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Effects of *In Vivo* and *In Vitro* Administration of Ghrelin, Leptin and Neuropeptide Mediators on Pulsatile Gonadotrophin-Releasing Hormone Secretion from Male Rat Hypothalamus Before and After Puberty

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ABSTRACT

The present study aimed to investigate the effects of leptin and ghrelin on pulsatile pulsatile gonadotrophin-releasing hormone (GnRH) secretion *in vitro* with emphasis on neuropeptide mediators and changes between prepuberty (15 days) and sexual maturity (50 days) in the male rat. When hypothalamic expiants were studied 90 min after an intraperitoneal injection of leptin, ghrelin or agouti-related protein (AgRP) at 15 days, the GnRH interpulse interval (IPI) was significantly increased by ghrelin and AgRP and decreased by leptin. At 50 days, an increase in GnRH IPI was also caused by ghrelin and AgRP. When the peptides were directly incubated with the expiants, the effects of leptin and AgRP *in vitro* were consistent with those seen after *in vivo* administration. By contrast, ghrelin resulted in a reduction of GnRH IPI and this was observed at 15 days only. To delineate the neuropeptide mediators of leptin and the effects of ghrelin in the hypothalamus, various hypothalamic neuropeptides and antagonists were used *in vitro.* At 15 days, the GnRH IPI was significantly decreased after incubation with cocaine and amphetamine-regulated transcript (CART), α-melanocyte-stimulating hormone, corticotrophin-releasing factor (CRF) and neuropeptide Y (NPY). The reduction of GnRH IPI caused by leptin was partially prevented by either an anti-CART antiserum or SHU 9119, a melanocortin MC3/MC4 receptor antagonist or a CRF receptor antagonist. The NPY-Y5 receptor antagonist did not influence the effects of leptin whereas that antagonist totally prevented the decrease in GnRH IPI caused by ghrelin. The ghrelin-induced reduction of GnRH IPI was partially prevented by SHU 9119. When used alone, SHU 9119 or a CRF-receptor antagonist resulted in increased GnRH IPI at 50 days while they had no effects at 15 days. The NPY-Y5 receptor antagonist resulted in increased GnRH IPI at 15 and 50 days. In conclusion, leptin and ghrelin show opposing effects on pulsatile GnRH secretion after administration *in vivo* whereas they both have stimulatory effects *in vitro.* Such effects involve consistently the anorectic peptides CART and CRF for leptin that are mainly active at 15 days. The melanocortigenic system appears to mediate the effects of both leptin and ghrelin. The effects of ghrelin also involve NPY receptors and operate effectively before and at sexual maturity.

Key words: gonadotrophin-releasing hormone, leptin, ghrelin, AgRP, α-MSH, CRF, rat.

In the hypothalamus, the control of energy balance is tightly regulated through complex interactions between peripheral nutrition-related hormones and hypothalamic neuropeptides (1, 2). Several decades ago, a link between fat mass and menstrual cyclicity already suggested that nutrition-related hormones could influence the hypothalamic control of puberty and reproduction (3). Further insight came from isolation of leptin, an adipocyte-derived anorectic hormone involved in the regulation of body weight, as well as a facilitatory or permissive control of puberty and reproduction (4, 5). We have previously shown that leptin incubation with prepubertal rat hypothalamic expiants could directly accelerate the pulsatile gonadotrophin-releasing hormone (GnRH) secretion (6). In addition, the effects of leptin *in vitro* on pulsatile GnRH secretion showed developmental changes because they disappeared with sexual maturation, except for an increased amplitude of GnRH pulses in the adult cycling female hypothalamus (7). More recently, ghrelin was isolated as an orexigenic peptide secreted by stomach cells (8, 9). Ghrelin was shown to be also expressed in hypothalamic neurones, providing an anatomical basis for local connection with central regulation of energy homeostasis (10). Ghrelin was shown to inhibit luteinising hormone (LH) secretion in adult individuals of different species (11-13) and to delay balano-preputial separation in the still maturing male rat (14). The first aim of the present study was to compare the effects of *in vivo* and *in vitro* administration of orexigenic and anorectic peptides on the interpulse interval (IPI) of GnRH secretion from hypothalamic expiants of prepubertal (15 days) and adult (50 days) male rats. Such a study was made possible by the availability of a model of hypothalamic expiant incubation where developmental variations in pulsatile GnRH secretion could be observed (15).

