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Gabaergic control of anxiety-like behavior, but not food intake, induced by ghrelin in the intermediate medial mesopallium of the neonatal chick

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ABSTRACT

Ghrelin (Grh) is an endogenous ligand of the growth hormone secretagogue receptor. In neonatal chicks, central Ghr induces anxiogenic-like behavior but strongly inhibits food intake. The intermediate medial mesopallium (IMM) of the chick forebrain has been identified to be a site of the memory formation, and the modulation of the $GABA_A$ receptors that are present here modifies the expression of behavior. Thus, the GABAergic system may constitute a central pathway for Ghr action in regulating the processes of food intake and stress-related behaviors. Therefore, we investigated if the effect of systemic administration of bicuculline ($GABA_A$ receptor antagonist) and diazepam (benzodiazepine receptor agonist) on the anxiety in an Open Field test and inhibition in food intake induced by Grh (30 pmol) when injected into IMM, were mediated by GABAergic transmission. In Open Field test, bicuculline was able to block the anxiogenic-like behavior induced by Ghr, whereas diazepam did not produce it. However, the co-administration of bicuculline or diazepam plus Ghr did not show any change in food intake at 30, 60 and 120 min after injection compared to Ghr alone. Our results indicate for the first time that Ghr, injected into the forebrain IMM area, induces an anxiogenic-like behavior, which was blocked by bicuculline but not diazepam, thus suggesting that Ghr plays an important role in the response pattern to acute stressor, involving the possible participation of the GABAergic system. Nevertheless, as neither drug affected the hypophagia induced by intra-IMM Ghr, this suggests that it may be mediated by different mechanisms.

Introduction

Ghrelin (Ghr) is a peptide of 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 (Hattori et al., 2001). It is an endogenous ligand of the growth hormone secretagogue receptor (GHS-R) and it is mainly produced in the rat stomach (Kojima et al., 1999). However, Ghr-producing cells have also been detected in the arcuate nuclei of the rat hypothalamus, which is a feeding control center (Cowley et al., 2003) with Ghr immunoreactivity also having been found in the chicken hypothalamus, although not in the arcuate nucleus as in rats (Ahmed and Harvey, 2002). Chicken Ghr was originally isolated from the proventriculus, the glandular portion of the avian stomach, indicating that this is the primary site of ghrelin production (Kaiya et al., 2002). In the chicken, GHS-R1 mRNA expression has also in fact been detected in several parts of the brain, suggesting a central action of Ghr (Geelissen et al., 2003).

At present, it is known that central Ghr plays an important role in various physiological functions and it has been reported that chicken Ghr can stimulate the release of growth hormone and corticosterone in chicks, as previously observed in rodents (Ahmed and Harvey, 2002; Kaiya et al., 2002). Furthermore, both peripheral and central Ghr rapidly increase food intake and body weight in mammals (Carlini et al., 2002; Nakazato et al., 2001; Wren et al., 2001a, 2001b). However, in neonatal chicks, the effect of central Ghr on feeding produces the opposite effect from that seen in mammals. Furuse et al. (2001) reported that an intracerebroventricular (i.c.v.) injection of Ghr strongly inhibited food intake in neonatal chicks. The underlying mechanism related to this is still unclear, although it has been reported that the anorexic effect of Ghr could be mediated by the corticotropin-releasing factor (CRF) and its receptor system (Saito et al., 2005). In rats, it has been observed that Ghr administration into the hippocampus, amygdala and dorsal raphe nucleus, induces an anxiogenic-like effect and an improvement in memory retention which has been measured in the plus-maze and in a step-down tests, respectively (Carlini et al., 2004; Currie et al., 2014). In chicks, Ghr also induced an anxiogenic-like

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behavior, but with decreased memory retention (Carvajal et al., 2009). This suggests that the peptide Ghr is a mediator of both behaviors being linked to food intake and body weight and behaviors associated with psychosocial stress, mood, and anxiety (Chuang and Zigman, 2010).

