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INTERACTION OF GHRELIN AND OPIOIDS IN LUTEINIZING HORMONE (LH) SECRETION BY PITUITARY CELLS OF COMMON CARP, *CYPRINUS CARPIO* (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE), CULTURED IN VITRO

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Background. Ghrelin, a newly discovered hormone is involved mainly in the regulation of body energy homeostasis. It has also been proved that ghrelin affects many other processes including the control of the hypothalamo-pituitary-gonadal axis in vertebrates. Ghrelin interacts also with other peptides and neurotransmitters, which are involved in gonadotropin release, such as endogenous opioid peptides. The aim of the experiment was to compare the effects of ghrelin and naltrexone, an opioid antagonist, on LH secretion by pituitary cells of mature female and male common carp, *Cyprinus carpio* L.

Materials and methods. In the in vitro experiment the time-dependent (10 and 24 h) action of ghrelin (10^{-7} or 10^{-6} M) and naltrexone at 10^{-6} M added alone or in combination on LH secretion by enzymatically dispersed pituitary cells of mature female and male carp (*Cyprinus carpio* L.) was studied.

Results. Ghrelin alone at a dose of 10^{-6} M stimulated LH secretion after 10 h of female- but not male-derived cell incubations. Ghrelin at 10^{-7} M had no effect on LH secretion from either female or male cells. Naltrexone alone stimulated LH secretion only after 24 h of female cell culture. In male cells no significant changes in LH secretion in response to naltrexone alone were found after 10 or 24 h incubation period. Combined treatment (ghrelin 10^{-7} or 10^{-6} M and naltrexone) stimulated LH secretion in female and male cell incubations at 10 and 24 h: LH levels were significantly higher in comparison to control, to ghrelin alone (in cells of both sexes) and to naltrexone alone (in male cells only).

Conclusion. The results suggest that opioids and ghrelin may control LH secretion in carp acting synergistically, probably through the same receptor type. The concept of opioid and ghrelin interaction in the gonadal steroid feedback on LH release is also discussed.

Keywords: common carp, pituitary cells, ghrelin, naltrexone, in vitro LH secretion

INTRODUCTION

The role of endogenous opioid peptides in the control of sexual maturation and reproduction of mammals is already well established (Yen et al. 1985, Piva et al. 1986, Ferin 1987, Gopalan et al. 1991, Kalra 1993). They act at all levels of the hypothalamo-pituitary-gonadal axis (Blank et al. 1986, Cacicedo and Sanchez-Franco 1986, Leposavic et al. 1991, Smith and Gallo 1997, Kaminski et al. 2004) and are also involved in gonadal steroid feedback on gonadotropin secretion from the pituitary (Barb et al. 1986, Martini et al. 1989, Behrens et al. 1993). The action of opioids on many aspects of reproduction in mammals is mainly inhibitory (Yilmaz et al. 1996, Smith et al. 1997, Kawamura et al. 2003).

In fish, opioids are also involved in the regulation of LH secretion but data on this aspect of fish reproduction are still very scarce. Opioids modulate GnRH and dopamine secretion, the two main factors controlling LH release in fish (Martini et al. 1989, Cheng 1996, Sokolowska-Mikolajczyk et al. 2002a,b, 2005, Socha et al. 2003). As in mammals, opioids may also mediate the gonadal steroid feedback on LH secretion in fish (Sokolowska-Mikolajczyk et al. 2002a, b, 2010).

Opioid regulation of LH release in higher vertebrates involves mediation of numerous neurotransmitter systems and other hormones which are also involved in LH synthesis and/or secretion, such as ghrelin or leptin (Kalra and Simpkins 1981, Aurich et al. 2002, Dudás and Merchenthaler 2004). This may also be true for fish.

