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Central ghrelin increases anxiety in the Open Field test and impairs retention memory in a passive avoidance task in neonatal chicks

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ABSTRACT

Ghrelin (Grh) is an endogenous ligand for the growth hormone secretagogue receptor. Although Ghr stimulates feeding in rats, it inhibits feeding in neonatal chicks. However, little is known about other central behavioral effects of Ghr. Therefore, we investigated the Ghr effects, injected intracerebroventricularly, on anxiety and memory retention of neonatal chicks in an Open Field test and in a one-trial passive avoidance task, respectively. In the Open Field test, the administration of Ghr in a dose-dependent manner increased the latency to ambulate but decreased ambulation activity, indicating an anxiogenic effect. Furthermore, chicks trained on a passive avoidance task and injected with a dose of 30 pmol of Ghr immediately after training showed an impairment of memory retention. However, there were no significant effects on the number of pecks during the pretraining, training, retention and discrimination. In addition, different doses of Ghr produced an inhibition in food intake at different times after injection. Our results indicate that Ghr induces anxiogenesis in chicks. Moreover, we have shown for the first time that Ghr can decrease memory retention in a non-mammalian species, suggesting that Ghr may play an important role in the processes of memory retention in birds.

1. Introduction

Ghrelin (Grh) is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). It is mainly produced in the rat stomach (Kojima et al., 1999) but 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). Ghr immunoreactivity was also found, in the chicken hypothalamus, although not in the arcuate nucleus, as in the case of rats (Ahmed & 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 Ghr production (Kaiya et al., 2002). In the chicken, Ghr mRNA expression has also been detected in several parts of the brain suggesting a central action of Ghr. However, the question why ghrelin is produced in several brain areas in addition to the hypothalamus remains unanswered (Saito et al., 2005).

Central Ghr plays an important role in various physiological functions in rats; for instance, in pituitary hormone secretion, gastrointestinal function and cardiovascular systems (Date et al., 2001; Kojima et al., 1999; Nagaya et al., 2001). However, little is known about its function in birds or in any of the other non-mammalian species. It has been reported that chicken Ghr can stimulate the release of growth hormone in chicks *in vivo* and *in vitro,* as previously seen in mammals (Ahmed & Harvey, 2002; Baudet & Harvey, 2003). Furthermore, both peripherally and centrally Ghr rapidly increases food intake and body weight in rats (Nakazato et al., 2001). However, the effect of Ghr on feeding produces the opposite effect from that seen in mammals, with an intracerebroventricular (icv) injection of Ghr reported to strongly inhibit food intake in neonatal chicks (Furuse et al., 2001). 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 and its receptor system (Saito et al., 2005).

In rodents, it has been shown that an icv administration of Ghr induces an anxiogenic effect (Carlini et al., 2002) and an improvement in memory retention in a step-down test (Carlini et al., 2002, 2004), in a spatial-dependent version of the novel object recognition test (Carlini, Gaydou, Schiöth, & de Barioglio, 2007; Diano et al., 2006) and in a T-maze footshock avoidance (Diano et al., 2006). These authors reported for the first time that showing Ghr promotes the formation of a hippocampal spine synapse density. Furthermore, Ghr knockout mice had a smaller number of hippocampal spine synapses than wild-type ones indicating that Ghr governs neuronal morphology of brain areas known responsible for memory (Diano et al., 2006). However, since Ghr inhibits food intake in chicks, it is possible that this peptide may have a different role to that found in rodents in the memory processes and anxiety

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behavior. Therefore, we investigated the action of distinct doses of centrally administered Ghr in neonatal chicks for different behavioral paradigms.