Gamma aminobutyric acid (GABA) is a major inhibitory neurotransmitter within the brain. In fact, neuronal inhibition is mediated primarily by ionotropic type A receptor ($GABA_AR$), which is expressed ubiquitously in most adult neurons (Korpi et al., 2002) and is a clinically relevant drug target for anticonvulsant, anxiolytic and sedative-hypnotic agents, including benzodiazepine, barbiturates, neurosteroids and general anesthetics (Vithlani et al., 2011). GABAergic synapses are critical for the development and coordination of the neuronal activity underlying the majority of physiological and behavioral processes in the brain (Luscher et al., 2011). Activity-dependent changes in the number of postsynaptic $GABA_AR$ represent one of the most powerful mechanisms underlying the functional plasticity of GABAergic synapses. Moreover, deficits in the functional expression of $GABA_AR$ have been implicated in the pathogenesis of a wide range of neuropsychiatric diseases as epilepsy, anxiety, depression, schizophrenia, and substance abuse (Vithlani et al., 2011). It has been reported that there is an increase in $GABA_AR$ density in synaptosomes after partial water immersion (Cid et al., 2008; Marin et al., 2002), Open Field (OF) test (Salvatierra and Arce, 2001) or novelty (Salvatierra et al., 1997, 2009).

In birds, GABA and $GABA_AR$ are present in several brain regions, such as the intermediate medial mesopallium (IMM) (Aller et al., 2003; Csillag et al., 1987). This region of the chick forebrain has been identified to be the site of the formation of memory for a one-trial passive avoidance task in the day-old chick (Gibbs, 2008; Gibbs and Ng, 1977). It has been observed that low doses of GABA inhibit memory, whereas higher doses result in memory enhancement in IMM, which may result from the differential activation of $GABA_A$ and $GABA_C$ receptors (Gibbs and Johnston, 2005). In addition, central or systemic administration of muscimol ($GABA_A$ receptor agonist) (Baldwin et al., 1990; Kamatchi and Rathanaswami, 2012; Pu et al., 1999) or diazepam (benzodiazepine receptor agonist) (Cooper, 2005; Patel and Ebenezer, 2008) increases food intake in mammals, and $GABA_A$ agonists also induce hyperphagia in birds (Bungo et al., 2003; Jonaidi et al., 2002, 2012; Zendehdel et al., 2009) and this effect attenuated by pretreatment with bicuculline or picrotoxin ($GABA_A$ antagonists). Recently, Cruz et al. (2013) showed that Ghr increased GABAergic transmission because its superfusion increased the amplitude of evoked inhibitory postsynaptic potentials and the frequency of miniature inhibitory postsynaptic currents in amygdala slices of naïve rat. Thus, the GABAergic system may constitute a central pathway for Ghr action in regulating the processes of food intake and stress-related behaviors. In this study, we examined whether the Ghr anxiogenic-like and hypophagic effects might be mediated by the GABAergic system in the IMM area of neonatal chicks.

Experimental procedures

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 housed in a white wooden box (90 × 40 × 60 cm) before performing the OF test, which was illuminated with an incandescent lamp hanging just above it and kept in a small room (3 × 3m) 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 performed at 9 a.m.

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

Drugs and injections

The Ghr peptide (purchased from Neosystem, France) was dissolved in 0.85% saline containing 0.1% Evans Blue solution, and administered in doses of 15, 30, 100, 300 and 900 pmol into the IMM area. Bicuculline methiodide (Sigma Chemical Co) $GABA_AR$ antagonist or Diazepam (Sigma Chemical Co) benzodiazepine agonist ($GABA_AR$ allosteric modulator) were dissolved in 0.85% saline and intraperitoneally (i.p.) injected at doses of 0.036 mg/kg and 0.05 mg/kg body weight, respectively, at a final volume of 100 μl. The final doses of bicuculline and diazepam used here produced no behavioral effects per se from separate experiments (data not shown). Then, intracerebral injections of Ghr were bilaterally given into the IMM (intra-IMM), previously called IMHV (Reiner et al., 2004), at a volume 3 μl/hem using a Hamilton syringe of 10 μl volume, according to the method of Davis et al. (1979). It was used an acrylic device to hold the head of chicks. The head holder with bilateral holes in the acrylic head-plate of the device was accommodated for the needle of the microsyringe.

This system does not require implantation of cannulae, and avoids the problems associated with methods such as that of ear bars. Moreover, the solution can be quickly injected into IMM with precision and security, and the stress suffered by this method is minimal (Furuse et al., 1999; Koutoku et al., 2005). 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. The depth of the brain injection was controlled by plastic tubing on the 27 gauge needle, which limits the depth of injection to 2.5 mm (Kuenzel and Masson, 1988). The needle was left inside during a period of 5 s in order to avoid reflux of the solution as well as any possible bleeding through the drilling of the epithelium and meningeal. As the chicks have soft unossified skulls, this procedure does not require an anesthetic and is routinely performed without administration of analgesics (Andrew, 1991).

