Leptin effects on pulsatile gonadotropin releasing hormone secretion from the adult rat hypothalamus and interaction with cocaine and amphetamine regulated transcript peptide and neuropeptide Y

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Abstract

Leptin may act as a negative feedback signal to the hypothalamic control of appetite through suppression of neuropeptide Y (NPY) secretion and stimulation of cocaine and amphetamine regulated transcript (CART). We aimed at studying the effects of leptin, CART and NPY on the hypothalamic control of the pituitary–gonadal system. Pulsatile gonadotropin-releasing hormone (GnRH) secretion was studied in vitro using retrochiasmatic hypothalamic explants from adult rats. In the female, GnRH pulse amplitude was significantly increased by leptin (10⁻⁷ M) and CART (10⁻⁶ M) irrespective of the estrus cycle phase while no such effects were seen in the male. The GnRH interpulse interval was not affected in both sexes. Passive immunoneutralization against CART caused a reduction in GnRH pulse amplitude in the female. A slight but significant increase in GnRH pulse amplitude was caused by NPY (10⁻⁶ M) in the female. However, GnRH pulse amplitude was not affected by a Y5-receptor antagonist (10⁻⁶ M) while the interpulse interval was significantly increased as shown previously in the male. The increase in GnRH pulse amplitude caused by leptin was totally prevented by coincubation with an anti-CART antiserum whereas it was not affected by coincubation with the NPY Y5-receptor antagonist (10⁻⁷ M). In conclusion, leptin and NPY show separate permissive effects on GnRH secretion in the adult rat hypothalamus. In both sexes, NPY is prominently involved in the control of the frequency of pulsatile GnRH secretion through the Y5 receptor subtype. Leptin causes a female-specific facilitatory effect on GnRH pulse amplitude which is mediated by CART and which occurs irrespective of the estrus cycle phase. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: NPY-receptor subtype; Estrus cycle; Pulsatility

1. Introduction

Alteration in energy balance may influence reproductive function in many species. Food restriction can alter adult reproductive function [1,2] and delay the timing of pubertal onset [3,4] by suppression of luteinizing hormone (LH) secretion. Many studies showed that leptin, a key regulator of food intake and energy balance, may play a regulatory role in the hypothalamic–pituitary–gonadal axis. In female ob/ob mice, leptin administration increased basal LH levels and restored fertility [5–7]. Other experiments have demonstrated that intracerebroventricular (i.c.v.) injection of leptin antiserum led to a decrease in LH pulsatility and an impairment of reproductive function [8].

Neuropeptide (NPY) and, more recently, the cocaine and amphetamine regulated transcript (CART) were shown to be involved in the mechanisms of leptin action on food intake. The hypothalamic transcripts of NPY, a potent stimulator of food intake, were increased by food restric-
tion [9] and reduced by i.c.v. administration of leptin [10,11]. Leptin administration to obese (ob/ob) mice stimulated the mRNA expression of CART, a hypothalamic inhibitor of food intake [12]. Therefore, it appeared interesting to study whether NPY and CART could be involved in leptin effects on the hypothalamic–pituitary–gonadal system.

Discrepant stimulatory and inhibitory effects of NPY on sexual maturation and reproduction were observed depending on species, steroidal environment [13], site of NPY administration in the brain [14] and chronic [15–17] versus acute pattern of infusion [18]. CART effects on the reproductive axis were unknown till we reported very recently a study of pulsatile GnRH secretion from hypothalamic explants of prepubertal male and female rats [19]. The frequency of GnRH pulsatility was stimulated by leptin. CART was likely to mediate such an effect. Using the same paradigm, NPY was found to accelerate GnRH pulsatility through the Y5-receptor subtype which appeared to be not involved in mediation of leptin effects [20]. Here, we used hypothalamic explants from adult male rats and female rats at different phases of the estrus cycle. We aimed at studying the effects of leptin and the possible mediating role of NPY and CART in the regulation of frequency and amplitude of pulsatile GnRH secretion.

