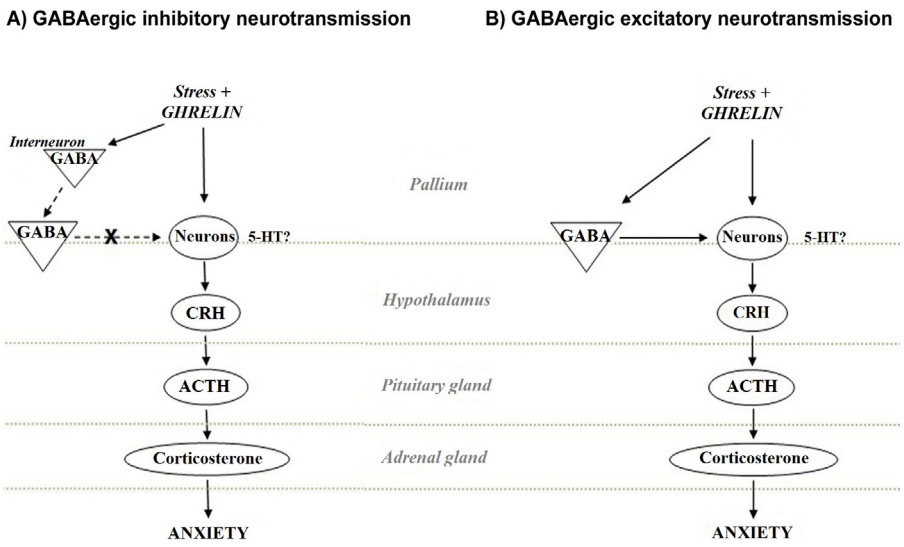

Fig. 4. A schematic overview of the possible regulation of the HPA axis by ghrelin, through $GABA_AR$, in neonatal chicks. As it was observed that bicuculline blocked the anxiogenic behavioral effect and HPA axis activation induced by ghrelin, we speculate that stress and ghrelin may directly stimulate some neurons (maybe the 5-HT neurons?) in the pallial or hypothalamus brain areas, which may activate the HPA axis and lead to anxiety. (A) If $GABA_AR$ activation caused neuronal inhibition, ghrelin could also act indirectly through GABAergic interneurons by inhibiting the GABAergic neurons (which regulate the neuronal cell activity that activates the HPA axis) causing anxiety. (B) If $GABA_AR$ activation caused neuronal excitation (such as that observed in immature brains and some mature neurons under certain conditions), then ghrelin could also act directly through the GABAergic neurons responsible for exciting the neuronal cells that activate the HPA axis, causing anxiety. For details, consult the main text. The dashed arrows indicate a neuronal inhibition; continuous arrows indicate a neuronal excitation. The cross indicates a disrupted action. ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; GABA, gamma-aminobutyric acid, 5-HT, serotonine.

GHS-R have been detected in the hypothalamus, pituitary, *cerebellum*, optic lobe and *striatum* [13,70,71], there are no reports published about their distribution in pallial areas. However, our findings suggest that GHS-R from pallial neurons may constitute a circuit with lower structures, such as the hypothalamus and/or the pituitary gland.

Recent studies have indicated a possible excitatory role of GABAergic neurotransmission in the mature brain [72], mainly due to a decrease in membrane KCC2 cotransporter, and with dephosphorylation being induced by an immobilization stress with a consequent increase in the intracellular Cl^- concentration [73]. Under this condition, $GABA_AR$ activation results in an outward Cl^- current and a subsequent membrane depolarization [74,75]. This still controversial but very interesting phenomenon has been observed in several areas of adult rodent brain, and it has been suggested to be part of the action circuit of the anxiogenic cholecystokinin peptide [72,76]. In addition, in early postnatal life, GABA can be an excitatory neurotransmitter, an action that is presumably important for neuronal development and network formation [75]. Therefore, we do not discard that ghrelin and $GABA_AR$ may be interacting in this way under an acute stress (Fig. 4B). Further detailed investigation is now necessary. Fig. 4

5. Conclusions

These results show that acyl-ghrelin may regulate HPA activation, which is also associated with an anxiogenic response in neonatal chicks exposed to an acute stressor. In addition, bicuculline, a $GABA_AR$ antagonist, blocked the HPA axis activation induced by ghrelin, under an acute stress. Thus, ghrelin and GABA-A-ergic neurotransmission have a complex functional interaction to regulate the anxiogenic response. Furthermore, our findings suggest that avian *mesopallium* with neurons expressing GHS-R may be a neural center of processing and integration of information, constituting neuroendocrine circuits with lower structures, such as the hypothalamus and/or the pituitary gland, among others.

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