Several hypothalamic anorectic or orexigenic neuropeptides have emerged as possible mediators because they are stimulated or inhibited by leptin, respectively. Among the anorectic peptides, we have previously shown a partial mediatory role of cocaine and amphetamine-regulated transcript (CART) in the effects of leptin on pulsatile GnRH secretion (16). In rodents, the orexigenic effect of ghrelin could be mediated by the neuropeptide Y (NPY) and agouti-related protein (AgRP), which are colocalised in the arcuate nucleus (ARC) (17). We reported previously that NPY could accelerate the pulsatile GnRH secretion *in vitro* via the NPY Y5-receptor subtype and this effect was independent of leptin action (6). The second aim of the study was to study *in vitro* the interactions of leptin and ghrelin with different intrahypothalamic anorectic and orexigenic peptide mediators, in an attempt to elucidate the neuromedi-ating pathways involved in the control of pulsatile GnRH secretion. The studied neuropeptides were α-melanocyte-stimulating hormone (MSH), corticotrophin-releasing factor (CRF) and CART as anorectic mediators and NPY and AgRP as orexigenic mediators. Those mechanistic studies, based on the manipulation of neuropeptide-medi-ating pathways by the addition of agonists or antagonists in culture medium, were performed at 15 days because both leptin and ghrelin appeared to regulate GnRH secretion *in vitro* at that age.

MATERIALS AND METHODS

Animals and reagents

Pregnants rats and male and female Wistar rats aged 15 and 50 days were purchased from the University of Liège and housed under standardised conditions (22 °C, 12 : 12 h light/dark cycle, lights on 06.30 h, food and water *ad libitum)*. Prepubertal pups were with lactating mother until weaning, which was at 3 weeks of age. All experiments were carried out with the approval of the Belgium Ministry of Agriculture and the Ethical Committee at the University of Liège.

The incubation medium MEM was purchased from Life Technology, Inc (Invitrogen Corporation, Merelbeke, Belgium). Anorectic peptides used were mouse recombinant leptin (R&D systems, Abingdon, UK), α-MSH (Sigma, St Louis, MO, USA), CRF (Phoenix Pharmaceuticals, Inc. Belmont, CA, USA) and CART$_{(52-102)}$-peptide (Novo Nordisk, Bagsvaerd, Denmark). Orexigenic peptides used were porcine NPY (Ferring Pharmaceutical Ltd, Copenhagen, Denmark), human AgRP (83-132)-NH2 (Phoenix Pharmaceuticals) and rat ghrelin (Neosystem, Strasbourg, France). The anti-CART antiserum was a kind gift from Novo Nordisk (Bagsvaerd, Denmark), SHU 9119, a pharmacological MC3/MC4 receptor antagonist was purchased from Bachem (Switzerland), and CRF antagonist, α-helical CRF 9-41 from Phoenix Pharmaceuticals, Inc. The NPY-Y5 receptor antagonist, *trans*-naphthalene-1-sulphonic acid-4-{(4-(3-dimethylamino-propylamino)-quinazolin-2-ylamino)-methyl}-aminde, was synthesised in the Medical Chemistry Department of Ferring Pharmaceutical Research Ltd (Chilworth, UK).

Hypothalamic expiant incubation and GnRH radioimmunoasay (RIA)

The animals were killed by decapitation between 10.00 h and 11.00 h. The retrochiasmatic hypothalamus was rapidly dissected and transferred into a static incubator as described previously (15). The medium consisted of phenol red free MEM supplemented with glucose, magnesium, glycine and bacitracin (25 mM, 1 mM, 10 nM, 20 μM retrospectively). In each experiment, 12-15 expiants were studied individually for 4 h through collection and renewal of the incubation medium (0.5 ml) every 7.5 min. The samples were frozen until assayed. Each experiment included 12-15 hypothalamic expiants which were incubated individually, each in a separate chamber. GnRH was measured in duplicate using a highly sensitive RIA with intra-and interassay coefficient of 14% and 18%, respectively (18). The GnRH antiserum was generously provided by Dr Y. F. Chen and V. D. Ramirez (Urbana, IL, USA) (19). The data below the limit of detection (5 pg/7.5 min) were assigned that value.

