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Effect of Orexin Infusion into Third Ventricle on the GnRH and LH Secretions in the Prepubertal Rat

H. Khazali and M. Behzadfar
College of Biological Sciences, Shahid Beheshti University, Tehran, Iran

Abstract: The goal of this study was to determine whether orexin affects gonadotropin releasing hormone (GnRH) and Luteinizing Hormone (LH) secretions in the prepubertal male and female rats. Forty prepubertal rats were randomly divided into 4 groups. Animals in group 1 and 2 were male and 3 and 4 were female. Animals in group 1 and 3 received infusions of 1 μg orexin and group 2 and 4 received infusions of 2 μg orexin into their third ventricle. Blood samples were collected from jugular veins, every 30 min from 4 h before the first infusion of orexin until 4 h after the last orexin infusion. Infusions of 1 and 2 μg orexin significantly ($p<0.01$) decreased the mean plasma concentrations and pulse amplitudes of GnRH and LH in prepubertal female animal. Also, infusions of 1 and 2 μg orexin significantly ($p<0.05$) decreased the mean plasma concentrations and pulse amplitudes of GnRH and LH in prepubertal male animal, but this decrease were lower in than female prepubertal rat. Infusions of 1 and 2 μg orexin did not change the mean plasma concentrations of FSH in the animal of all groups. Infusions of 1 and 2 μg, orexin significantly ($p<0.01$) decreased the glucose levels of the prepubertal female animals. The conclusion of this experiment indicated that orexin may negatively affect the GnRH and LH in the prepubertal rats with negative energy balance, but not in those with the positive energy balance.

Key words: Orexin, GnRH, LH, rat

INTRODUCTION

Orexin is a 33-amino-acid neuropeptide that is mostly found in hypothalamus (Antunes *et al*., 2001; Arihara *et al*., 2000; Backberg *et al*., 2000). Based on its neuron distributions in the hypothalamus, orexin coexists with many other neurons. For example, orexin neurons are found in high concentrations in hypothalamic areas considered to be important in the regulation of many physiological effects such as food intake, adrenal secretions and reproduction (Antunes *et al*., 2001; Balasko *et al*., 1999; Kohsaka *et al*., 2001). The effect of orexin on reproduction is not very clear. For example, it was shown that orexin decreases or increases GnRH and LH secretions. In humans, it showed that plasma LH concentration is reduced in hypocretin-deficient narcoleptic men, whereas gonadal steroid hormone levels are normal (Kok *et al*., 2004). The mechanism of this inhibitory effect of decreased orexin is through the decrease in GnRH secretion. This data suggests that orexin increases LH secretions through the GnRH secretions. Moreover, other studies show that intra-cerebroventicular or rostal POA administrations of orexin induces GnRH and LH concentrations in rats (Kohsaka *et al*., 2001; Pu *et al*., 1998), whereas administrations of orexin in medial POA and ARC/ME decrease GnRH and LH concentrations in rats (Pu *et al*., 1998; Russell *et al*., 2001, 2003). Furthermore, other studies showed that orexins suppressed the pulsatile secretion of LH in ovariectomized female rats (Furuta *et al*., 2002; Tamura *et al*., 1999). All of the above studies with the contradictory results were conducted in pubertal human and rats. There are no reports about the orexigenic effect of orexin on the GnRH and LH secretions in prepubertal animals. Therefore, the goal of this experiment was to determine whether orexin affects the GnRH and LH secretions in the prepubertal rats.

MATERIALS AND METHODS

This experiment was conducted in Shahid Beheshti University at Tehran, Iran from April 2006 till November 2008.

Experimental design: Forty rats were randomly divided into 4 groups. Animals in group 1 and 2 were males and animals in group 3 and 4 were females. During the course of the experiment, daily feed was weighed based on body weight and individually given to each rat every morning.

Corresponding Author: Dr. Homayoun Khazali, College of Biological Sciences, Shahid Beheshti University, Tehran, Iran
Tel: 0098-912-1254041 Fax: 0098-21-22403041

Surgery: All animals were prepared for surgery. Rats were anesthetized throughout the surgery for third ventricle cannulation under stereotaxic methods and jugular vein cannulations. The guide cannula was fixed to the skull with stainless steel screws and dental cement. The external opening to the canal was closed with a stainless steel cap. Orexin (Tabeshyarnoor Co. Ltd.) was dissolved in ethanol and stored at -20°C as a stock solution (10 mg/500 μL) for no longer than 2 days. Each rat was kept in a single cage for a 5 days recovery period. During the recovery period, cannules were washed by PBS solution to prevent from clotting. Immediately before the experiment it was dissolved in Ringer- Locke solution and a similar solution one without orexin was prepared as the control vehicle. During the experiments, rats were kept in comfortable cages. Rats in group 1 and 3 received 1 μg orexin and in group 2 and 4 received 2 μg orexin into their third ventricles every hour for 4 h. The perfusions flow rate was 5 μL min^{-1} and the volume of perfusates collected at 30 min intervals was about 10 μL. The tubes for perfusates contained 5 μL of aprotinine (250 IU, a proteolytic enzyme inhibitor) and were kept in an ice bath during sampling. Immediately after filling, they were frozen in liquid nitrogen and stored at -80°C until assayed for GnRH.