2. Materials and methods

2.1. Animals

Male and female Wistar rats were used. They were housed in temperature and light-controlled conditions (22°C, lights on between 07:00 and 19:00) with water and standard rat pellet ad libitum. The protocols were approved by the University Committee on animal research.

2.2. Hypothalamic explants incubation

The animals were sacrificed by decapitation between 10:00 and 11:00 for all experiments except during the estrous cycle when some experiments were started in the afternoon around 16:00. The retrochiasmatic hypothalamus was rapidly dissected after decapitation and transferred into a static incubator as described previously [21,22]. The studied retrochiasmatic explants did contain GnRH axons and terminals but virtually no GnRH cell bodies [23]. In each experiments, 12–15 explants were studied individually for 4–6 h, through collection and renewal of the incubation medium (0.5 ml) every 7.5 min. The samples were frozen until the specific GnRH radioimmunoassay was performed.

2.3. GnRH RIA

GnRH was measured in the collected fractions using a highly sensitive RIA [21,22]. The values below the limit of detection (5 pg/7.5 min) were assigned that value. The GnRH antiserum was generously gifted by Dr. Y.F. Chen and V.D. Ramirez (Urbana, IL, USA) [24]. This antiserum was highly specific for GnRH without significant cross reactivity of any of the peptides used in the experiments (leptin, NPY and CART).

2.4. Study protocols

Using hypothalamic explants from adult (50-day-old) male and female rats, the frequency and amplitude of pulsatile GnRH secretion was studied without (control) or in the presence of different compounds: mouse recombinant leptin (R&D system, Abingdon, UK), Porcine NPY (Ferring, Copenhagen, Denmark), CART (Nordisk, Bagsvaerd, Denmark) and an anti-CART rabbit antiserum (Novo Nordisk) were used at a 1:1000 dilution. A NPY-Y5 receptor antagonist, trans-naphthalene-1-sulfonic acid-4-{4-(3-dimethylamino-propylamino)-quinazolin-2-ylamino}-methyl]-cyclohexylmethyl]-amide [25] was synthesized in the Medicinal Chemistry Department of Ferring Research (Chilworth, UK).

The frequency and amplitude of spontaneous pulsatile GnRH secretion and the effects of leptin (10⁻⁷ M) were studied using explants from 50-day-old female rats which were obtained in the morning or in the afternoon of each phase of the estrous cycle. Vaginal smears were performed at the time of experiments to determine the estrous cycle phase. A similar study was performed using explants from 50-day-old male rats aged 50 days. Using explants of 50-day-old female rats which were obtained at 10:00, the effects of CART (10⁻⁶ M) were studied. In this protocol, each explant was used as its own control since it was incubated for 3 h without CART (control) and for the subsequent 3-h period, with 10⁻⁶ M of CART. Following the same protocol, the effects of an anti-CART antiserum used alone or together with leptin (10⁻⁷ M) were studied.

Using explants from adult female rats, the effects of NPY (10⁻⁷ M) and of the NPY-Y5 receptor antagonist (10⁻⁶ M) on the frequency and the amplitude of pulsatile GnRH secretion were studied. Each explant was used as its own control.

2.5. Statistical analysis

The occurrence of significant pulses of GnRH secretion was determined using the Pulsar program as described previously [26]. The mean (±S.D.) interpulse interval and pulse amplitude were calculated. Comparisons were performed using the unpaired, two-tailed Student’s t-test, with P < 0.05 as the threshold for significance of differences.
3. Results

3.1. Pulsatile GnRH secretion at different phases of the estrus cycle

Using hypothalamic explants of adult female rats obtained in the morning as well as in the afternoon (Table 1), the mean GnRH interpulse interval was around 40 min and did not change throughout the estrus cycle. Using explants obtained in the morning, the mean GnRH pulse amplitude did not change significantly throughout the cycle. Using explants obtained in the afternoon, when the preovulatory LH surge is known to occur on proestrus, a significantly increased pulse amplitude was seen on proestrus compared to the other phases. Since no differences were observed between estrus, metestrus and diestrus in these and the following experiments, the data obtained during these three phases were pooled in the subsequent analysis.

