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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

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