2. Materials and methods

2.1. Animals

Groups of 20 chicks (*Gallus gallus domesticus*) of both sexes were obtained immediately after hatching from a commercial hatchery INDACOR (Argentina) when they were only a few hours old. On arrival, this procedure was repeated three times and in all, a total of sixty chicks were housed in a white wooden box that measured 90 × 40 × 60 cm (length × width × height) before performing the Open Field test. This box was illuminated with an incandescent lamp hanging just above it and kept in a small room (3 × 3 m) at 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. The chicks were socially reared until they reached 3 days of age. Daily food replenishment (Cargill, broiler BB, and 20% minimum crude protein 12.34 MJ/kg) and maintenance chores were performed at 9 a.m.

For the passive avoidance task, another 20 chicks on a total of 58 ones were obtained immediately after hatching from a commercial hatchery and then were housed in pairs in 24 × 20 cm cages and kept under quiet conditions under a dim red light with water and food freely available for 24 h. 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 keep the number of animals used to a minimum.

2.2. Peptide and icv injection

The Ghr peptide (purchased from Neostystem, France) was dissolved in 0.85% saline containing 0.1% Evans Blue solution, and then administered in doses of 20, 30, 40, 80 and 200 pmol. Intracerebroventricular injections were given freehand at a volume of 10 μl using a Hamilton syringe (Andrew, 1991; Johnston, Clements, & Rose, 1999). The depth of the injection was 3 mm, controlled by using a plastic sleeve on a 27-gauge needle. 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 control group was given saline containing Evans Blue solution. The regional targets are the forebrain hemispheres, such as telencephalic structures, neurochemically and functionally comparable to the mammalian neocortex, claustrum, and pallial amygdale, in addition to other pallial areas such as hippocampus (Reiner et al., 2004).

2.3. Open Field test

Sixty chicks (3 days old) were individually gently captured, injected with the different doses of Ghr indicated above or with saline, and immediately after placed in a cardboard box before being carried to a separate room where the chick was placed in the center of a 60 × 60 cm Open Field (OF) apparatus with sides 30 cm high. This OF was made of white wood and the floor was marked off into 25 squares of 12 cm × 12 cm each, illuminated by a 100 W overhead bulb (Gallup & Suarez, 1980). The following types of behaviors were analyzed for 10 min: latency to ambulate, locomotor activity (number of squares crossed), latency for defecation, number of defecations and number of escapes. After testing, the floor of the OF apparatus was cleaned with towels wetted with 70% ethanol. Spontaneous activity was recorded by a digital camera suspended 1.5 m above the center of the apparatus (Day 1 of the experiment). The monitoring system was set up in a separate room to avoid disturbing the birds.

Twenty four hours later (Day 2 of the experiment), each chick was again placed in the center of the OF and its behavior was analyzed as described above. The birds were immediately decapitated after the experiment. The brains were then removed and inspected in order to control the accuracy of the placement of the injection.

2.4. One-trial passive avoidance task

Fifty-eight chicks were trained in a one-trial passive avoidance task at the age of 24 h as described by Lössner and Rose (1983) according to a model first introduced by Cherkin (1969). Chicks were pretrained by three 10 s presentations of a small (2.5 mm) white bead. This bead was positioned directly in front of and between 0.5 and 1 cm from the tip of each chicks beak. Chicks that did no peck at the bead at least two times out of the three pretraining trials were excluded from further testing (approximately 11%). After 30 min, half of the birds were trained by a presentation of a 4 mm chrome bead coated with 100% solution of methylantranilate (MeA) for 30 s. The other half of the chicks was trained by the presentation of the same bead coated with water. Chicks that did not peck at the chrome bead during training were also excluded from further experimentation (approximately 4%). Immediately after training, chicks from two groups (water or MeA-trained chicks) were injected with saline or 30 pmol of Ghr, in order to measure memory retention. Retention was tested 24 h after training to avoid the confounding effects of residual short-term memory, pro-active performance deficits, and circadian variations (Cherkin, 1969). Testing involved the presentation of a dry chrome bead to each chick for 30 s followed at least 5 min later by a 10-s presentation of a white bead. Chicks were considered to be amnesic if they responded to the test by pecking at both the previously aversive dry chrome and white beads. However, chicks which pecked exclusively at the white bead and avoided the chrome bead were considered to show recall of the training experience. Finally, the number of pecks directed towards the bead by each chick was recorded during the pretraining, training, retention and discrimination in order to check for any non-specific effects which Ghr may have had on pecking. All birds were immediately decapitated after the discrimination test. The brains were then removed and inspected in order to control the accuracy of the placement of the injection.