Ghrelin is a newly discovered pleiotropic hormone that is involved mainly in the regulation of body energy home-

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ostasis. It has also been proved that ghrelin affects many other processes including the control of the hypothalamo-pituitary-gonadal axis in higher vertebrates. In primates, sheep, and rats ghrelin has an inhibitory impact on the reproductive system (Furuta et al. 2001, Vulliémoz et al. 2004, Fernandez-Fernandez et al. 2006, Iqbal et al. 2006). In fish, like in mammals, ghrelin controls food intake and regulates the release of pituitary hormones (GH and LH) (Unniappan et al. 2002, 2004, Sokolowska-Mikolajczyk et al. 2009). Limited data on the role of ghrelin in sexual maturation or reproduction of fish show that, unlike in mammals, ghrelin stimulates LH release, acting directly on the pituitary (Grey and Chang 2008, Sokolowska-Mikolajczyk et al. 2009) and/or at the level of hypothalamus (Unniappan and Peter 2004). It seems possible that at this level ghrelin interacts with other peptides and neurotransmitters, which are also involved in gonadotropin release, such as endogenous opioid peptides.

The concept of the endogenous opioid interaction with ghrelin in mammals is based on the similarity of the receptors for both hormones: ghrelin receptors belong to a small subset of 7TM (seven transmembrane domain) receptors. Some other members of this subset are the motilin receptor and, to a certain degree, the somatostatin and opioid receptors (Holst et al. 2003). There are also anatomical examples of such interaction. It has recently been observed that ghrelin immunoreactive hypothalamic neurons innervate other peptidergic systems such as proopiomelanocortin (POMC) neurons (Cowley et al. 2003), which synthesize β-endorphin. Guan et al. (2008) suggest that POMC-producing neurons are modulated via synaptic communication with ghrelin-containing neurons and that these neurons may have a positive feedback effect on POMC-containing neurons through direct synaptic contacts.

Also physiological examples exist of the interactions between these two regulating systems: ghrelin produces excitatory effects on the neurons of the arcuate nucleus (Riediger et al. 2003), where endogenous opioids containing neurons are located (Bloom et al. 1979). There are reports showing a positive influence of ghrelin on opioid peptide release and/or activity (Kamegai et al. 2000). Pierzchała-Koziec and Zubel (2008) found an interaction of opioids and ghrelin in response to the activation of the immune system in lambs and Pierzchała-Koziec et al. (2006) demonstrated that ghrelin is involved in regulation of opioids and leptin release in mice.

As regards the relationship between ghrelin and opioid peptides in the regulation of gonadotropin release in fish, we know of no data in the scientific literature. Therefore, the aim of the present in vitro study was to examine the effects of human ghrelin and opioid receptor antagonist naltrexone on LH secretion from common carp pituitary cells.

MATERIALS AND METHODS

Fish. The experiment was conducted on sexually mature common carp, *Cyprinus carpio* L., in June 2005. Because one part of this experiment concerning the spontaneous and sGnRH-analogue-stimulated LH release under the influence of ghrelin has already been published (Sokolowska-Mikolajczyk et al. 2009), the control values are the same for both reports. Eight females and 10 males weighing an average of 1.95 ± 0.30 and 1.46 ± 0.06 kg, respectively were netted from the outdoor ponds of the Fishery Station belonging to the Department of Ichthyobiology and Fisheries, University of Agriculture in Krakow, Poland. Gonad maturity was specified as a percentage of body weight (gonadosomatic index GSI). Male gonads (GSI = 4.2 ± 0,8) were at the stage of spermiation, GSI for female gonad was 22.13 ± 2.64. Fish were transferred to flow-through basins (1.5 m^3 volume). Fish were exposed to a simulated natural photoperiod (16L : 8D) and water temperature of 18 ± 2°C for three days. On the day of the in vitro experiment fish were anaesthetised with 2-phenoxy-ethanol (Merck, Germany) at 0.3 mL · $L^{-1}$of water, killed by decapitation and the pituitary gland were collected and placed in sterile ice-cold medium (MEM-Eagle, Sigma-Aldrich, USA) buffered with 15 nM Hepes (Sigma-Aldrich, USA) and 9 mM sodium bicarbonate (P.O.Ch., Poland).

Culture technique. The enzymatic dispersion of the pituitary glands (separate pools of female and male pituitaries) and the technique of cell culture were described in detail elsewhere (Mikołajczyk et al. 1990, Sokolowska-Mikolajczyk et al. 2009). In brief, collected glands (separately from males and females) were chopped into small pieces and subjected to dispersion for 6–8 h at 20°C in the medium containing 0.1% (w/v) collagenase H (Boehringer Mannheim, Germany) and 1% BSA (Sigma-Aldrich, USA). The cells were harvested by 10-min centrifugation (200 m· s^{-2}) at 20°C and washed twice with pre-incubation medium containing 2% (v/v) serum substitute (Ultroser SF, Sepracor S.A., France) and 1% (v/v) antibiotic-antimycotic (Sigma-Aldrich, USA).