The forebrain hemispheres, such as the telencephalic structures, are neurochemically and functionally comparable to the mammalian neocortex, claustrum, and pallial amygdale, in addition to other pallial areas such as the hippocampus (Reiner et al., 2004). Immediately after Ghr administration, one group of birds was evaluated in the OF test and another was used for evaluating the food intake.

Experimental design

Chicks of 4–6 days old in total of 5 experiments were used. In the experiment 1, chicks were individually gently captured and placed in a cardboard box before being taken to a separate room where injected intra-IMM with Ghr to make the dose–response curve on the OF behavior. In the experiments 2 and 3, chicks were individually gently captured and placed in a cardboard box before being taken to a separate room where previously injected i.p. with bicuculline or diazepam and 20 min later were injected intra-IMM with Ghr or saline as shown in Table 1. Immediately then, they exposed to OF test during 10 min. In the feeding experiments (4 and 5), 4–6-day-old chicks were placed previously overnight in an individual box without access to food but with

Table 1
Summary of the experimental treatments.

Group	i.p.	Intra-IMM
1	Saline	Saline
2	Bicuculline	Saline
3	Diazepam	Saline
4	Saline	Ghrelin
5	Bicuculline	Ghrelin
6	Diazepam	Ghrelin

i.p.: intraperitoneally injection; intra-IMM: bilateral injection intra intermediate mesopallium

free access to water and in the day of the experiment were injected as described above (Table 1).

Open field test

Immediately after treatments, chicks were placed in the center of a 60 × 60 cm OF apparatus with sides 30 cm high, which was made of white wood and had the floor was marked off into 25 squares of 12 × 12 cm each, illuminated by a 100 W overhead bulb (Gallup and Suarez, 1980). 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. After testing, the floor of the OF apparatus was cleaned with towels wet with 70% ethanol. All birds were immediately decapitated and their brains were removed and fixed in 4% formaldehyde solution to verify the site of the injection by optical microscope. Chicks that were not injected correctly into the IMM were discarded from the analysis (Fig. 1).

Food intake

On the day of the experiment, followed the intra-IMM injection each chick was replaced in a box with food. The quantity of food intake was determined 30, 60 and 120 min after the injection, by measuring the disappearance of diet from the pre-weighed feeder with a digital balance of a precision of 0.01 g. In most cases, no spillage was observed due to the fact that a limited amount of food was available in the feeder. However, if spillage was observed, this was taken into account.

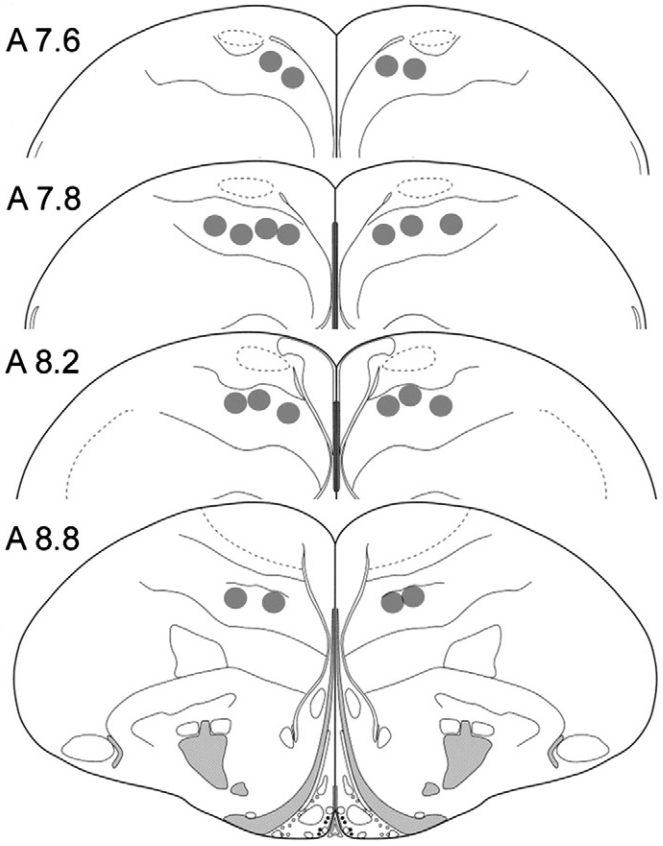

Fig. 1. A schematic representation of the microinjection sites within the intermediate mesopallium (IMM) of the chick. Correct bilateral injection placements are indicated as closed circles. Numbers correspond to anterior–posterior references (in mm) in the atlas developed by Kuenzel and Masson (1988).

