The effect of leptin on the tonic secretion of gonadotropins in the female rats

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Abstract

OBJECTIVES: This study is an attempt to determine, the *in vivo* action of leptin on this hypophysiotropic hypothalamic area, by evaluating the concentrations of luteinizing hormone (LH) and follicular stimulating hormone (FSH) in the serum.

METHODS: The experiments were done by stereotaxic injection of recombinant rat leptin (rrleptin) into the third cerebral ventricle (V3) of adult female Wistar rats. Subjects were divided into five groups. Group A included normal intact animals. In Group B, the rats were stereotaxically administered with rrleptin in the V3. The rats, in Groups C, D and E, were subjected to electrolytic lesion of the arcuate nucleus (ARC), of the ventromedial nucleus (VMH) and of both of these hypothalamic nuclei, respectively. Immediately after the electric lesion, they were intracranially injected with rrleptin. Blood samplings for serum LH and FSH levels estimation were performed three times: i) just before any stereotaxic procedure, ii) six hours, and iii) twenty-four hours after leptin administration.

RESULTS: The results showed that serum LH levels increased dramatically in group B, six hours after leptin administration. The LH levels in Groups C, D and E presented the same pattern with a lower peak. The FSH levels were doubled six hours after leptin administration in all groups without any exception. Both LH and FSH serum levels reverted to the initial basic levels after 24 hours.

DISCUSSION: The significant conclusion derived from this study is that ARC and VMH, which are responsible for controlling the tonic secretion of gonadotropins, respond in a different way for the FSH and LH secretion. This also suggests that some other mechanism(s) or factor(s) may additionally participate in the control of the tonic component of FSH secretion.

ORIGINAL ARTICLE

Abbreviations:

LH:	luteinizing hormone
FSH:	follicular stimulating hormone
GnRH:	gonadotropin-releasing hormone
ARC:	arcuate nucleus of hypothalamus
VMH:	ventromedial nucleus of hypothalamus
V3:	third cerebral ventricle
HPA:	hypothalamus-pituitary axis
POA:	preoptic area
OVLT:	organum vasculosum lamina terminalis
CNS:	central nervous system
rrleptin:	recombinant rat leptin

INTRODUCTION

Leptin (from the Greek word "leptos" meaning slim), also called ob protein, is an 167 aminoacid adipocyte-secreted protein, which serves as a signal, referring to the amount of energy stores to the brain (Zhang, Proenca *et al*., 1994). Leptin is also considered to be a key link between the adipose tissue and the reproductive system, since it is thought to play a significant role in regulating several neuroendocrine functions.

The search for leptin action sites revealed the presence of multiple splice variants class I cytokine receptors (Ob-Rs) in several brain areas (Lee, Proenca *et al*., 1996; Tartaglia, 1997; Tartaglia, Dembski *et al*., 1995). Only one in five known (Ob-Rs) splice variants, the long or signalling form of the leptin receptor b (Ob-R_b), has been shown to be essential for signalling energy reserves (Uotani, Bjorbaek *et al*., 1999). It is also especially abundant in the arcuate nucleus (ARC), the dorsomedial, paraventricular and ventromedial nuclei (VMH) and the lateral hypothalamus (Elmquist, Bjorbaek *et al*., 1998; Fei, Okano *et al*., 1997; Mercer, Hoggard *et al*., 1996).

Research has revealed the participation of leptin in the reproduction control. This is implied by the presence of Ob-R at all levels of the hypothalamus-pituitary-gonadal axis (Baranowska, Wolinska-Witort *et al*., 2005; Chan & Mantzoros, 2001). Moreover, leptin's influence on the ovarian follicular development (Barash, Cheung *et al*., 1996) and its restorative action on fertility following its administration (Chehab, Lim *et al*., 1996) are findings supporting this role. However, the mechanism(s) by which this is accomplished still remain unknown.

Previous studies in vitro reported that leptin acts directly in both the hypothalamus and the pituitary, in order to stimulate the release of gonadotropin-releasing hormone (GnRH) and luteinising hormone, respectively.

Although distribution of Ob-R in the brain varies among different species, all species express Ob-R mRNA in the arcuate and ventromedial nuclei of the hypothalamus, two areas acknowledged to influence feeding, as well as reproductive behaviour (Finn, Cunningham *et al*., 1998).