Study protocols

In vivo experiments

Four groups of six rats aged 15 or 50 days and raised in similar conditions were studied. Leptin, ghrelin, AgRP or saline were injected intraperitoneally between 09.00 h and 09.20 h. The amounts injected were 30 μg/kg for leptin and 20 μg/kg for ghrelin and AgRP dissolved in 100 μ of 0.9% saline. These amounts were consistent with the lowest effective doses in dose-response feeding studies (20, 21). The animals were decapitated 90 min after peptide injection to study the retrochiasmatic expiants all in the same conditions (i.e. in the absence of any agonist or antagonist added *in vitro).* These comprised the ex *vivo* conditions.

In vitro experiments

Using retrochiasmatic hypothalamic expiants obtained at 15 or 50 days, the frequency of pulsatile GnRH secretion was studied during incubation without (control) or with different substances. The studied anorectic peptides were leptin, CRF and α-MSH used at 10^{-7} M and CART ($_{52-102}$) peptide used at 10^{-6} M. The orexigenic peptides ghrelin, AgRP and NPY were all used at 10^{-7} M. To antagonise specifically the different peptides or receptors, the anti-CART antiserum was used at 1 : 1000 dilution. The SHU 9199, the CRF antagonist and the NPY-Y5 receptor antagonist were used at 10^{-7} M. These antagonist effects in our conditions were investigated by studying the effect of anti-CART antiserum, SHU 9119 and a CRF antagonist, α-helical CRF 9-41 on the changes in pulsatile GnRH secretion induced by CART, α-MSH and CRF, respectively.

To study the effects of leptin and ghrelin *in vitro,* as well as the effects of the antagonists on leptin and ghrelin-induced changes in pulsatile GnRH frequency, the expiants were incubated in three different conditions: leptin or ghrelin alone, each antagonist alone and both reagents together. The different conditions (control, agonist, antagonist, agonist + antagonist) were applied throughout the entire expiant incubation period.

Statistical analysis

The occurrence of significant pulses of GnRH secretion was determined using the Pulsar programme as described previously (15). The mean ± SD interpulse interval was calculated. In some instances, all the expiants in a group showed a similar GnRH IPI. Then, SD was zero and could not be represented in the figures. Significant effect of treatment was determined by a t-test (control versus peptide). The effect of antagonists on control and treated group was determined by one-way ANOVA followed by Newman-Keuls post test when the threshold for significance of difference ($P < 0.05$) was reached. In previous studies between birth and puberty, we observed changes in GnRH pulse frequency without changes in amplitude (15). Only when using hypothalamic expiants from normally cycling adult female rats was an increase in amplitude of pulsatile GnRH secretion observed in the afternoon of pro-oestrous, and both leptin and NPY showed a stimulatory effect on the amplitude of GnRH secretion irrespective of the cycle phase (7). In this study, GnRH pulse amplitude was not significantly affected in any of the study conditions (data not shown).

RESULTS

As shown in Fig. 1(A), using hypothalamic expiants obtained 90 min after intraperitoneal administration of ghrelin or AgRP in 15-day-old rats, the mean ± SD GnRH IPI was significantly increased (73.8 ± 2.9 or 75 ± 3.2 min, respectively) versus vehicle-injected groups (59.1 ± 2.5 min). Intraperitoneal injection of leptin in 15-day-old rats resulted in a significant decrease of the GnRH IPI (45.5 ± 0 min). At 50 days (Fig. 1B), similar effects were seen after intraperitoneal administration of ghrelin and AgRP but not after leptin. When the effects of the three peptides were directly studied *in vitro* during incubation with hypothalamic ex-plants (Fig. 1C,D), leptin caused a reduction of the GnRH IPI at 15 days and no change at 50 days. AgRP resulted in an increase in GnRH IPI which was significant at 50 days only (53.5 ± 2.7 min versus 39.2 ± 3.8 min in controls). As opposed to the effects after administration *in vivo,* expiant incubation with ghrelin *in vitro* caused a significant decrease in GnRH IPI at 15 days (50.2 ± 4.2 min).