Statistical analysis

Data from OF behaviors assumed a non-normal distribution and were analyzed using the Kruskal–Wallis nonparametric tests. Whenever the test indicated significant effects ($p < 0.05$), a pairwise comparison (Dunn test) was carried out. Data from food intake measures were analyzed using a two-way ANOVA (treatment x time), and when the test indicated significant effects, a Bonferroni post test was also carried out. A p value <0.05 was considered to represent a significant difference in all cases.

Results

Experiment 1. Effect of IMM administration of ghrelin on anxiety-like behavior

The Kruskal–Wallis test showed a significant effect of a Ghr dose administered in the IMM area on ambulation latency ($H = 32.91$, $p < 0.0001$), for the number of ambulations ($H = 21.56$, $p < 0.0006$) and the defecation latency ($H = 13.61$, $p = 0.0183$), Dunn's post-hoc test revealed a significant increase in the ambulation latency for all doses used (30, 100, 300 and 900 pmol) respect to saline ($p < 0.05$) (Fig. 2). Also, the post-hoc test revealed significantly decreased ambulation for Ghr doses of 30, 300 and 900 pmol respect to saline ($p < 0.05$), but showed a significant reduction in defecation latency only at the Ghr dose of 300 pmol respect to saline ($p < 0.05$) (Table 2). Taken together, these data suggest that Ghr induced an anxiogenic-like effect when injected into the IMM area. However, the Kruskal–Wallis test did not indicate any significant differences in the number of defecations ($H = 9.60$, $p = 0.0872$) or in the number of escapes ($H = 2.45$, $p = 0.7835$) (Table 2).

Experiment 2. Effect of bicuculline administration on anxiety-like behavior induced by ghrelin

The Kruskal–Wallis test revealed a significant effect of the co-administration of 0.036 mg/kg of i.p. bicuculline plus intra-IMM Ghr (30 pmol) on ambulation latency ($H = 32.07$, $p < 0.0001$), on number of ambulations ($H = 24.81$, $p < 0.0001$) and also on defecation latency ($H = 28.89$, $p < 0.0001$). Dunn's post-hoc test revealed that the

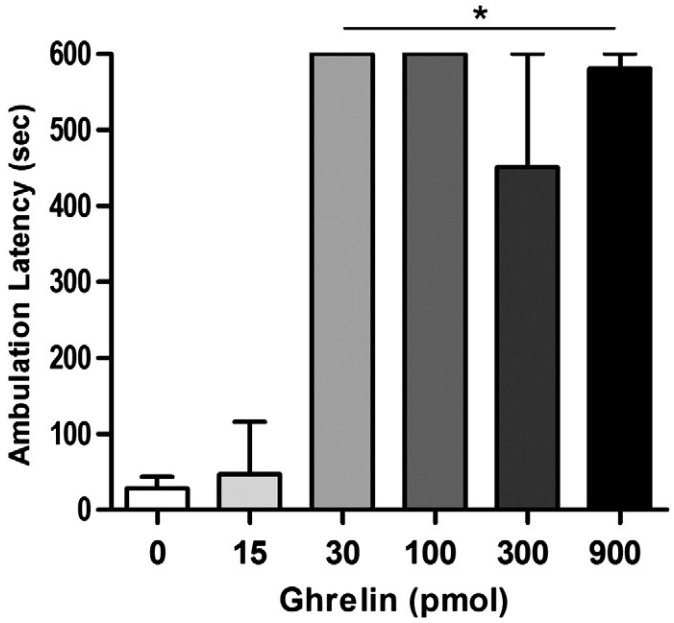

Fig. 2. Effect of intra-IMM administration of different doses of Ghr on the latency to ambulate (Open Field) in 4–6 day-old chicks. Bars represent median (interquartile range). n = 6–10. *p < 0.05 compared to saline (Dunn's post hoc test).

Table 2
Effect of administration of ghrelin into the IMM on anxiety-like behavior.