On the other hand, it has been demonstrated that leptin from rat pituitaries stimulates in vitro gonadotropin release, thus indicating the ability of leptin to directly affect the pituitary tissue (Borowiec, Wasilewska-Dziubinska *et al*., 2002; Yu, Kimura *et al*., 1997).

The objective of this study was to evaluate, *in vivo*, the leptin action on the tonic secretion of the two gonadotropins (LH and FSH), by stereotaxically injecting leptin to the 3rd cerebral ventricle (V3). The vicinity of the V3 to the ARC, to the VMH and to the pituitary gland permits the injected leptin to act: a) on all surrounding areas (if leptin is administered without damaging the hypophysiotropic hypothalamic area), or b) to the pituitary and all other hypothalamic areas, except the ARC and the VMH, when electrolytic lesion of the above mentioned nuclei is performed.

MATERIALS AND METHODS

Animals

All animal studies were done according to the EEC regulation and administrative provision for the protection of animals used for experimental and other scientific purposes (86/609/EEC, 24.11.1986) and were approved by the Bioethical Committee of the Medical School, Aristotle University of Thessaloniki, Greece.

The stereotaxic rat model was established using adult female Wistar rats of ~250g body weight. Rats were housed and fed ad libitum under stable standard environmental conditions (22±2 °C temperature and 14:10hr light-dark cycle). All subjects had regular oestrous cycle, confirmed by two consecutive normal cycles before the experiment. Daily collection of vaginal discharges with swabs confirmed the oestric phase and the regularity of the cycle. The oestric phase was determined by examining the discharges (dyed with Methylene Blue stain) under direct microscopy. Animals that did not meet all requirements were excluded from the study.

Stereotaxic Procedure and Experimental Protocol

The animals were sedated, under general anaesthesia administered intraperitoneally 10 mg/kg Xylazine (Rompum®; Bayer AG, Leverkusen, Germany) and then, 10 to 15minutes later, 50 mg/kg Ketamine Chlorhydrate (Imalgen® 1000; Merial, Lyon, France).

Stereotaxy was performed with the Lab Standard stereotaxic device (Stoelting Co.; Illinois, U.S.A.) and stereotaxic co-ordinates were estimated from the atlas of Pellegrino *et al*. (1979). The co-ordinates for the V3 were 0.2 mm anterior to and 0.5 mm lateral to the bregma, and 10.39 mm ventral to the dura at 29° degrees from the vertical plane. The co-ordinates for the ARC were 0.2 mm anterior to and 0.5 mm lateral to the bregma, and 9.93 mm ventral to the dura at 29° degrees from the vertical plane. The co-ordinates for the VMH were 0.2 mm anterior to and 0.5 mm lateral to the bregma, and 9.71 mm ventral to the dura at 25° degrees from the vertical plane. The above co-ordinates resulted in only two scull punches required to reach all three mentioned

areas. In addition, the tilted stereotaxic technique eliminated the risk of haemorrhage, often observed in vertical techniques.

The electrolytic lesions were performed using DC currents of 650 μA for 20 seconds, generated by an electronic lesion maker (Ugo-Basile 3500, Comerio, Italy). The two electrical poles of the device were connected to the tip of the electrodes and the tail of the rat, respectively.

All experiments started simultaneously at 9.00 p.m. during the animals' dioestrus I phase. The animals (n=20) were randomly divided into five groups (A, B, C, D and E). Group A (n=4) were normal intact rats (control group). In group B (specific control group), leptin administration (2 μl of 125 mg/100 ml) was performed into the V3, without damaging the hypopsychotropic-hypothalamic area. In groups C (n=4), D (n=4) and E (n=4) the rats were subjected to bilateral electrolytic lesion of the ARC, the VMH and both of these hypothalamic nuclei, respectively and then leptin was immediately injected into the V3. All subjects were given free access to food and drinking water throughout the whole experimental procedure.

Reagents and Hormone Assays

Rat recombinant leptin (rrleptin) (Cat no: 598-LP, R&D Systems Europe; Oxon, United Kingdom) was reconstituted according to the manufacturer guidelines to a final concentration of 125 mg/100 ml (pH=5.2). Then, 2 μl of rrleptin were injected, which had similar concentration to the one found in normally fed dioestrous female rats (Watanobe & Suda, 1999). It had been decided not to use concentrations above this value, since it has been proven that such concentrations do not further elevate LH secretion (Watanobe, 2002). The rrleptin was injected with a micro-syringe (1801RN, Hammilton; Bonaduz, Switzerland) which held a 26s gauge stainless steel needle.