2.5. Control of the food intake

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.

2.6. Statistical analysis

Data from OF behavior assumed a non-normal distribution and were analyzed using Kruskal–Wallis nonparametric tests. Whenever the test indicated significant effects ($p < 0.05$), a pairwise comparison Dunn test was carried out. A p value < 0.05 was considered to represent a significant difference. An avoidance score for each experimental group was calculated by dividing the number of chicks in that group which did not peck at the chrome bead in the test by the total number of chicks allocated to the group, expressed as a percentage. Avoidance scores of Ghr treatment were

performed using χ^2 test, in order to compare between the avoidance of a group of chicks given a specific treatment (MeA training) and another group given a Ghr treatment. Thus the suppression of learning (or decreased avoidance) was defined as amnesia. The data of number of pecks during pretraining, training, testing and discrimination were expressed as the mean ± S.E.M. Data of training were analyzed by a one-way ANOVA that examined the main effect of training (water vs. MeA), and data of testing and discrimination were analyzed by a two-way ANOVA with a 2×2 factorial arrangement of treatments (training, water vs. MeA with treatment, saline vs. Ghr and their interaction). Whenever ANOVA indicated significant effects ($p < 0.05$), a pairwise comparison of means by the Newman Keuls test was carried out. In all cases, the assumptions of the analysis of variance (homogeneity of variance and normal distribution) were attained.

3. Results

3.1. Open Field behavior

During the OF test described above, the chicks suffered stress by isolation and novelty (Salvatierra & Arce, 2001). On Day 1 of the experiment, the Kruskal–Wallis test showed a significant effect of Ghr on the latency to ambulate in OF ($H = 37.415$; $p < 0.001$). Low doses of Ghr (30 and 80 pmol/per chick) induced an increase in the threshold latency to ambulate compared to saline ($p < 0.01$ and $p < 0.001$, respectively). Furthermore, higher doses of Ghr induced a greater latency to ambulate, with this corresponding to the total time of duration of the test for the 200 pmol dose ($p < 0.001$) (Fig. 1).

Fig. 2 shows the exploratory activity in OF test. The Kruskal–Wallis test showed a significant effect of Ghr on ambulatory activity ($H = 21.652$; $p < 0.0001$). The Dunn post hoc test revealed that high doses induced a significant decrease of ambulation until a complete inhibition of activity was reached (200 pmol, $p < 0.05$). Furthermore, significant differences were observed in the latency to defecate ($H = 13.031$; $p < 0.05$) and the number of defecations ($H = 14.289$; $p < 0.02$) during Day 1 of the experiment. The Dunn post hoc test demonstrated a total disappearance of the physiological responses, in agreement with the ambulatory inhibition observed at the dose of 200 pmol ($p < 0.05$). However, no significant differences between other parameters recorded were observed (Table 1).

On Day 2 of the experiment, the Kruskal–Wallis test no significant difference in the latency to ambulate was observed ($H = 8.015$; $p = 0.155$) (Fig. 3). In addition, no significant differences between the other parameters recorded were observed (Table 1).