Ghrelin (pmol)	Number of ambulations	Defecation latency (s)	Number of defecations	Attempted escapes
0 (Saline)	40 (27–137)	22 (11–345)	2 (1–3)	0 (0–10.5)
15	17 (4–45.5)	412 (102.5–600)	1.5 (0–2)	0 (0–0.5)
30	0 (0–10) *	486 (363–600)	1 (0–2)	0 (0–0.5)
100	0 (0–49)	600 (481–600)	0 (0–0.5)	0 (0–5)
300	4 (0–6)*	600 (535–600) *	0 (0–1)	0 (0–0.5)
900	2 (0–4.5)*	341 (6.5–600)	1 (0–1.5)	0 (0–0)

Each value is expressed as median (interquartile range). n = 6–10. *p < 0.05 compared to saline group (Dunn's post-hoc test).

significant increase on the latency of ambulation induced by Ghr was completely eliminated by bicuculline ($p < 0.05$) (Fig. 3). This suppressed action of bicuculline was also observed by an increased locomotor activity ($p < 0.05$) and a decreased time to start to defecate ($p < 0.05$) (Table 3). However, the Kruskal–Wallis test did not indicate any significant effects on the number of defecations ($H = 11.92$, $p = 0.06$) or on the attempts to escape ($H = 4.879$, $p = 0.1809$) induced by Ghr (Table 3). Overall, this suggests that bicuculline was able to block the anxiogenic like-behavior induced by Ghr.

Experiment 3. Effect of diazepam administration on anxiety-like behavior induced by ghrelin

The Kruskal–Wallis test showed a significant effect of 30 pmol of intra-IMM Ghr on ambulation latency ($H = 17.60$, $p = 0.0005$), on the number of ambulations ($H = 15.40$, $p = 0.0015$) and attempts to escape ($H = 16.35$, $p = 0.001$). Dunn's post-hoc test revealed a significant increase in latency to ambulate in Ghr-injected chicks compared to saline-injected ones ($p < 0.05$). However, co-administration of 0.05 mg/kg of i.p. diazepam plus intra-IMM Ghr (30 pmol) did not reverse the increase induced by Ghr alone ($p > 0.05$) (Fig. 4). No significant changes in latency to defecate ($H = 10.34$, $p = 0.05$) or number of defecations ($H = 8.141$, $p = 0.05$) were observed (Table 4). Thus, diazepam did not eliminate the changes induced by Ghr on the behavioral pattern measured in the OF test.

Table 3
Effect of bicuculline administration on anxiety-like behavior induced by ghrelin.

Treatment	Number of ambulations	Defecation latency (s)	Number of defecations	Attempted escapes
Saline	68 (35–127)	183 (16–419)	1 (0.5–2)	2 (0–5)
BIC 0.036 mg/kg	35 (22–91)	11 (6–56)	1 (1–2)	0 (0–4)
Ghr 30pmol	2.5 (0–17) *	600 (600–600) * *	0 (0–1)	0 (0–1.5)
BIC + Ghr	28 (7–42)	60 (25–113)	2 (1–2)	0 (0–2)

Each value is expressed as median (interquartile range). n = 11–17. *p < 0.05 compared to saline group (Dunn's post-hoc test).

Experiment 4. Effect of bicuculline intraperitoneal administration on hypophagia induced by ghrelin

A two-way ANOVA revealed an independent significant effect of treatments ($F_{3,68} = 15.66$, $p < 0.0001$) and time after injection ($F_{2,68} = 239.5$, $p < 0.0001$). The Bonferroni test revealed that 30 pmol of Ghr significantly ($p < 0.05$) inhibited feeding and continued to do so up to 120 min after the injection into the IMM compared to saline. However, co-administration of 0.036 mg/kg bicuculline plus Ghr did not produce any changes in food intake compared to Ghr alone ($p > 0.05$) (Fig. 5A).

Experiment 5. Effect of diazepam intraperitoneal administration on hypophagia induced by ghrelin

A two-way ANOVA revealed an independent significant effect of treatments ($F_{3,45} = 14.61$, $p < 0.0001$) and time after injection ($F_{2,45} = 223.3$, $p < 0.0001$), with the Bonferroni test showing that the inhibitory effect on food intake induced by 30 pmol of Ghr alone ($p < 0.01$) compared to saline was not reversed by the co-administration of diazepam (0.05 mg/kg) at 30, 60 and 120 min ($p > 0.05$) (Fig. 5B).