Table 1 show the mean ± SD GnRH IPI observed during incubation of hypothalamic expiants obtained in 15-day-old male rats with different peptides and antagonists used alone or both together. When used alone, the studied antagonists did not change the GnRH IPI, except that the NPY-Y5 receptor antagonist caused a slight but significant increase in GnRH IPI. A significant reduction of the GnRH IPI was observed during incubation with the anorexigenic peptides CART, α-MSH and CRF, as well as the orexigenic peptide NPY. When each of the four peptides was coincubated together with its respective antagonist, the reduction in GnRH IPI was totally prevented (Table 1). The leptin-induced reduction in GnRH IPI (46.5 ± 2.8 min) was significantly but only partially overcome by anti-CART AS, SHU 9119 or CRF antagonist. As shown previously (6), the NPY-Y5 receptor antagonist did not change the reduction in GnRH interpulse interval caused by leptin. The stimulatory ghrelin effect on GnRH IPI was not changed in the presence of anti-CART AS or CRF antagonist. By contrast, ghrelin effect was totally overcome by the NPY-Y5 receptor antagonist and partially by SHU 9119. Fig. 2 shows comparisons of the effects of expiant incubation with anorectic and orexigenic intrahypothalamic peptide mediators or their antagonists in prepubertal and adult male rats. Although all the studied peptides caused a reduction in GnRH IPI at 15 days, they did not show any effect at 50 days. As shown previously (6), a significant increase in GnRH IPI was observed during incubation with the NPY-Y5 receptor antagonist (10^{-6} M), both at 15 and 50 days. No effect was observed with the anti-CART AS at a 1 : 1000 dilution. The CRF antagonist and SHU9119 did not change the GnRH IPI at 15 days whereas they resulted in increased GnRH IPI

at 50 days.

Fig. 1. *Effects of in vivo administration and in vitro incubation of leptin, ghrelin, agouti-related protein (AgRP) and saline on the gonadotrophin-releasing hormone (GnRH) interpulse interval observed using hypothalamic expiants of 15- and 50-day-old male rats. In the ex vivo conditions, hypothalamic expiants were studied starting 90 min after in vivo administration. *P < 0.05 versus control (saline).*

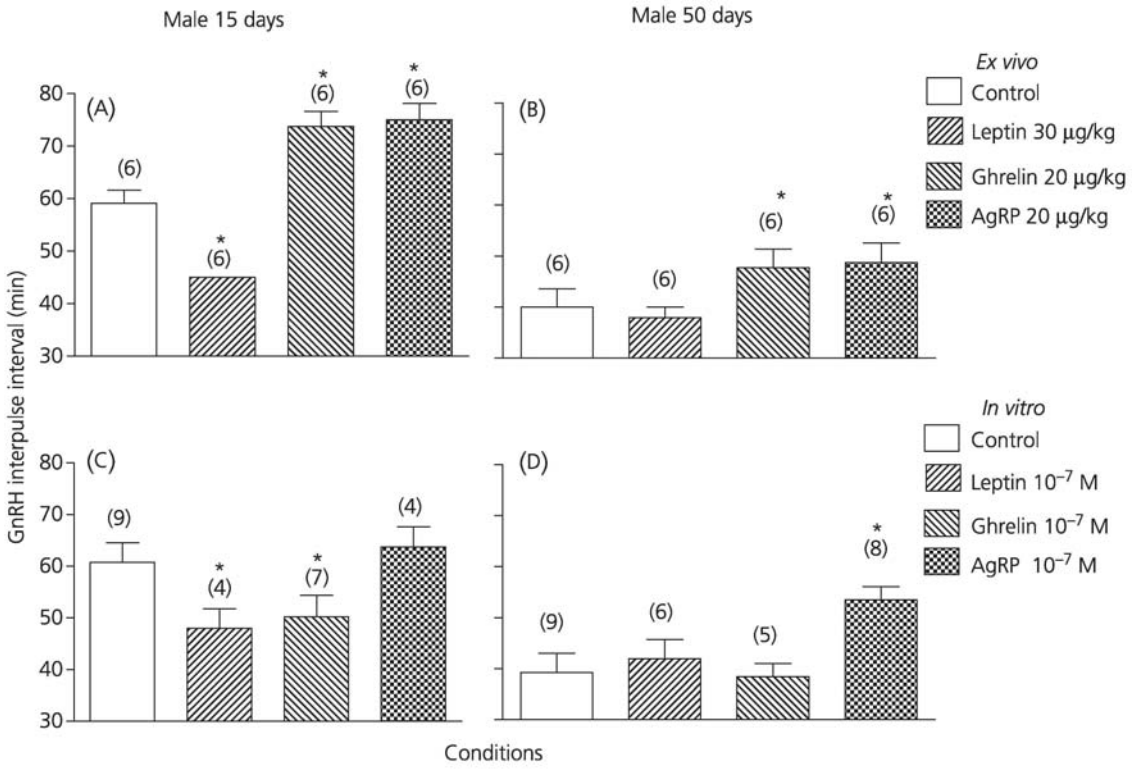


Table 1. *Effects of Anorectic and Orexigenic Peptides and Antagonists on Gonadotrophin-Releasing Hormone Interpulse Interval (min) in vitro Using Hypothalamic Expiants of 15 Day-Old Male Rats.*