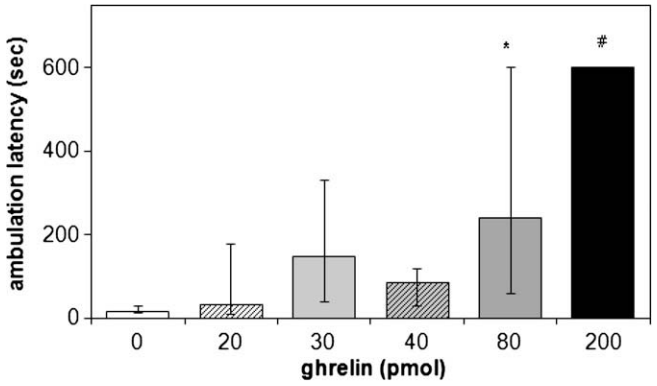

Fig. 1. Effect of icv administration of different doses of Ghr on the latency to ambulate (Open Field) in chicks during Day 1 of the test. Bars represent median (min–max). $n = 9$–11. $^{*}p < 0.01$ and $^{\#}p < 0.001$ Compared to saline (Dunn post hoc test).

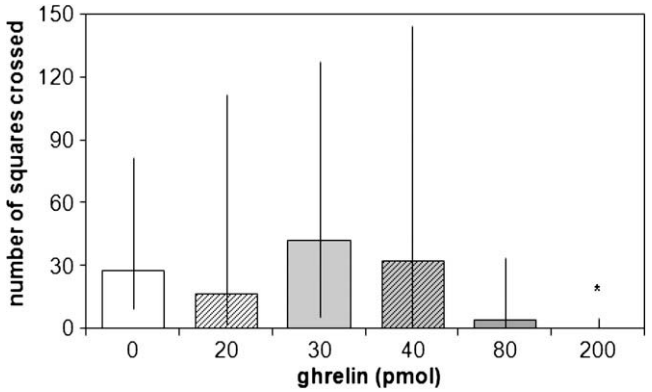

Fig. 2. Effect of icv administration of different doses of Ghr on ambulation activity (Open Field) in chicks during Day 1 of the test. Bars represent median (min–max). $n = 9$–11. $^{*}p < 0.05$ Compared to saline (Dunn post hoc test).

Table 1
Open Field test: behavioral changes induced by injection of Ghr.

Treatment	Am	L_{def}	ND	NE
Day 1				
Saline	28 (10–82)	39.5 (0–150)	1.5 (0–2)	0.5 (0–6)
Ghr, 20 pmol	17 (2–112)	0 (0–162)	0 (0–2)	0 (0–7)
Ghr, 30 pmol	42 (6–128)	111.5 (0–440)	1 (0–2)	0 (0–13)
Ghr, 40 pmol	32.5 (1–145)	72.5 (0–210)	1 (0–1)	0 (0–12)
Ghr, 80 pmol	4 (0–34)	0 (0–95)	0 (0–1)	0 (0–1)
Ghr, 200 pmol	0 (0–5)*	1 (0–2)	0 (0–1)	0 (0–1)
Day 2				
Saline	34 (7–241)	35 (9–540)	1 (1–3)	4 (0–36)
Ghr, 20 pmol	585 (18–270)	70 (0–124)	1 (0–3)	0 (0–44)
Ghr, 30 pmol	120 (5–188)	120 (58–300)	2 (1–3)	0 (0–31)
Ghr, 40 pmol	17 (0–236)	15 (0–457)	1 (0–3)	0 (0–23)
Ghr, 80 pmol	66 (0–179)	0 (0–460)	0 (0–3)	2.5 (0–8)
Ghr, 200 pmol	21.5 (0–181)	0 (0–280)	0 (0–1)	0 (0–10)

Behaviors were scored for 10 min after drug administration (20, 30, 40, 80 and 200 pmol) or saline (control). Day 1 corresponds to the day of injection. Day 2, 24 h after injection. Am, ambulation, as an index of exploratory activity. L_{def}, latency to defecate. ND, number of defecations. NE, number of escapes. Values are expressed as median (min–max). $n = 9$–11.
$^{*}p < 0.05$ compared to saline (control) (Dunn post hoc test).