The LH RIA assay kit (ref.: AHR002) and the rat serum FSH ELISA assay kit (ref.: AER004) were both provided by Biocode-Hycel; Liege, Belgium. Both hormone assays were perfomed according to the manufacturer guidelines.

Blood Sampling

Blood sampling was performed before any stereotaxical procedure, at 9:00 a.m and was repeated 6 and 24 hours after leptin administration. Blood specimens (500 μl) were sampled from the lateral tail vein. There was no need for anaesthesia yet good illumination and dilation were normally required. Dipping the tail in warm water helped to dilate the underline vessels. Blood serum was separated by centrifugation (4000 rpm for 15 min) and then stored at -20°C.

Histological Confirmation

At the end of every experimental procedure, the subjects were perfused transcardially with 0.9% saline for 1 minute, followed by 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4) for 10 minutes. Brains were removed from the skull, postfixed in 4% paraformaldehyde and stored at -20°C. Then, they were cut to paraffin-embedded sections (4 μm) at coronal plane and Nissl stained. Only animals with histological confirmation of correct stereotaxic procedure (location of the track marks and lesions) contributed to the data given in the Results.

RESULTS

Results, expressed as mean ± S.D., are demonstrated in Table 1 and the fluctuations of the median concentrations in Figure 1. All data for the two variables, FSH and LH, were summarized graphically using box plots (Figures 2 and 3). The interaction effect between group and sample was statistically significant for both LH

Table 1: Descriptive statistics for the consequent LH and FSH serum concentrations in the five experimental groups.

LH SERUM CONCENTRATION (NG/ML)

	Group A		Group B		Group C		Group D		Group E	
	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD
1st Sample	0.58	0.08	0.46	0.13	0.82	0.1	0.77	0.05	0.86	0.09
2nd Sample	0.61	0.07	1.57	0.05	1.21	0.05	1.21	0.04	1.06	0.05
3rd Sample	0.66	0.04	0.66	0.05	0.76	0.07	0.74	0.07	0.88	0.04

FSH SERUM CONCENTRATION (NG/ML)

	Group A		Group B		Group C		Group D		Group E	
	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD
1st Sample	4.89	0.38	4.5	0.13	4.95	0.68	4.93	0.53	4.78	0.63
2nd Sample	4.98	1.7	9.59	3.23	10.13	0.47	10.1	0.73	10.21	0.5
3rd Sample	5.02	0.13	5.7	1.52	4.3	1.34	4.24	1.03	4.1	0.5