	No antagonist	Anti-CART AS (10 : 1000)	SHU 9119 (10^{-7} M)	CRF antagonist (10^{-7} M)	NPY-Y5R antagonist
No peptide	61.0 ± 2.2 (19)	60.7 ± 2.2 (4)	60.8 ± 2.5 (3)	61.5 ± 3.3 (4)	64.6 ± 3.8 (4)*
CART 10^{-6} M	45.7 ± 2.7 (4)*	58.5 ± 3.0 (4) †	-	-	-
α-MSH 10^{-7} M	48.0 ± 3.8 (4)*	-	60.0 ± 0.0 (4)†	-	-
CRF 10^{-7} M	47.3 ± 3.4 (4)*	-	-	57.8 ± 3.7 (3)†	-
NPY 10^{-7} M	45.6 ± 2.3 (3)*	-	-	-	60.5 ± 3.7 (5)†
Leptin 10^{-7} M	46.5 ± 2.8 (11)*	56.3 ± 3.9 (4)* †	51.0 ± 4.3 (10)*†	54.0 ±2.9 (4)*†	46.50 ± 3.30 (5)*
Ghrelin 10^{-7} M	50.3 ± 4.2 (7)*	49.5 ± 3.8 (6)*	56.3 ± 3.9 (4)*†	49.5 ± 3.8 (4)*	57.8 ± 3.5 (5)†

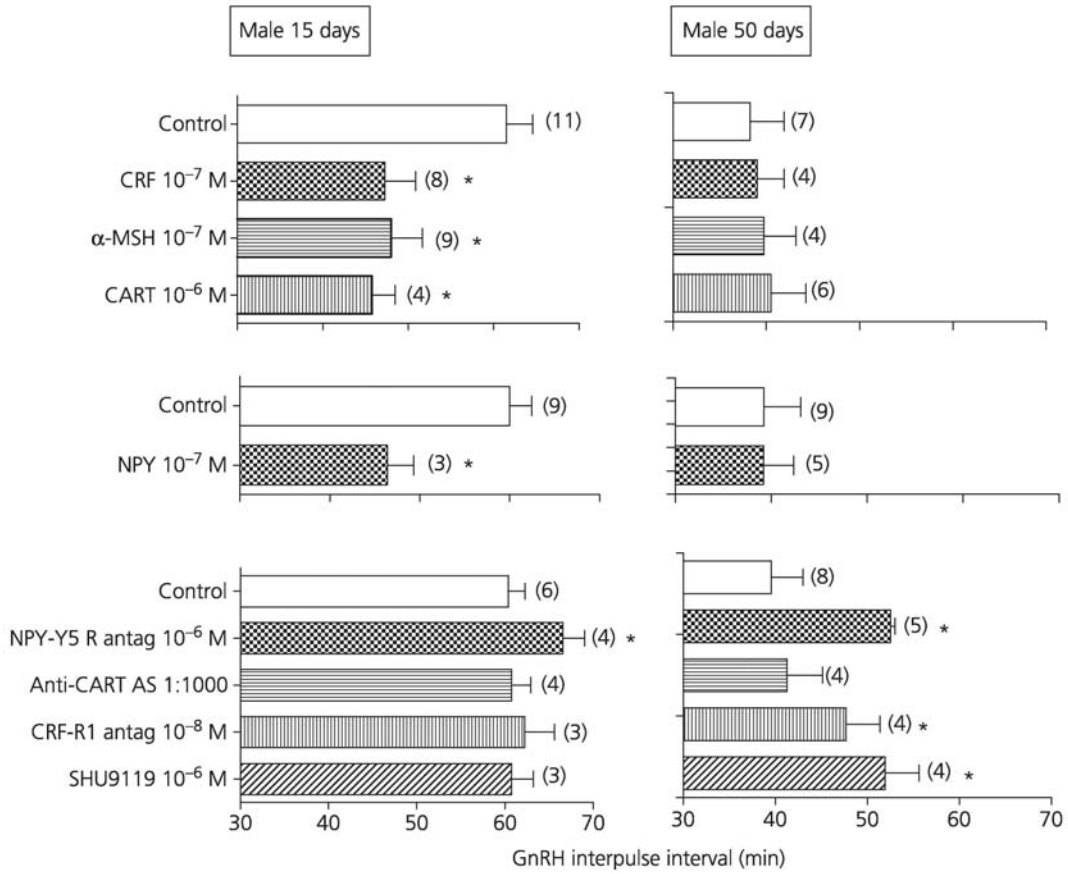
Data are shown as the mean ± SD; the number of expiants is given between parentheses. *P < 0.05 *vs* no peptide and no antagonist; †P < 0.05 *vs* no antagonist. CART, Cocaine and amphetamine-regulated transcript; α-MSH, α-melanocyte-stimulating hormone; corticotrophin-releasing factor (CRF); NPY, neuropeptide Y.