Cell viability test and cell counting were performed with a Thoma haemocytometer. Cells were resuspended in the pre-incubation medium and transferred into four 96-well microplates (Nunc A/S Denmark) coated with Poly-L-lysine (Sigma-Aldrich, USA) (two plates for cells of female pituitary pool and two for male pituitary pool). Each well contained approximately 5 × 10^4 cells in 250 µL of medium. Then the plates were sealed and incubated for 48 h at 22°C.

On the third day of culture the pre-incubation medium was replaced with medium containing ghrelin (Human, 1–18, Peptides International-Louisville, USA) at a concentration of 10^{-7} or 10^{-6} M, the opioid antagonist naltrexone (Sigma-Aldrich, USA) at a concentration of 10^{-6} M or the combination of ghrelin (both concentrations) and naltrexone. Control wells were filled up with medium without any supplementation. Each treatment group consisted of five wells containing cells from either the pool of male or female pituitaries. Two identical microplates were prepared. One of these was incubated for 10 h and the other for 24 h at 22°C. At the end of the incubation periods the plates were centrifuged (200 m· s^{-2}) for 10 min at 20°C, and the media were collected and frozen at –20°C until LH determination by ELISA (Kah et al. 1989). Sensitivity of the performed ELISA

was in the range 0.6–100 ng · mL^{-1} with the intra- and inter-assay coefficients of variance at 5% and 9%, respectively.

Statistics. Mean basal LH concentrations (LH secretion in the presence of the control medium only) were 3.147 ng · mL^{-1} for females and 2.146 ng · mL^{-1} for males. LH levels measured in the medium samples were recalculated and presented as a percentage of basal secretion (secretion in the presence of the control medium). Then data were analysed using the nonparametric two-tailed Mann–Whitney U-test. The differences between the means were determined as significant for $P < 0.05$.

The experiment was conducted according to the guidelines given by the Institutional Ethical Committee.

RESULTS

Effects of ghrelin on LH secretion

Pituitary cells from females. Cells treated with ghrelin at 10^{-6} M for 10 h released 72.2 percentage points more LH than cells incubated with control medium. Lower concentration of ghrelin (10^{-7} M) had no significant effect on LH secretion to the medium, though LH concentrations increased by 62.9 percentage points in comparison to control (Fig. 1). After 24-h incubation there were no significant differences in LH secretion between cells incubated in the presence of ghrelin and in control medium (Fig. 2).

Pituitary cells from males. The incubation of male cells for 10 or 24 h in the presence of ghrelin (both concentrations) did not cause any significant changes in LH secretion in comparison to the control, after either 10 or 24 h of culture (Figs. 3, 4).

Effects of naltrexone on LH secretion

Pituitary cells from females. Ten hours of cell culture in the presence of naltrexone did not change the LH release into the medium (Fig. 1). A significant 52.2-percentage-point increase of LH secretion in comparison to control incubations was found after 24-h incubation (Fig. 2).

Pituitary cells from males. Pituitary cells did not respond to naltrexone presence in the medium and the changes in LH secretion after 10- or 24-h incubation were not significant in relation to the control (Figs. 3, 4).

Effects of ghrelin and naltrexone on LH secretion

Pituitary cells from females. Ten hours of cell incubation in the presence of the combination of ghrelin (both concentrations) and naltrexone showed a significant increase of LH secretion in comparison to the control (by 141.8 and 126.7 percentage points, respectively). After 24 h of incubation the differences between ghrelin- and naltrexone-treated cells and control incubations were still significant, since LH levels in the presence of ghrelin (10^{-7} M) + naltrexone and ghrelin (10^{-6} M) + naltrexone were 132.6 and 105.2 percentage points higher, respectively (Figs. 1, 2). The combination of ghrelin and naltrexone stimulated LH release also in comparison to the appropriate concentration of ghrelin alone by 122.2 and 110.57 percentage points, respectively.