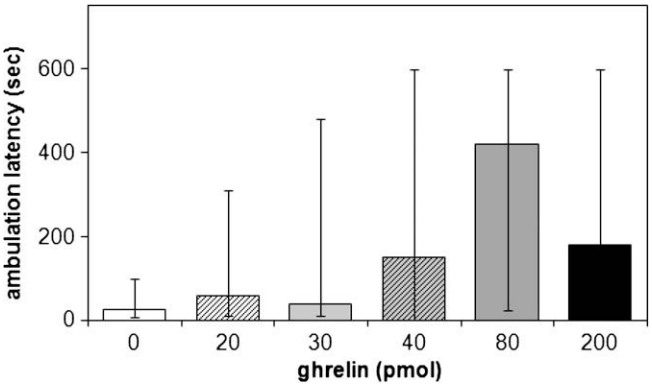

Fig. 3. Effect of icv administration of different doses of Ghr on the latency to ambulate (Open Field) in chicks during Day 2 of the test. Bars represent median (min–max). $n = 9$–11.

3.2. One trial passive avoidance learning

Fig. 4 shows the effects of an icv injection of 30 pmol of Ghr on avoidance scores 30 min after training for the passive avoidance task. This dose was chosen because it did not produce a complete inhibition of locomotor activity. There was no significant difference

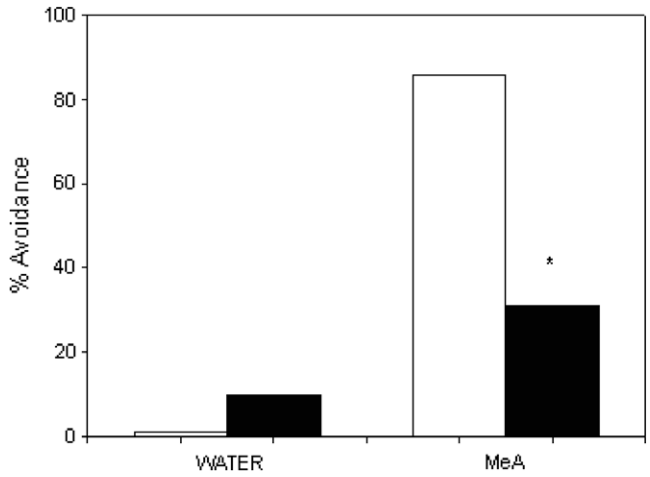

Fig. 4. Effect of training condition on retention for the passive avoidance task by saline-injected (□) and 30 pmol Ghr-injected (■) chicks. $n = 13–16$. $^{*}p < 0.01$ Compared to saline-injected chicks in MeA group (χ^2 test).

Table 2
Number of pecks after 30 pmol of Ghr for passive avoidance task in 2-day-old chicks.

Treatment	Pretraining	Training	Testing	Discrimination
Water/saline	4.35 ± 0.44	8.95 ± 1.39	7.53 ± 0.66	12.35 ± 1.37
Water/Ghr	3.97 ± 0.50	10.17 ± 1.76	7.08 ± 1.OS	10.08 ± 1.49
MeA/saline	5.95 ± 0.65	1.63 ± 0.26*	0.32 ± 0.01*,+	9.57 ± 1.52
MeA/Gftr	7.32 ± 0.75	1.73 ± 0.19*	3.11 ± 0.51	8.59 ± 122

Values are expressed as means ± SEM. $n = 13–16$.
* $p < 0.01$ compared to same treatment in water-trained chicks.
+ $p < 0.05$ compared to Ghr-injected chicks in MeA group (Newman–Keuls test).

in avoidance between Ghr-injected and saline-injected chicks trained with water ($\chi^2 = 12.5$, d.f. = 1, $p = 0.39$). However, significantly lower percentage of avoidance to peck at the chrome dry bead in Ghr-injected chicks compared to saline-injected ones ($\chi^2 = 5.76$, d.f. = 1, $p < 0.01$) was observed.