DISCUSSION

In the present study, rat hypothalamic expiants are used because they retain *in vitro* the capacity to secrete GnRH in a reproducible pulsatile manner with developmental changes in GnRH pulse frequency between birth and adult age (15). This enabled the effects of leptin and ghrelin on pulsatile GnRH secretion and maturational changes to be studied directly. Although our previous data on the effects of leptin *in vitro* (6, 7) are consistent with the observations obtained *in vivo* by others (4, 5), the unexpected stimulatory effects of NPY that we obtained *in vitro* (6, 7) are discordant from the inhibitory effects reported in different conditions *in vivo* (22-24). Therefore, the effects of leptin and ghrelin have been compared after either *in vivo* administration or *in vitro* incubation with the peptides. After peripheral administration *in vivo,* the stimulatory effects of leptin and the

inhibitory effects of ghrelin on the GnRH pulse generator are consistent with the opposing effects of leptin and ghrelin on energy balance as well as on LH levels in different species (4, 5, 11-14). However, *in vitro,* ghrelin shows puzzling stimulatory effects on pulsatile GnRH secretion, which are in contradiction to our observations after *in vivo* administration. Because some effects of ghrelin could be mediated via NPY neurones, the unexpected stimulatory effects of ghrelin *in vitro* could be related to the similarly unexpected effects of NPY that we reported previously (6, 7). The discrepancy between the effects obtained both *in vitro* and *in vivo* could depend on our experimental *in vitro* conditions. Regional differences in response to ghrelin are suggested by a recent electrophysiological study in the arcuate nucleus showing that ghrelin causes neuronal excitation in the ventromedial area, possibly through NPY neurones as opposed to inhibition in the ventrolateral area, and possibly through pro-opiomelanocortin (POMC) neurones (25). Accordingly, it could be that peripherally administered ghrelin activates some neurones that are not reached and/or not activated in our hypothalamic expiants. Alternatively, the NPY receptors activated in the *in vitro* conditions (i.e. the NPY-Y5 subtype as shown in this study) could be different from the receptors mediating NPY inhibition of the pituitary-gonadal axis *in vivo* (i.e. the NPY-Y1 and NPY-Y2 receptors) (23, 24, 26). The absence of an effect of NPY and ghrelin when incubated with ex-plants obtained at 50 days could suggest a developmental variation in expression of the NPY-Y5 receptor subtype. As previously shown (6), no effect could be observed with the NPY-Y5 receptor antagonist at day 5, whereas an increase of GnRH IPI was observed at 15, 25 and 50 days. Moreover, the hypothalamic expiants could not exhibit the inhibitory response to NPY if they do not retain the ult-radian and cyclic patterns of NPY discharge governing the basal and cyclic variations in GnRH-LH secretion (22) possibly involved in such a response. Whatever the explanation, the physiological or functional significance of data obtained *in vitro* should be considered as limited, particularly when peripheral hormones are used and the data are discordant from *in vivo* observations. However, the reliability of the data obtained *in vitro* supports the relevance of the *in vitro* paradigm to study the intrahypothalamic mediators involved in peptide effects on GnRH secretion, even if the activity of such mediators could not necessarily reflect the *in vivo* conditions.