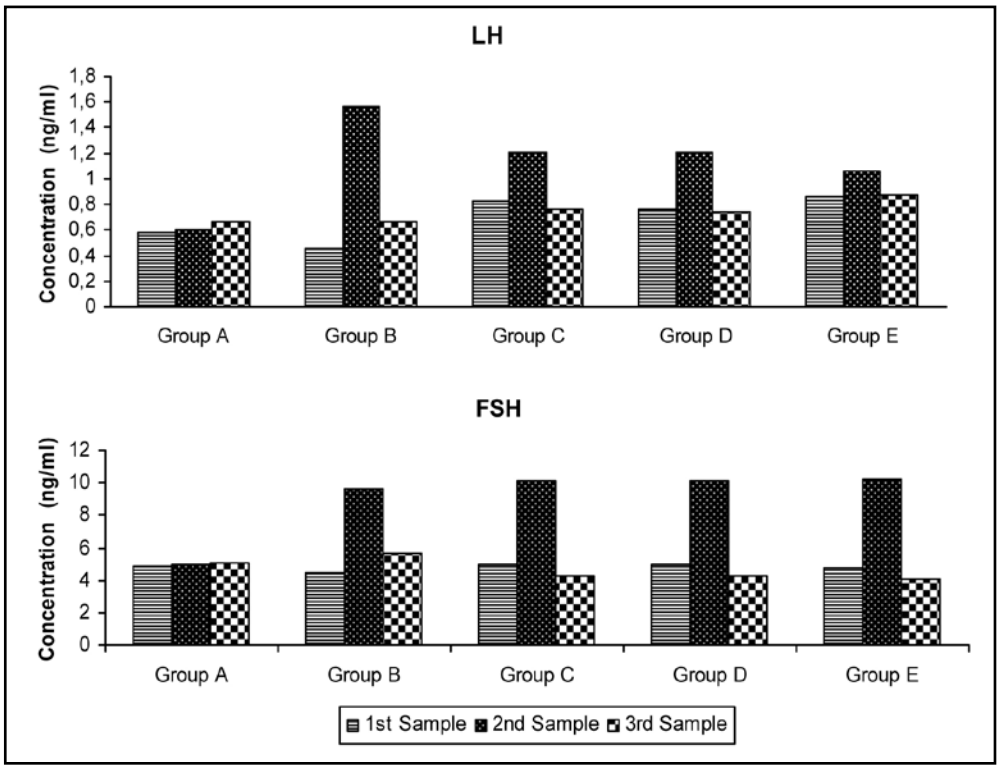

Figure 1: Demonstration of the median concentration of serum LH and FSH for all groups during the 3 consecutive measurements

(RMANOVA, f=42.84, p<0.0001) and FSH (RMANOVA, f=6.88, p<0.0001). Further exploration using the Tukey-adjusted comparisons for interaction term evidenced that:

In **Group A**, the consecutive measurements of the concentration of serum LH (Tukey's Test, p=1.000) and FSH (Tukey's Test, p=1.000) remained stable. This ought to be expected, though, since earlier studies (Ramirez VD & McCann, 1964) have already shown that the levels of LH and FSH are stable during the dioestrus I phase. It also confirmed that the time of the experiment was not coinciding with the LH/FSH surge of the oestrus cycle, which could bias the results.

In **Group B**, the levels of serum LH increased dramatically (340%) (Tukey's Test, p=<0.0001), and FSH levels was more than doubled (213%) (Tukey's Test, p=<0.0001), six hours after leptin administration. The comparison of LH and FSH levels with the control subjects (Group A) clearly showed how leptin acts and affects, 6 hours after the administration at the V3, LH and FSH levels (Tukey's Test, p=0.0001 for both variables).

In **Group C**, the concentration of serum LH increased significantly (148%), (Tukey's Test, p<0.0001), but much less than in Group B. Nevertheless, FSH serum levels also doubled (204%) (Tukey's Test, p<0.0001), six hours after the leptin administration. The comparison of LH levels with the specific control subjects (Group B) demonstrated the repressed action of leptin on the HPA (Tukey's Test, p<0.0001) when electrolytic lesion is performed to ARC prior to leptin administration in the V3. On the other hand, this action remained consistent as far as the FSH secretion was concerned (Tukey's Test, p=1.000).

In **Group D**, the concentration of serum LH increased significantly (157%) (Tukey's Test, p<0.0001) and similarly to that of Group C (Tukey's Test, p=1.000), while FSH serum levels also doubled (205%) (Tukey's Test, p<0.0001), six hours after leptin administration. The comparison of LH and FSH levels with Group B (the specific control group) indicated that an electrolytic lesion performed to VMH prior to leptin administration affected Group D similarly to group C (Tukey's Test, p=1.000 for both variables) where the electrolytic lesion was performed at the ARC. Consequently, the location of the lesions did not cause different effects.

In **Group E**, the concentration of serum LH increased (Tukey's Test, p<0.0001) more or less the same as in Groups C (123%) (Tukey's Test, p=0.1675) and D (Tukey's Test, p=0.1367), six hours after leptin administration. The FSH serum levels were more than double (213%) (Tukey's Test, p<0.0001) six hours after leptin administration, presenting almost the same pattern as in Groups B, C and D (Tukey's Test, p=1.000 for all groups) (Figure 3).

The LH level in Groups C, D and E presented the same pattern six hours after leptin administration, although Group B showed higher peak values (Figure 3).The damage caused to the hypothalamic nucleus/ei affected the secretion of the hormone (Tukey's Test, p<0.0001 for all groups) six hours after leptin admin-

Table 2: Tukey-Adjusted comparisons for interaction term (comparisons of the five experimental groups within samples). *Comparison is significant at $p<0.01$ level.