Table 2 shows the number of pecks at the bead during sessions of the one-trial passive avoidance task. A one-way ANOVA of number of pecks during training revealed a significant effect of MeA on the number of pecks occurred during the training session ($F_{1,54} = 42.571$, $p < 0.0001$) indicating an immediate potency of suppressive action of MeA. Newman–Keuls test showed a significant decrease in the number of pecks ($p < 0.001$) in the MeA-trained chicks compared to water-trained ones. In addition, a two-way ANOVA revealed a significant effect of training during testing session ($F_{1,54} = 27.71$; $p < 0.0002$). Newman–Keuls test showed a significant increase in the number of pecks in Ghr-injected chicks ($p < 0.05$) compared to saline-injected ones, in the MeA-trained group, indicating a decrease in the memory retention. Furthermore, a lower number of pecks ($p < 0.01$) in the saline-injected chicks of water-trained group compared to MeA-trained ones were observed. No change in water-trained chicks was observed after Ghr administration, indicating that the amnesic effect of Ghr seems specific to memory. However, no significant differences in the number of pecks during the discrimination session were observed (Table 2).

3.3. Food intake

The Ghr effect on food intake was used in order to verify that Ghr acts in the central nervous system. Our results showing that different Ghr doses inhibited the food intake to different times after injection (30 min after Ghr treatment, control = 2.42 ± 0.15, Ghr 20 pmol = 1.43 ± 0.07, Ghr 30 pmol = 1.14 ± 0.19, Ghr 40 pmol = 0.63 ± 0.13, Ghr 80 pmol = 0.28 ± 0.16. 60 min after Ghr treatment, control = 2.73 ± 0.15, Ghr 20 pmol = 2.24 ± 0.09, Ghr 30 pmol =2.05 ± 0.07, Ghr 40 pmol = 1.56 ± 0.13, Ghr 80 pmol = 1.42 ± 0.16 and 120 min after Ghr treatment, control = 3.66 ± 0.31, Ghr 20 pmol = 2.79 ± 0.23, Ghr 30 pmol = 2.93 ± 0.12, Ghr 40 pmol = 2.40 ± 0.10, Ghr 80 pmol = 1.95 ± 0.13) are in accordance with those previously demonstrated by Furuse et al. (2001). A two-way ANOVA revealed an independent significant effect of different Ghr doses ($F_{5,105} = 50.15$; $p < 0.0001$) and time after injection ($F_{5,105} = 119.02$; $p < 0.0001$). The Newman–Keuls test showed that all the doses of Ghr significantly ($p < 0.001$) inhibited feeding and continued to do up to 120 min after the injection.

4. Discussion

The present results show for first time that Ghr administered in neonatal chicks can induce a fearful and/or anxious behavior in the Open Field test and an impairment of memory retention in the passive avoidance task. This is also the first time that effects of Ghr on memory in a non-mammalian species have been revealed, suggesting that Ghr may have had a conserved evolutionary role for regulating memory in vertebrates for at least 250 million years.

It is well known that the Open Field responses, in chicks, primarily represent a fear reaction to a novel environment and isolation, and later a social motivation to reinstall contact with conspecifics (Faure, Jones, & Bessei, 1983; Gallup & Suarez, 1980). Thus, the latency to ambulate may be used to determine fear in the task, while the number of crossed squares and escapes may be interpreted as a behavior pattern socially motivated in order to reinstall contact for isolated chicks. Fig. 1 shows that the acute icv administration of Ghr increased, in a dose-dependent manner, the latency to begin to ambulate which had been induced by fear and/or anxiety, suggesting that Ghr might act an anxiogenic agent. Related to this, we have previously described that anxiolytic doses of diazepam induced a decrease in the latency to ambulate in an Open Field test on subpopulations categorized that exhibited a high level of anxiety (Marin, Martijena, and Arce(1997); Salvatierra and Arce (2001)). In addition, Carlini et al. (2002) observed that icv Ghr induced an anxiogenic effect in rats exposed to an elevated plus–maze test. However, higher doses of Ghr (80 and 200 pmol) increased the latency and decreased ambulatory activity to such an extent that it became completely inhibited.