3.2. Effects of leptin on frequency and amplitude of pulsatile GnRH secretion

Using hypothalamic explants obtained from adult female rats in the morning, the interpulse interval was not affected by leptin (10^{-7} M) while the GnRH pulse amplitude was significantly increased, irrespective of the cycle phase (Table 2). Using explants obtained in the afternoon, the GnRH interpulse interval was not affected by leptin. The GnRH pulse amplitude was significantly increased by leptin (10^{-7} M) irrespective of the estrus cycle phases (Fig. 1). The total GnRH secretion was significantly increased by leptin except in the afternoon of proestrus when GnRH secretion was already increased in control conditions. Since leptin effects were seen unequivocally in the morning at all phases of the cycle, the subsequent experiments were carried out using explants obtained in the morning, irrespective of the cycle phase.

Using hypothalamic explants from adult male rats, leptin affected neither the GnRH interpulse interval (39.5±3.4 vs. 40.3±3.7 min, leptin vs. control) nor the GnRH pulse amplitude (10.8±1.0 vs. 11.3±1.3 pg/7.5 min, leptin vs. control).

3.3. Effect of CART-peptide and NPY on frequency and amplitude of pulsatile GnRH secretion

As shown in Fig. 1, using explants from adult female rats, the GnRH pulse amplitude was significantly increased by 10^{-5} M of CART (13.6±4.5 vs. 9.5±2.2 pg/7.5 min, CART vs. control) while the interpulse interval was not affected (data not shown). A less important but significant increase in GnRH pulse amplitude was caused by 10^{-7} M of NPY (12.2±1.7 vs. 10.6±0.9 pg/7.5 min, NPY vs. control) while the interpulse interval was not affected (40.9±3.8 vs. 39.6±3.4 min).

As shown in Fig. 2, the anti-CART antiserum significantly reduced GnRH pulse amplitude (9.2±1.7 vs. 11.8±1.6 pg/7.5 min, anti-CART vs. control) while the

<table>
<thead>
<tr>
<th>Cycle phase</th>
<th>Proestrus</th>
<th>Estrus</th>
<th>Metestrus</th>
<th>Diestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interpulse interval (min)</td>
<td>41.2±3.9</td>
<td>45.0±8.2</td>
<td>41.4±3.8</td>
<td>41.2±3.9</td>
</tr>
<tr>
<td>Pulse amplitude (pg/7.5 min)</td>
<td>9.6±2.6</td>
<td>7.6±4.5</td>
<td>6.3±4.1</td>
<td>6.5±4.6</td>
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</table>

* Data are mean±S.D. with a mean of four explants studied in each group.
* P < 0.05 versus the other cycle phases.

Table 2

<table>
<thead>
<tr>
<th>Cycle phase</th>
<th>Interpulse interval (min)</th>
<th>Amplitude (pg/7.5 min)</th>
<th>Total secretion (pg/3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Leptin</td>
<td>Control</td>
<td>Leptin</td>
</tr>
<tr>
<td>Morning Proestrus</td>
<td>41.2±3.9</td>
<td>39.7±3.5</td>
<td>9.6±2.6</td>
</tr>
<tr>
<td>Other phases</td>
<td>42.0±4.9</td>
<td>41.5±4.2</td>
<td>6.5±4.2</td>
</tr>
<tr>
<td>Afternoon Proestrus</td>
<td>40.5±3.9</td>
<td>41.2±3.9</td>
<td>11.1±0.8</td>
</tr>
<tr>
<td>Other phases</td>
<td>39.7±3.5</td>
<td>40.1±3.6</td>
<td>7.5±0.7</td>
</tr>
</tbody>
</table>