Discussion

In the present study, we have shown for the first time that Ghr injected into the forebrain IMM area may induce an anxiogenic-like behavior, which was blocked by an $RGABA_A$ antagonist (bicuculline)

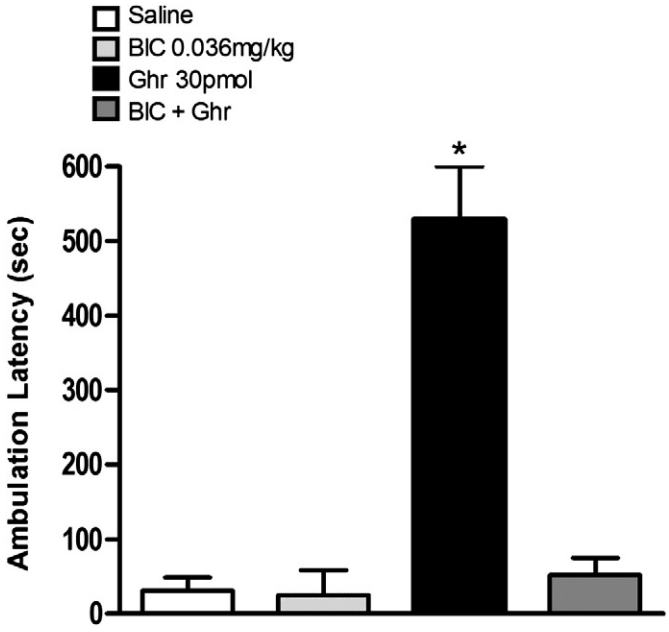

Fig. 3. Effect of i.p. bicuculline (BIC) administration on the latency to ambulate (Open Field) induced by intra-IMM Ghr, in 4–6 day-old chicks. Bars represent median (interquartile range). n = 11–17. *p < 0.05 compared to saline (Dunn's post hoc test).

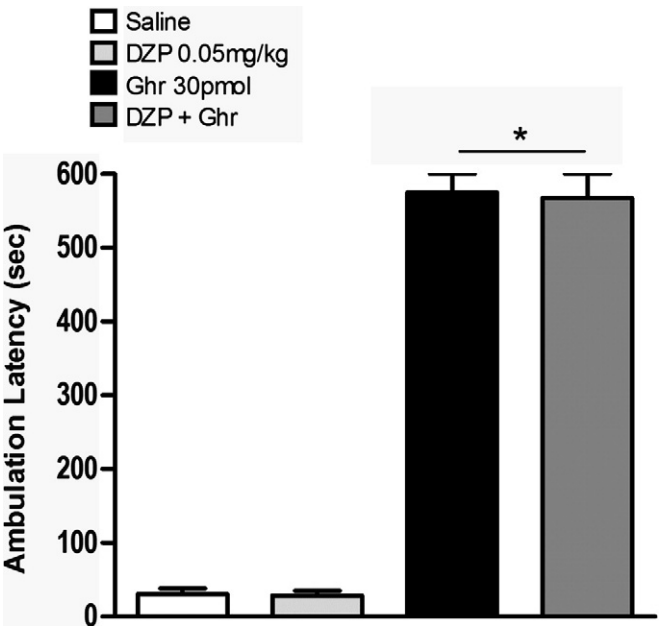

Fig. 4. Effect of i.p. diazepam (DZP) administration on the latency to ambulate (Open Field) induced by intra-IMM Ghr, in 4–6 day-old chicks. Bars represent median (interquartile range). n = 6–8. *p < 0.05 compared to saline (Dunn's post hoc test).

Table 4
Effect of diazepam administration on anxiety-like behavior induced by ghrelin.

Treatment	Number of ambulations	Defecation latency (s)	Number of defecations	Attempted escapes
Saline	87.5 (39–113)	212.5 (17–519.5)	2 (0.5–2)	4 (2.5–9.5)
DZP 0.05 mg/kg	59 (12–115)	24 (20–37)	1 (1–2)	1 (0–7)
Ghr 30pmol	1 (0–22.5) *	600 (547.5–600)	0 (0–1)	0 (0–0) *
DZP + Ghr	3 (0–7.5) *	526 (230–600)	0.5 (0–1)	0 (0–0) *

Each value is expressed as median (interquartile range). n = 6–8. *p < 0.05 compared to saline group (Dunn's post-hoc test).

without behavioral changes when a benzodiazepine receptor agonist (diazepam) was injected. However, this did not affect the decrease in food intake induced by Ghr.