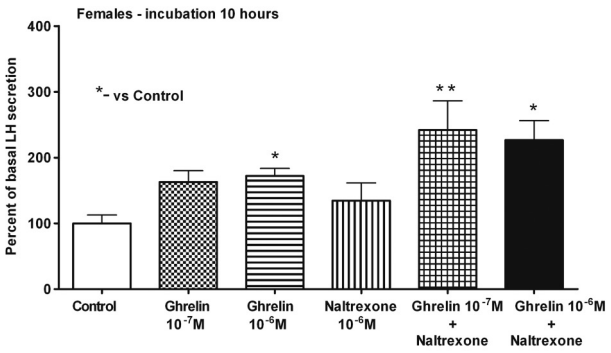

Fig. 1. Percentage of basal LH secretion from female pituitary cells incubated for 10 h with control medium, human ghrelin at 10^{-7} or 10^{-6} M, naltrexone (10^{-6} M) or the combination of naltrexone with ghrelin at 10^{-7} or 10^{-6} M; Bars represent means and SEM are given as vertical error bars ($n = 5$); *($P < 0.05$), **($P < 0.01$) vs. control

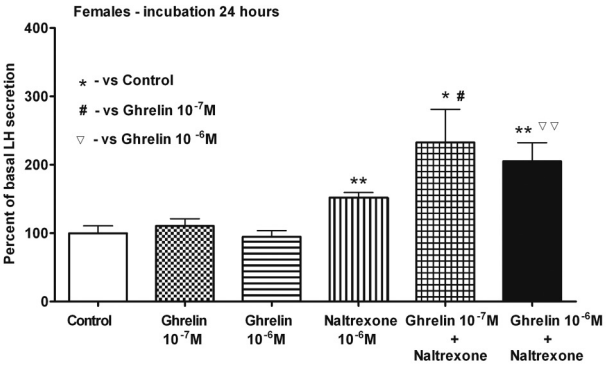

Fig. 2. Percentage of basal LH secretion from female pituitary cells incubated for 24 h with control medium, human ghrelin at 10^{-7} or 10^{-6} M, naltrexone (10^{-6} M) or the combination of naltrexone with ghrelin at 10^{-7} or 10^{-6} M; Bars represent means and SEM are given as vertical error bars ($n = 5$); *($P < 0.05$), **($P < 0.01$) vs. control; #($P < 0.05$) vs. ghrelin 10^{-7} M;▽▽($P < 0.01$) vs. ghrelin 10^{-6} M

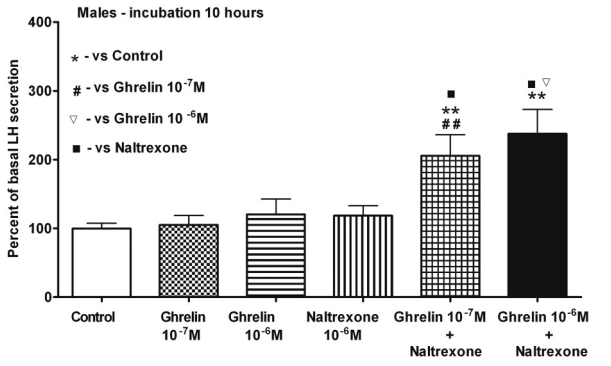

Fig. 3. Percentage of basal LH secretion from male pituitary cells incubated for 10 h with control medium, human ghrelin at 10^{-7} or 10^{-6} M, naltrexone (10^{-6} M) or the combination of naltrexone with ghrelin at 10^{-7} or 10^{-6} M; Bars represent means and SEM are given as vertical error bars ($n = 5$); **($P < 0.01$) vs. control; ##($P < 0.01$) vs. ghrelin 10^{-7} M;▽($P < 0.05$) vs. ghrelin 10^{-6} M; ■($P < 0.05$) vs. naltrexone