* Leptin was used at a 10^{-7} M concentration. The data are mean±S.D., a mean of four explants being studied at each phase.
* P < 0.05 versus control.
4. Discussion

The preovulatory LH surge was known to take place during the afternoon of proestrus [27–29]. In this study, using explants obtained in the afternoon of the different cycle phases, we showed that the frequency of GnRH pulsatility did not change whereas the amplitude showed about a two fold increase on the afternoon of proestrus. Several in vivo studies showed that GnRH secretion in pituitary stalk plasma was markedly increased at the time of the preovulatory LH surge [30–32]. It was interesting to observe that this process was associated with an increased GnRH pulse amplitude in vitro whereas we showed that changes in pulse frequency without changes in amplitude characterized the period preceding onset of puberty [33].

An interplay between various inhibitory and excitatory neurotransmitters may occur on proestrus, leading to the preovulatory GnRH discharge [34]. Leptin might be involved since it was shown to stimulate GnRH secretion from the median eminence and arcuate nucleus [35,36]. Using explants from the prepubertal female rat, we found that the frequency of GnRH pulsatility was accelerated by leptin in a dose-dependent manner [20]. Intracerebroventricular administration of leptin antiserum to adult female rats resulted in an impairment of reproductive function with persistent anestrus [8]. Here, using hypothalamic explants from adult female rats, leptin was found to increase GnRH pulse amplitude whatever the estrus cycle phase and the time (morning or afternoon) when the explant was obtained. These data indicated an overall facilitatory role of leptin on the amplitude of GnRH secretion instead of a specific contribution to the occurrence of the preovulatory LH surge. In agreement with our in vitro data, it was shown in vivo that i.c.v. administration of a leptin antiserum caused reduction of LH pulse amplitude without a change in frequency [8].

Since the increase in GnRH pulse amplitude caused by leptin in the female was not seen in the male, it is possible that this effect is dependent on sex steroids. Such a concept is consistent with the facilitatory effects of estrogens on leptin receptor gene expression [37]. However, because leptin had stimulatory effects in the prepubertal hypothalamus from rats of both sexes, we may hypothesize that testosterone might account for disappearance of leptin effects in the male instead of estrogen for persistent effects in the female.

Leptin could influence GnRH secretion by acting trans-synaptically rather than by a direct effect [38]. Indeed, little coexpression of ob-receptor and GnRH was found in the rat at the protein or mRNA levels [39]. CART might be a hypothalamic mediator of leptin effects on GnRH neurons. Using explants from 15-day-old rats, our previous studies showed that CART could be involved in mediating leptin stimulatory effects on the frequency of GnRH pulsatility [19]. Using hypothalamic explants from Zucker rats homozygous for the leptin receptor mutation, we

3.4. Effect of anti-CART antiserum and NPY Y5-receptor antagonist on leptin-induced stimulation of GnRH secretion

Using explants of adult female rats, the increase in GnRH pulse amplitude caused by leptin (10−7 M) was totally prevented by coincubation with an anti-CART antiserum whereas it was not affected by coincubation with the NPY Y5-receptor antagonist (Fig. 3).
found that GnRH pulse frequency was not affected by leptin but significantly accelerated by the CART-peptide. In the present study, using explants from adult female rats, the GnRH pulse amplitude was similarly affected by CART and leptin, further suggesting the mediating role of CART. A role for the endogenous CART-peptide is supported by the decreased GnRH pulse amplitude after CART immunoneutralization. The antiserum could totally prevent leptin effects on GnRH pulse amplitude at 50 days whereas, used at the same concentration, the antisera could only partially overcome leptins effects on GnRH pulse frequency at 15 days. Thus, though CART appeared to be involved in mediating leptin effects at different stages throughout life, the absolute activity or the importance of CART relative to other mediators may change with age.