		FSH Concentration (ng/ml)				LH Concentration (ng/ml)			
		B	C	D	E	B	C	D	E
1st Sample	A	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	B		1.0000	1.0000	1.0000		0.9999	0.9998	0.9992
	C			1.0000	1.0000			1.0000	1.0000
	D				1.0000				0.9998
2nd Sample	A	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
	B		1.0000	1.0000	0.9999		<0.0001*	<0.0001*	<0.0001*
	C			1.0000	1.0000			1.000	0.1675
	D				1.0000				0.1367
3rd Sample	A	0.9998	0.9997	0.9992	0.9997	1.000	0.7571	0.9209	0.0760
	B		0.8811	0.8489	0.8850		0.6953	0.8842	0.5800
	C			1.000	1.000			1.000	0.5300
	D				1.000				0.3181

istration (Table 2). Nevertheless, examination of serum FSH concentrations in all the above groups did not demonstrate any difference (Tukey's Test, p=1.000 for all groups).

The comparison of the LH levels between group C, D or E indicated that electrolytic lesions performed to both hypothalamic nuclei and to each one individually, prior the leptin administration, did not in fact have additional effect to the secretion of LH, even though it did not present any significant divergence (Tukey's Test, p =0.1675 for C, Tukey's Test, p=0. 0.1367 for D) (Table 2).

Consequently, the location and the number of the lesions did not cause different action. On the other hand, FSH secretion for all these groups was similar, proving that the lesions did not cause any alteration to the function of the pituitary (Tukey's Test, p=1.000 for all groups). However, the LH and FSH levels reverted to the initial basic level 24 hours after leptin administration in all groups (Figure 1).

DISCUSSION

The reproductive process, which demands high energy consumption, is correlated with the nutritional status and energy stores. On the other hand, leptin, as a controller of the fat stores and an energy state messenger to the CNS, is also involved in the functions of the reproductive system.

Several studies suggest that leptin modulates hypothalamic–pituitary–gonadal axis functions. *In vivo* and in vitro experiments have explored the subject and proved the above statement. Leptin receptors have been isolated at all levels of the reproductive axis, covering the hypothalamus, the pituitary gland and the gonads. It has been shown that leptin stimulates Gonadotropins-releasing hormone release from the hypothalamus, and luteinizing hormone and follicle stimulating hormone release from the pituitary (Yu, Kimura *et al*., 1997), probably by activating its own receptors. Other studies support the notion that leptin acts on multiple hypothalamic targets and it is consistent with its suggested role as an important physiological signal in the secretion of gonadotropins. The action of leptin on the hypothalamus has been investigated by measuring leptin receptors and the GnRH levels, and attempts have been made to describe the mechanism(s) of leptin action on the reproductive system.

The controversies, observed in previous studies as far as the role of leptin on the reproductive axis is concerned, are due to different factors. The leptin dose as well as the way of its administration seems to be crucial (Baranowska, Wolinska-Witort *et al*., 2005). It has already been proven that, although LH and FSH secretion has a different peak value, the increase in their levels is definitely related to the leptin dose (Ogura, Irahara *et al*., 2001). However, leptin expression has a quantitative cut off limit (Watanobe, 2002), since high leptin concentrations lead to a decrease of GnRH secretion in vitro (Burcelin, Thorens *et al*., 2003). The gender difference in leptin levels, the oestrus cycle occurrence in females and the role of gonads in controlling the hypothalamus-pituitary axis (HPA) contribute to the various observations.

The mode of leptin administration by injecting into the blood stream or directly into the CNS seems to be another important factor, since the serum concentrations of leptin versus the cerebrospinal fluid ones, regulated by the blood-brain barrier, are kept at a ratio of