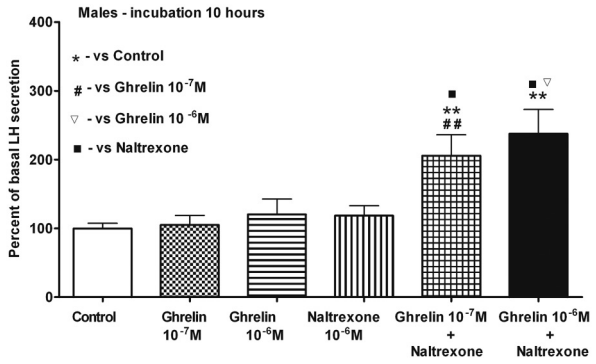

Fig. 4. Percentage of basal LH secretion from male pituitary cells incubated for 24 h with control medium, human ghrelin at 10^{-7} or 10^{-6} M, naltrexone (10^{-6} M) or the combination of naltrexone with ghrelin at 10^{-7} or 10^{-6} M; Bars represent means and SEM are given as vertical error bars ($n = 5$). **($P < 0.01$) vs. control; # ($P < 0.05$) vs. ghrelin 10^{-7} M

Pituitary cells from males. The combination of both tested agents resulted in the highest LH secretion after 10 h of incubation (Figs. 3, 4). Indeed, LH levels increased by 105.7 and 137.5 percentage points and by 100.8 and 117.3 percentage points in comparison with the control and the respective ghrelin concentration. After a 24-h incubation period, only the lower ghrelin concentration combined with naltrexone induced a significant increase in LH release (79.2 and 80.3 percentage points, compared to control and ghrelin, respectively).

DISCUSSION

Gonadotropin secretion in fish is regulated by many factors acting at all levels of the hypothalamo-pituitary-gonadal axis. The most important factors that received the most attention are gonadotropin-releasing hormone and dopamine, which inhibits gonadotropin release (Chang and Peter 1983, 1984, Peter et al.1986, 1991). Other factors like norepinephrine, g-amino butyric acid, melatonin, neuropeptide Y, cholecystokinin (Van der Kraak et al. 1998), opioids (Cheng unpublished*) Rosenblum and Peter 1989, Sokolowska-Mikolajczyk et al. 2002a, b, 2005), ghrelin (Unniappan et al. 2002, Sokolowska-Mikolajczyk et al. 2009) and leptin (Peyon et al. 2001, Weil et al. 2003) are also involved in the regulation of gonadotropin secretion. They can act directly at the level of the pituitary gonadotrophs, but also by the modulation of GnRH and dopamine secretion from the hypothalamus. Usually they act at both levels (Trudeau 1997). The in vivo experiments conducted on goldfish and common carp demonstrated the involvement of opioids or ghrelin in gonadotropin release, but they failed to identify the exact site of their action (Rosenblum and Peter 1989, Sokolowska-Mikolajczyk et al. 2002a,b, Unniappan and Peter 2005). The in vitro approach demonstrated that both peptides act directly on gonadotrophs of goldfish and common carp (Sokolowska-Mikolajczyk et al. 2002a, b, Socha et al. 2003, Unniappan and Peter 2004, Sokolowska-Mikolajczyk et al. 2005, 2009). The present work investigated the interaction of opioids with ghrelin, because ghrelin is implicated in the regulation of opioid release in mice (Pierzchała-Koziec et al. 2006). It has recently been found that ghrelin immunoreactive hypothalamic neurons innervate other peptidergic systems such as proopiomelanocortin neurons (Cowley et al. 2003).

In the presently reported experiment female carp pituitary cells in the presence of ghrelin alone at a concentration of 10^{-6} M significantly ($P < 0.05$) increased LH levels in comparison to the control medium by 72.2 percentage points after 10 h of incubation. A ten times lower concentration of ghrelin also increased LH secretion (by 62.9 percentage points), but this difference was not significant (Fig. 1). Male pituitary cells incubated and treated in the same way as female-derived cells did not respond to ghrelin (Figs. 3, 4). These in vitro effects of ghrelin on LH secretion in female and male carp have already been described by Sokolowska-Mikolajczyk et al. (2009). Unniappan and Peter (2004) also observed stimulatory influence of ghrelin in goldfish pituitary cell incubation system, but with a 100 times lower dose than in our experiment. In the same publication the authors showed that also in vivo ghrelin caused LH to rise in goldfish. In rats, ghrelin predominantly inhibits in vivo LH secretion (Furuta et al. 2001, Kawamura et al. 2003, Vulliémoz et al. 2004), but in vitro data are not so consistent (Fernandez-Fernandez et al. 2005) and show that the response is related to the steroid levels in the investigated animals. In our experiment, differential response to ghrelin depending on the sex of the fish-donor of pituitary cells may indicate that the steroid levels, which vary by sex, are responsible for a particular response or lack thereof.