Neuropeptide Y, a potent stimulator of food intake, was shown to affect the reproductive axis in a different number of species including rats, though discrepant stimulatory and inhibitory effects were observed [13–18]. In the rat, the involvement of NPY in the prepubertal GnRH and LH surges was supported by the concordant increase of NPY gene expression in the arcuate nucleus [40] and NPY tissue content in the median eminence [41]. In addition, NPY stimulation of GnRH release was maximal under conditions leading to GnRH surges [42,43]. The pituitary responsiveness to pulsatile GnRH stimulation was enhanced by NPY in proestrus rats [44]. Thus, the contribution of NPY to the preovulatory LH surge could involve both a direct action on GnRH secretion [13,45] and a potentiation of the pituitary response to GnRH [43,46]. The actions of NPY are mediated through distinct receptor subtypes showing different affinity for NPY agonists which have been cloned [47]. The Y5-receptor [48,49] and Y1-receptor [50] were shown to be involved in NPY-induced food intake. Our previous data obtained with different NPY agonists were consistent with the involvement of the Y5-receptor subtype in the stimulatory effect on the frequency of GnRH pulsatility in the prepubertal hypothalamus [20]. In the present study, the contribution of NPY to the preovulatory increase in GnRH pulse amplitude appeared to be equivocal since exogenous NPY had only modest effect and prevention of endogenous NPY effects through a Y5 receptor antagonist did not alter GnRH pulse amplitude. We did however not separate NPY effects in relation to the cycle phase. Such experiments as well as studies using antagonists for different receptor subtypes (Y1, Y4) possibly involved in the adult female [51] are warranted. It is noteworthy that the Y5 receptor antagonist revealed an endogenous facilitatory NPY effect on the frequency of pulsatile GnRH secretion in the adult female hypothalamus which was similar to that recently
confirmed to be involved in the leptin effect on pulsatile GnRH secretion in the adult female as it was shown earlier in the prepubertal hypothalamus in both sexes.

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References

[3] Foster DL, Olster DH. Effect of restricted nutrition on puberty in the effect of an anti-CART antiserum (upper panel) and of a NPY Y5-receptor antagonist (lower panel) on leptin-induced stimulation of GnRH secretion from explants of the adult female rats hypothalamus. These data are mean ± S.D. with a mean of six explants studied in each group. *, P < 0.05 versus control.
[13] Khorram O, Pau KYF, Spies HG. Bimodal effects of neuropeptide Y reported in the adult male [20]. Such an NPY effect which is not sexually dimorphic and different from the female-specific leptin stimulation of GnRH pulse amplitude, added to the dissociation between NPY and leptin effects. This dissociation was critically observed using explants from prepubertal rats where NPY could accelerate the frequency of pulsatile GnRH secretion through a mechanism not involving the leptin–CART pathway [20]. Here, a similar dissociation was found in the adult female hypothalamus.

In conclusion, using a hypothalamic explant model, we demonstrated an increase in amplitude of pulsatile GnRH secretion in the afternoon of proestrus. Both leptin and NPY showed a permissive effect on the amplitude of GnRH secretion in the adult female hypothalamus with distinct mechanisms for the two peptides. Endogenous NPY was found to control the frequency of pulsatile GnRH secretion through the Y5 receptor subtype in the adult hypothalamus of both sexes. In contrast, leptin showed a female-specific facilitatory effect on GnRH pulse amplitude, irrespective of the estrus cycle phase. CART was


[16] Pierroz DD, Cruz NM, d’Alleves C, Aubert ML. Chronic administration of neuropeptide Y into the lateral ventricle starting at 30 days of life delays sexual maturation in the female rat. Neuroendocrinology 1995;61:293–300.

[17] Pierroz DD, Cattezlis C, Aebi AC, Rivier JE, Aubert ML. Chronic administration of Neuropeptide Y into the lateral ventricle inhibits both the pituitary-testicular axis and growth hormone and insulin-like growth factor I secretion in intact adult male rats. Endocrinology 1996;137:3–12.