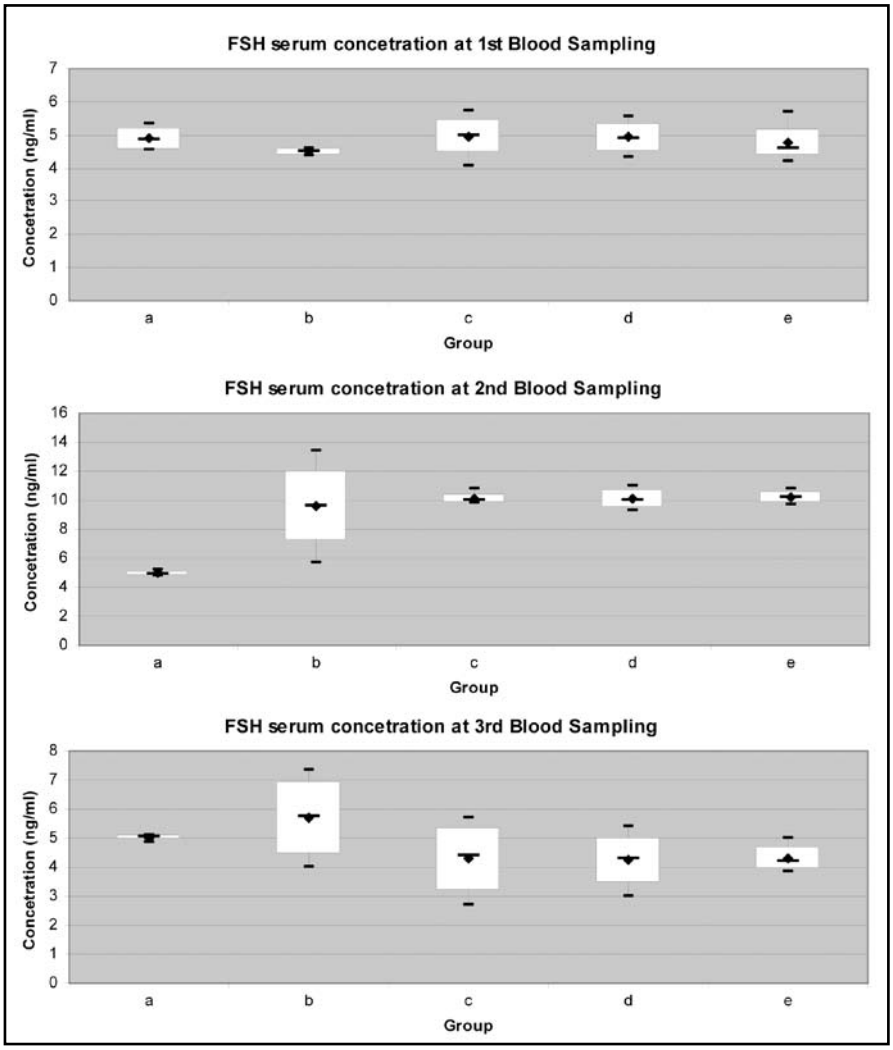

Figure 2: Box plots summarizing graphically the three consecutive FSH serum concentrations for all groups.

1:10. The energy state of animals is another factor contributing to the controversies. Fasted animals show hypoleptineamia and administration of leptin via the circulatory system can induce a rise in the CNS levels of leptin, although always keeping the same above mentioned ratio.

This study was designed as an attempt to distinguish and assess the quantitative *in vivo* action of leptin on the tonic secretion of gonadotropins. The experiments were planned to be performed on non-ovariectomized female rats, which were stereotaxically injected with leptin into the V3. In this way, the blood-brain barrier was abolished and the gonad steroid inhibition of LH and FSH was conserved. The performed lesions intended to rule out tonic GnRH secretion and to observe the leptin effect on other hypothalamic areas and on the pituitary, six hours after the instillation.

It is well known that, lesions of the hypothalamus and consequently cell damage, result in the acute rise of LH and FSH levels (Bishop, Fawcett *et al.*, 1972) probably because of GnRH release. Blood sampling six hours after the leptin administration would avoid including this explosion-type elevation of gonadotropin levels because of their small life turnover. Furthermore, 24 hours later, the fact that LH and FSH returned to their initial levels, indicates that no significant de novo synthesis occurred and that the elevation of the hormones, observed earlier was due to release of stored hormone forms by the pituitary.

We observed that LH and FSH secretion was induced by leptin administration into the third cerebral ventricle of normal rats. Its action was understrengthened regarding the release of LH, when lesions performed to the above nucleus indicate that GnRH, secreted from the above nuclei, participates in the LH secretion. The elimination of only one of the above nuclei was enough to decrease significantly the levels of LH. However, when both nuclei were destroyed, LH was not secreted in a lower level, indicating that the nuclei did not have additional role over the secretion but rather that leptin acted directly to the pituitary or other brain areas responsible for GnRH secretion.