Fig. 2. *Effect of anorectic, orexigenic and antagonist peptides on the gonadotrophin-releasing hormone (GnRH) interpulse interval using hypothalamic expiants from prepubertal (15 days) and adult (50 days) male rats. Data are shown as mean ± SD. The number of expiants in each experiment is given in parentheses. *P < 0.05 versus control. CRF, Corticotrophin-releasing factor (CRF); α-MSH, α-melanocyte-stimulating hormone; CART, cocaine and amphetamine-regulated transcript; NPY, neuropeptide Y.*



Thus, we took advantage of the hypothalamic expiant model to study some anorectic or orexigenic neuropeptide mediators involved in the effects of leptin and ghrelin. Here, expiants obtained in 15-day-old animals were used because GnRH pulse frequency is affected by both leptin and ghrelin at that age whereas only ghrelin results in some effects at 50 days. In a previous work, CART was shown to mediate part of the effects of leptin (6). A role of the melanocortin system in mediating the effects of leptin is supported by leptin receptor expression in POMC neurones in the arcuate nucleus (27). POMC expression and the frequency of action potentials in the POMC neurones are stimulated by leptin (28, 29).α-MSH synthesised in POMC neurones binds to the MC3/MC4 receptors, which mediate a decrease in food intake caused by agonists of MC4-R and leptin (30), with this effect being blocked by SHU 9119, a pharmacological MC3/MC4-R antagonist (31). In the present study, α-MSH stimulation of pulsatile GnRH secretion is totally prevented using SHU 9119 whereas this antagonist partially overcomes the acceleration of pulsatile GnRH secretion caused by leptin. Thus, a pathway involving POMC neurones, α-MSH and MC4-receptors partly mediates the control of GnRH secretion by leptin. A direct α-MSH stimulation of GnRH neurones is suggested by the synaptic contacts shown between α-MSH-containing neurones and GnRH neurones (32) and the expression of functional MC4-R by GT1-cells (33). Other studies have provided evidence of melanocortin system involvement in leptin stimulation of GnRH secretion *in vivo* (34). However, in the male ob/ob mouse, the MC4-R agonist MTII has no effect on plasma gonadotrophin levels (35). Inactivation or blockade of the MC4-R is unable to alter leptin stimulatory effects on the reproductive axis in that mutant mouse (35) or to alter LH secretion in nonmutant rodents (36, 37). The discrepancies between these observations may be explained by experimental conditions: acute versus chronic peptide administration, steroid environment, *in vitro* versus *in vivo* settings. Also, the existence of multiple pathways for the effects of leptin on GnRH secretion might account for the absence of any dysfunction *in vivo* following inactivation of MC4-R. The developmental stage may influence the response as discussed below.

The CRF-containing neurones in the paraventricular nucleus are another target of leptin action in the regulation of food intake (38, 39). For example, the CRF1-9 α-helical antagonist prevents the inhibition of feeding by leptin (40). In the present study, at 15 days, the frequency of pulsatile GnRH secretion was stimulated by CRF, with this effect being prevented by the CRF antagonist α-helical CRF 9-41. CRF acts as an important inhibitory neuromodulator of GnRH-LH secretion and appears to be a possible mediator of the inhibitory effect of stress on the hypothalamic-pituitary-gonadal axis (41-44). However, CRF does not appear to be a prerequisite for that mechanism because the stressor-induced suppression of LH surge occurs in the CRF knockout female mouse (45). Thus, depending on experimental conditions, including the steroid environment (46), CRF could result in either inhibitory or stimulatory or no effects on GnRH-LH secretion, such as previously discussed for α-MSH. Our observation that the increase of pulsatile GnRH secretion induced by leptin is partially prevented by a CRF antagonist suggests a mediating pathway involving leptin and CRF-containing neurones in the regulation of GnRH secretion. Anatomical connections between CRF terminals and GnRH-secreting neurones have been reported (47). Taken together, our previous data and that of the present study indicate that CART-, CRF- and POMC-expressing neurones are possible additive and alternative mediators of the effects of leptin on GnRH secretion. Such redundant pathways might explain the gonadal disruption in leptin-deficient rodent whereas inactivation or blockade of any peptide that mediates the effects of leptin does not affect the reproductive axis *in vivo.* In addition, a direct effect of leptin on GnRH neurones is possible because leptin receptors are expressed in GT1-7 immortalised GnRH cells (48).