In the presently reported in vitro experiment naltrexone alone at 10^{-6} M did not change LH secretion from female carp pituitary cells at 10 h of culture, but after 24 h LH secretion under the influence of naltrexone was higher ($P < 0.01$) than in the control incubations. In the case of male-derived cells incubated and treated in the same way as female cells, no response to naltrexone after either 10 or 24 h of incubation was noted (Figs. 3, 4). The choice of naltrexone dose used in the experiment was based on the results of Socha et al. (2003) who showed, in the in vitro carp pituitary cell culture, the dose-dependent increase of LH levels under 10^{-8}, 10^{-6}, 10^{-4}, or 10^{-2} M of naltrexone with a concentration of 10^{-6} M being the minimal effective one. However the lack of the reaction to naltrexone in the in vitro experiment with carp has already been recorded in another experiment with female and male carp pituitary cell incubations performed during the late gonadal recrudescence (Sokolowska-Mikolajczyk et al. 2005). Also in vivo results on common carp (Sokolowska-Mikolajczyk et al. 2002a, b) confirm that opioid influence on gonadotropin release is affected by the actual sex steroid milieu. It may be true also for ghrelin in fish, but there are not yet enough data to support such a statement.

Carp pituitary cells treated with the combination of ghrelin (10^{-7} or 10^{-6} M) and naltrexone released the high-

* Cheng K.W. 1996. Molecular studies of proopiomelanocortin in goldfish brain. M.Phil. Thesis. The University of Hong Kong, Hong Kong.

est amount of LH into the cultured medium (Figs. 1–4). In female cells after 10 h of incubation, the amounts of LH were significantly higher than in the control incubations by 148.1 or 126.7 percentage points, respectively (Fig. 1). In 24-h incubations LH concentrations were still higher than in the control (132.6 and 105.2 percentage points) and also compared to ghrelin alone (by 122.2 or 110.6 percentage points, respectively) (Fig. 2). A similar phenomenon was observed in male cells: 10-h incubation caused a significant increase of LH secretion into the culture media in comparison to the control (by 105.7 and 137.5 percentage points), to ghrelin (100.8 and 117.3 percentage points) and naltrexone alone (87.1 and 118.9 percentage points, respectively) (Fig. 3). After 24-h culture only ghrelin at a dose of 10^{-7} M with naltrexone significantly increased LH secretion by 79.2 percentage points in relation to the control and by 80.34 percentage points in relation to ghrelin alone (Fig. 4). To summarize the results of the present investigation, if ghrelin and naltrexone act alone on the carp pituitary cells the effect is sex dependent—there is a stimulation of LH secretion in the case of female cells but no response of male cells. If both hormones are applied together a significant increase of LH secretion to the medium is observed for cells of female and male origin, indicating a possible potentiation of naltrexone on ghrelin LH-releasing action.

It is interesting that even if there is no response to naltrexone or to ghrelin alone, like in male cells, the stimulation of LH is so strong when both hormones are present in the medium (Figs. 3, 4). These effects are not easy to explain because of insufficient amount of data, which comes from the in vitro study only. It is presumed that both hormones may act through the same receptor type localised on gonadotrophic cells and the sum of ligand concentrations stimulates this receptor type more strongly than a single dose of each of the hormones. It is also possible that one hormone can up-regulate the number of other hormone receptors. This explanation is only speculation and to be closer to the real situation many more in vivo and in vitro studies should be done. They would explain the relationships between opioids and ghrelin in LH secretion control at all levels of the hypothalamo-pituitary-gonadal axis. In this context the problem of opioid and ghrelin participation in gonadal steroid feedback on gonadotropin release in fish could be clarified, because to date there has been no convincing data explaining the mediation of which hormone and at which level is responsible for this feedback. Evidence on opioid involvement in this process in fish is scarce and no data on ghrelin exist to our knowledge. As opioids and ghrelin can act through the same receptor type in mammals (Pierzchała-Koziec 2004) and probably also in fish (differential response to ghrelin depending on the sex of donor fish cells in the present findings), we cannot rule out the possibility that gonadal steroids affect the hypothalamus and pituitary indirectly through several factors (in this case ghrelin and opioids) which interact between each other. However, without further research on this subject such a statement is still a matter of conjecture.

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