On the other hand, FSH levels remained the same, even when the GnRH secretion was blocked from the

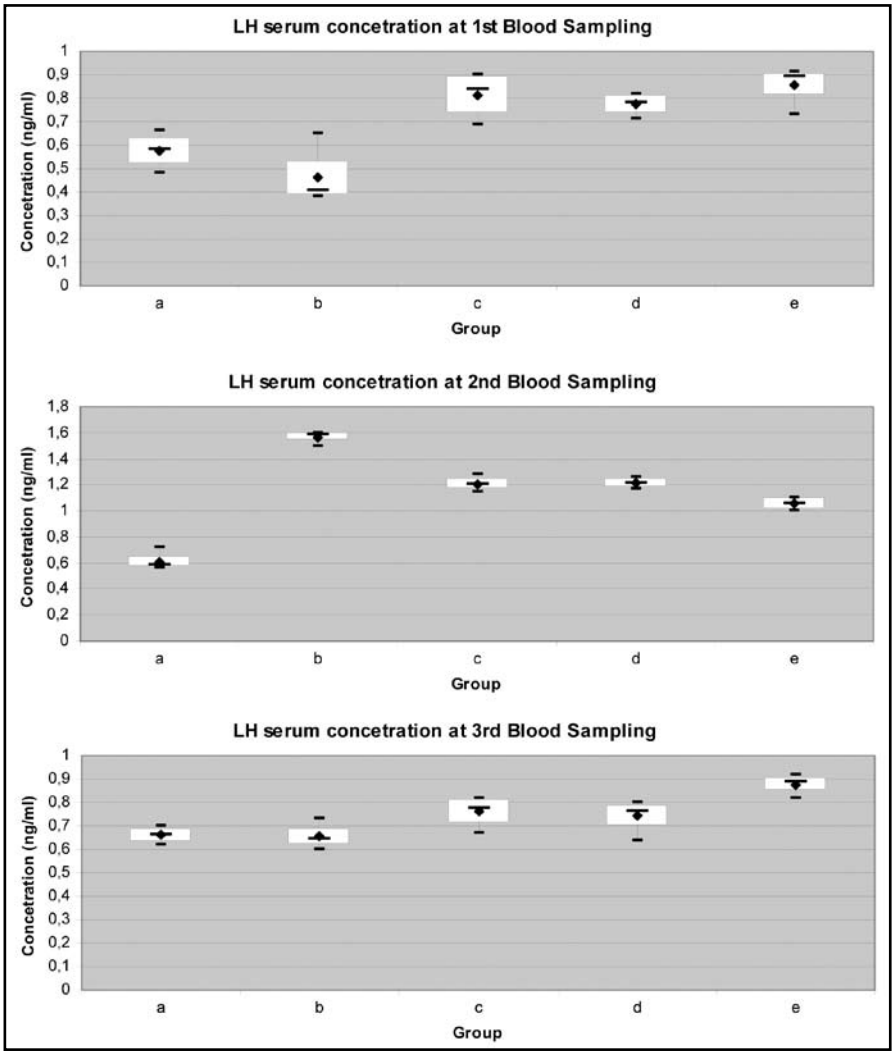

Figure 3: Box plots summarizing graphically the three consecutive LH serum concentrations for all groups.

ARC and VMH. We can therefore assume that leptin can act directly on the pituitary and trigger off maximum secretion of FSH or to activate different mechanism(s) in other areas of the brain containing leptin receptors. Such an area could be the preoptic area (POA), a key area for the production of GnRH regarding the pre-ovulatory secretion of the gonadotropins, indicating that the tonic secretion of FSH is not affected from the leptin administration. We also have to consider the possibility that a selective FSH-releasing factor may participate in the pathway. Such agents have been identified; lamprey gonadotropin-releasing hormone III (l-GnRH-III), has been detected within the organum vasculosum lamina terminalis (OVLT) region of the POA (Hiney, Sower *et al.*, 2002) of the rostral hypothalamus and is reported to selectively induce follicle-stimulating hormone release (Dees, Hiney *et al.*, 1999).

The significant conclusion derived from this study is that ARC and VMH, which are responsible for controlling the tonic secretion of gonadotropins, respond in a different way to FSH and LH secretion. This also suggests that some other mechanism(s) or factor(s) may additionally participate in the control of the tonic component of FSH secretion.

STATISTICAL ANALYSIS

Repeated measures analysis of variance (RMANOVA) techniques were used to explore the effects of the various groups and samples on the two variables of interest, FSH and LH.

Finally, if the main model showed a statistically significant effect *(i.e., $p \leq 0.05$)* for the group factor, sample factor or the interaction term of group by sample, then further analysis was needed to identify which levels were different from each other. This follow-up analysis of multiple comparisons was completed using Tukey's analysis methods.

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