Ghrelin is an endogenous ligand for the growth hormone secret-agogue receptors (GHS-R) which are expressed in the arcuate nucleus and the ventromedial hypothalamus (49). GHS-R mRNAs are expressed highly in NPY/AgRP-neurones and weakly in POMC-neurones. However, a recent study suggested that ghrelin could inhibit LH secretion independent of the GHS-R because the unacyl-ated form of the peptide caused such an effect (14). The reduction in GnRH pulse frequency reported in the present study after systemic administration of ghrelin is consistent with the reduction in LH secretion found in different species (11-14, 50). The involvement of NPY in the mediation of the effects of ghrelin on pulsatile GnRH secretion is indicated by the complete abolition of the effects of ghrelin by the NPY-Y5 receptor antagonist, consistent with the ghrelin-NPY-NPY-Y5 receptor pathway operating in the control of food intake (17). An increased expression of CRF mRNA occurs after peripheral administration of ghrelin and the ghrelin-induced orexigenic effect in mice is inhibited by a CRF antagonist (51). However, our data obtained using a CRF antagonist do not support a connection between ghrelin and CRF-expressing neurones in the regulation of pulsatile GnRH secretion. The partial but significant prevention by SHU 9119 of the effects of ghrelin on pulsatile GnRH secretion suggested an unexpected link between ghrelin and POMC-expressing neurones. Although electrophysiological studies show that ghrelin hyperpolarises POMC neurones in the arcuate nucleus, most likely through a NPY effect, a paradoxical ghrelin-induced depolari-sation of POMC neurones could be due to the effects of AgRP (10). Such a mediating pathway was investigated in the present study through the action of AgRP, an endogenous MC3/MC4 receptor antagonist, which is synthesized and secreted in the arcuate nucleus and colocalised with NPY neurones (52). Systemically-

administred AgRP was as effective as ghrelin in reducing the GnRH pulse frequency and a similar effect was obtained during incubation with AgRP *in vitro.* These effects are consistent with the suppression of pulsatile LH release after central infusion of AGRP in the monkey (53). Despite the inhibitory effect of AGRP on pulsatile GnRH secretion, SHU 9119 had no effects. This could be due to either a more potent action of AGRP than SHU 9119 on MC3/MC4-receptors or the existence of other AGRP receptors, as already suggested (54).

The mediation of leptin and the effects of ghrelin could also involve neuropeptides known to be critical for the GnRH neurone function such as kisspeptin. Kiss-1 neurones have been shown to express the leptin receptor and to be less active in the o6/ob mouse (55). These neurones could thus provide a more direct pathway of leptin connexion to the GnRH neurones. However, the Kiss-1 neurones could also be involved in the regulation of the effects of ghrelin because the LH secretory response to kisspeptin is reduced by ghrelin (14).

Because developmental variations in pulsatile GnRH secretion were observed in our model and nutrition-related neuropeptides could be involved in the mechanism of sexual maturation, the effects of anorectic and orexigenic peptides were compared in immature and adult male rats. After systemic administration at 50 days, leptin no longer shows the stimulatory effect seen at 15 days whereas a persistent inhibitory effect is observed with ghrelin and AGRP. Consistent with the data *obtained in vivo,* leptin, α-MSH, CART and CRF show no effects when directly incubated *in vitro* at 50 days as opposed to the effects seen at 15 days. A fully effective endogenous level of activity of leptin and mediating neuropeptides in the adult hypothalamus could account for the absence of effects of the anorectic neuropeptides at 50 days as opposed to 15 days. In accordance with that concept, pulsatile GnRH secretion can be influenced at 50 days by prevention of the effects of endogenous neuropeptides using the CRF antagonist and SHU 9119 whereas no effects were observed at 15 days. Because the stimulatory effects of α-MSH on pulsatile GnRH secretion disappeared at 50 days, whereas inhibitory effects of SHU9119 appeared, this suggests that maturation results in increased endogenous stimulation at MC4 receptors. The absence of anti-CART AS effects could be explained by a subtotal neutralisation due to insufficient concentration of the antiserum. It was interesting to observe a potent and age-independent effect of AgRP, which appeared to inhibit GnRH pulse frequency to a similar extent after peripheral administration in the immature and adult rat. Thus, the developmental changes in the regulation of GnRH pulse frequency primarily involved the anorectic peptides as opposed to the orexigenic peptides.

In conclusion, an opposite effect of leptin and ghrelin is shown on the frequency of pulsatile GnRH secretion, consistent with the pathophysiological observations linking energy balance and reproductive function. Those two hormones act through distinct neuromediators, with CART and CRF-expressing neurones being primary targets for leptin and NPY-containing neurones for ghrelin. By contrast, the melanocortigenic system appears to be a common pathway for mediation of the actions of both leptin and ghrelin. Between prepuberty and adulthood, the stimulatory activity of the anorectic mediators appears to increase whereas the inhibitory effects of the orexigenic mediators appear to operate both before and after puberty.

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