The IMM would be a homologous area to the mammalian neocortex and constitutes an important center of integration that relates the sensory and motor system and receives afferents from different brain regions related to the motivational aspects of behavior (Atoji and Karim, 2014; Bradley et al., 1985; Jarvis et al, 2005). Thus, this area may respond to auditory and visual stimuli such as those generated by a novel environment and isolation in the case of OF. Interestingly, we showed that the IMM could be an important Ghr action site in response to an acute stressor induced by the OF test. In chicks, an OF response is primarily a fear of novelty and isolation, in addition to a tendency to reinstate contact with conspecifics (Faure et al., 1983), and represents a compromise between opposing tendencies to reinstate contact and to avoid detection by potential predators (Gallup and Suarez, 1980). Thus, changes in the latencies to ambulate and defecate may be established as an outline of fear in the task, while the number of crossed squares and escape attempts can be interpreted as a socially motivated behavior pattern in order to reinstall contact for isolated chicks (Carvajal et al., 2009; Gallup and Suarez, 1980).

In our study, Ghr (30 pmol) injected into IMM significantly increased the latencies to ambulate (Fig. 2) and defecate, and decreased the number of ambulations (Table 1) respect to saline in chicks exposed to the OF test, indicating that IMM may be involved in these behavioral changes. Related to this, it has been previously described that IMM is a multi-modal sensory integration area (equivalent to the avian cortex) that has well-defined role in memory processing in chicks (Csillag, 1999; Gibbs, 2008) and also shows an increased metabolic activity following to social stress (Müller and Scheich, 1986).

Several studies have shown that IMM has a high density of GABAergic neurons and $GABA_AR$ (Aller et al., 2003; Csillag et al., 1987). Also, it is an area that participates in short-term memory processes and is vulnerable after training to $GABA_AR$ antagonist (bicuculline) (Gibbs and Johnston, 2005). We observed that the previous administration of an ineffective dose of bicuculline (0.036 mg/kg) blocked the behavioral response elicited by Ghr (30 pmol) in the OF test (Fig. 3 and Table 2). Moreover, a non-anxiolytic dose of diazepam (0.05 mg/kg) did not change the increase induced by Ghr on behavioral response (Fig. 4 and Table 3). Our previous studies demonstrated that centrally administered Ghr induced in neonatal chicks a fearful and/or anxious behavior in the OF test, suggesting that this peptide plays an important role in the processes of memory formation associated with stress response to novelty and isolation (Carvajal et al., 2009). Similar response was also observed after i.p. injection of an anxiogenic β-carboline (inverse agonist of benzodiazepine receptor) (Marin et al., 1997). In rodents, it has been demonstrated that the i.c.v. administration Grh as well as into the amygdala, hippocampus or raphe nucleus induced anxiogenic response by reduced activity in the open arm of an elevated plus maze (Carlini et al., 2002, 2004) by increasing the release of CRF from the hypothalamus (Asakawa et al., 2001). In agreement with this, Saito et al. (2005) demonstrated that astressin (CRF_2R antagonist) attenuated the rise in plasma corticosterone induced by central Ghr in neonatal chicks. Besides, Roberto et al. (2010) reported that CRF augments GABAergic transmission and more recently, Cruz et al. (2013) found that Ghr administered in rat central amygdala may induce an increase in the GABAergic activity, such as the amplitude of evoked inhibitory postsynaptic potentials (IPSPs) and the frequency of miniature inhibitory postsynaptic currents (mIPSCs) suggesting a potential role of Ghr in regulating GABAergic neurotransmission. Therefore, the anxiogenic action induced by Ghr might be modulated by GABAergic system by involving the possible activation of the hypothalamic–pituitary–adrenal (HPA) axis.

On the other hand, GABAergic pathways exerted an inhibitory influence upon the release of serotonin at both the cell body and terminal level of corticolimbic serotonergic projections, of which a hyperactivity was implicated in the induction of anxious states (Millan, 2003) suggesting to central serotonergic system as a potential target in the expression of anxiety-like behavior induced by Ghr (Hansson et al, 2014). Thus, Ghr could also increase anxiety by activating serotonergic pathways whereas co-administration with bicuculline could suppress release of serotonin and consequently the anxiogenic effect of Ghr via GABAergic interneurons, although further investigations would be required to demonstrate underlying mechanisms of Ghr effects on anxiety.