Faure et al. (1983) also described a sleep-like behavior (sitting with eyes closed) during the first phase of an Open Field test, which reflected an inhibition of the behavior pattern induced by fear in the task. Therefore, sleep-like behaviors seem to play a fundamental role in the response to stress. On the other hand, Tachibana, Ohgushi, and Furuse (2001) reported that a central infusion of 600 pmol of Ghr induced sleep-like behavior. Thus, the immobility observed at the highest doses of Ghr may also be considered a response to fear and/or anxiety induced by a task.

Similar effects on the latency to defecate and the number of defecations for high doses were also observed, indicating that greater doses of Ghr induced a complete inhibition of physiological responses. However, no significant differences in the other parameters at anxiogenic doses were observed, possibly due to the fact that the interpretation of defecation is difficult in chicks, in contrast to the case of rats where it constitutes a primary fear index (Faure et al., 1983).

On Day 2 of the OF task, Ghr tended to inhibit ambulation but only at high doses. However, the total exploratory activity was higher than on Day 1, indicating a habituation to repeated exposure to the same environment (Lat, 1973). Habituation procedures have also been shown to attenuate stress responses to novelty in chicks (Taukulis & McKay, 1992). Hence, the increase in the latency

to ambulate suggested that Ghr could modify the response to novelty stress during the first day but did not influence habituation during the second day.

It has also been reported that the effects of a strong passive avoidance task (100% MeA) were retained for 24 h following the task. The fact that Ghr decreased the percentage of avoidance when administrated immediately after a training session indicates that this peptide modifies mainly memory retention. However, our results are not in agreement with previous data showing that Ghr improved memory retention in rats (Carlini et al., 2002, 2004) and mice (Diano et al., 2006) and reversed the decreased memory for novel object recognition in chronically food-restricted mice (Carlini et al., 2008).

Ghr-induced hyperphagia is controlled by orexigenic peptides such as neuropeptide Y and orexin in rats (Nakazato et al., 2001; Toshinai et al., 2003). However, in neonatal chicks, these peptides do not mediate food intake, suggesting that different mechanisms control feeding behavior (Furuse et al., 1999). It has been reported that the inhibitory effect of central Ghr on food intake is caused by activation of the endogenous corticotropin-releasing factor system observed by increased plasma corticosterone levels after icv Ghr administration (Kaiya et al., 2002; Saito et al., 2005) suggesting that this peptide in addition to its growth hormone-releasing activity, have an important function as regulator of adrenal function in birds (Kaiya et al., 2002). These results are consistent with Kühn, Geelissen, Van der Geyten, and Darras (2005) indicating that Ghr controls growth hormone release and profoundly increases plasma concentrations of corticosterone suggesting that the Ghr effects on anxious/fear behavior in Open Field could be mediated by corticotropic axis. On the other hand, it has been observed that absorption of yolk sac ingredients in young chickens during the first 5 days of life is intensive (Jamroz, Wertelecki, Wiliczkiewicz, Orda, & Skorupinska, 2004). Thus, loss of yolk sac commonly maximized around 3–4 days posthatch is in phase with the ability of the gastrointestinal system to recover nutrients from feed (Morán, 2007; Noy & Pinchasov, 1993). Hence, the impaired effect on memory of Ghr might be attributed to these different mechanisms of feeding behavior associated to inhibition of food intake.

Moreover, an examination of the number of pecks during the pretraining, training and discrimination indicated that the Ghr treatment did not produce a significantly deleterious effect on other aspects of the chickś behavior; thus, the amnesic effect of Ghr seems to be specific to memory, rather than a non-specific behavioral alteration, thereby supporting the notion that Ghr could act specifically on processes of memory.

In summary, we have demonstrated that acute the administration of Ghr in the central nervous system induces anxiogenic effects and decreases memory retention, suggesting that this peptide may play an important role in the processes of memory formation in birds although its role in metabolic processes and homeostatic regulation should not be discarded.

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