Our findings showed that administration of 30 pmol of Ghr into IMM decreased food intake up to 2 h after injection. Similar results were also

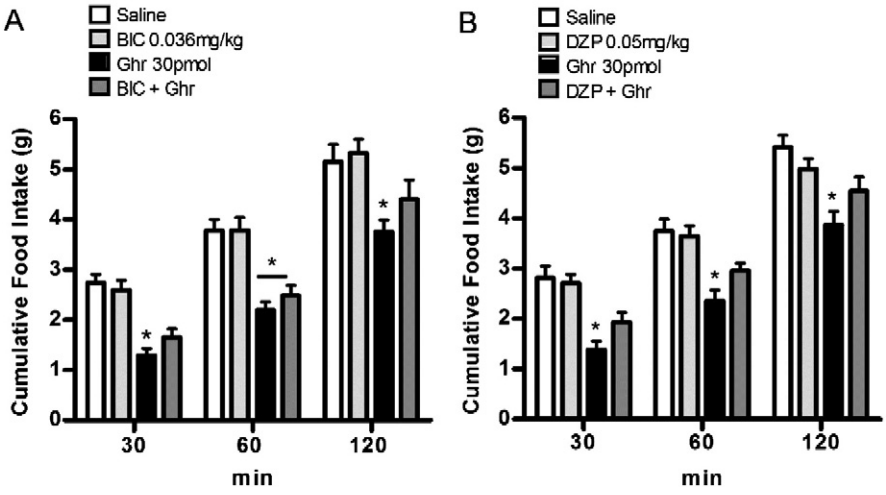

Fig. 5. Effect of BIC (A, n = 13–21) or DZP (B, n = 10–15) i.p. administration on hypophagia induced by intra-IMM Ghr in 4–6 day-old chicks. Bars represent media ± SEM. *p < 0.05 compared to saline (Bonferroni's test).

reported by Carvajal et al. (2009), Furuse et al. (2001) and Saito et al. (2002). Here, neither co-administration of 0.036 mg/kg of bicuculline nor of 0.05 mg/kg of diazepam did change the hypophagia induced by Ghr (Fig. 5). According to this, Jonaidi et al. (2012) observed that i.c.v. injection of GABAergic agents partially attenuated the decrease in food intake induced by Ghr suggesting that the contribution of the GABAergic system is limited if not irrelevant. This would indicate that GABAergic neurotransmission does not act as the main modulator of the inhibition of food intake produced by Ghr, at least in the IMM area. These authors reported that anorexic effect of Ghr centrally administered was due to a decrease in the synthesis of GABA, by reducing the expression of the gene encoding GAD_2 (glutamate decarboxylase enzyme), whereas $GABA_AR$ might not be involved in this action (Jonaidi et al, 2012). In addition, it has been observed that emotional changes evoked by the pharmacological manipulation of GABAergic neurotransmission in rat nucleus accumbens are not related to changes in food intake (Lopes et al., 2012). Interestingly, in chicks, CRF appears to play a crucial role in the inhibition of food intake and Ghr-induced anorexia may be mediated by the CRF system with consequent activation of the hypothalamic–pituitary–adrenal axis (Denbow et al., 1999; Saito et al., 2005). This system is regulated by the GABAergic fibers that modulate secreting-CRF hypothalamic neurons (Cullinan et al., 2008), suggesting that a diminished release of GABA induced by Ghr resulted in activation of the CRF system, which could lead to a state of hypophagia in the animal. However, the decrease in food intake induced by Ghr may have occurred through an alternative pathway to the GABAergic system. A recent finding reported that hypophagia induced by Ghr in chickens involved participation of the serotonergic system (Zendehdel et al., 2013). Together, we do not discard that this hypophagia may not be associated with a change in state of anxiety of the bird, at least in IMM, or that it could be mediated by a different mechanism to that observed in rodents. However, it should be investigated through further studies.

Conclusion

In summary, our results provide neuroanatomical and behavioral data indicating that IMM, a area with large homology to the mammalian neocortex, would be a brain target for Ghr action. Additionally, the $GABA_AR$ may participate in the anxiety-like behavior induced by Ghr suggesting that this peptide and GABA-A-ergic neurotransmission exert a complex functional interaction, at least, in the IMM to modulate the anxiety response. Although, it might not be strongly involved in hypophagia, we do not discard that both behavioral responses occur through different mechanisms.

Declaration of interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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