Blood collection: Blood samples were collected from cannules that were put into the jugular veins, every 30 min from 4 h before first infusion of orexin until 4 h after the last orexin infusion following injection with GnRH. Blood samples were kept at 4°C until centrifugation. A saturated sodium citrate solution (40 μL sodium citrate solution mL^{-1} blood) was added to the samples before centrifugation to prevent clotting of plasma during storage. Plasma was stored at -20°C until assayed for LH.

Hormone assays: Plasma LH were measured by a homologous double-antibody radioimmunoassay (RIA) as described earlier by Pelletier *et al.* (1982). For LH assay, rat LH (TYN-rLH) and antisera against LH were provided by Tabeshyarnoor Co. (Industrial City of Bu-Ali, Hamadan, Iran). Rat LH (TYN-rLH) was used for iodination. A seven-point standard curve ranging from 0.04 to 10 ng LH was used. An average assay binding of 40% was achieved using an initial 1:20000 dilution of LH antiserum for LH assays. The inter- and intra-assay variations were 6 and 9%, respectively. Plasma GnRH was measured by a homologous double-antibody radioimmunoassay (RIA) as described earlier by Caraty *et al.* (1997). For GnRH assay, concentrations of GnRH in portal plasma were measured by RIA in duplicate aliquots after methanol extraction using a specific antibody against the C-terminal portion of the molecule as described earlier by Caraty *et al.* (1997). GnRH assay sensitivity averaged 0.2 pg $tube^{-1}$ (10 assays) and the inter- and intra-assay variations were 9 and 12%, respectively. The pulse amplitude of GnRH and LH were determined by pulsar program (Merriam and Wachter, 1982).

Statistical analysis: All analysis were conducted using General Linear Model procedures. Data was analyzed using an analysis of variance for a repeated measure design. Mean comparisons were evaluated by least significant difference with single degree of freedom. The number of LH pulses was determined by the PC-PULSAR computer program according to the method of Merriam and Wachter (1982) with the following G parameters: G1 = 3.98, G2 = 2.40, G3 = 1.68, G4 = 1.24 and G5 = 0.93.

RESULTS

GnRH and LH: Infusions of 1 and 2 μg orexin significantly ($p<0.01$) decreased the mean plasma concentrations and pulse amplitudes of GnRH and LH in prepubertal female animal. Also, infusions of 1 and 2 μg orexin significantly ($p<0.05$) decreased the mean plasma concentrations and pulse amplitudes of GnRH and LH in prepubertal male animal, but this decrease were lower in than female prepubertal rat (Fig. 1, 2).

Mean plasma LH levels of the animals in group 3 and 4 were about 2.8, 1.4, 2.9 and 2.8, 1.5, 3 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 1). Also, mean pulse amplitudes of LH of the animals in group 3 and 4 were about 0.6, 0.7, 0. 7 and 0.7, 0.5, 0.7 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 1). Mean portal plasma GnRH concentrations levels of the animals in group 3 and 4 were about 95, 77, 102 and 99, 75, 102 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 2). Mean pulse amplitudes of GnRH of the animals in group 3 and 4 were about 29, 22, 32 and 32. 21, 33 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 2). Infusions of 1 and 2 μg orexin into third ventricle decreased mean plasma concentrations and pulse amplitudes of GnRH and LH of the animals in groups 1 and 2. Mean plasma LH levels of the animals in group 1 and 2 were about 4.1, 31, 4 and 4, 3, 4 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 1). Also, mean pulse amplitudes of LH of the animals in group 1 and 2 were about 0.97, 0.75, 0. 8 and 0.95. 0.75 and

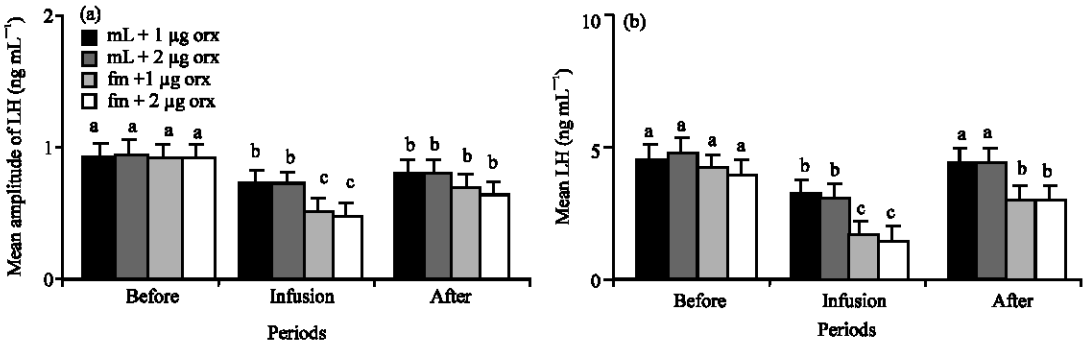

Fig. 1: (a) Mean pulse amplitude and (b) mean plasma concentrations of LH of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 µg orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of orexin (NE: Normal Energy; LE: Low Energy). Treatments with different letter(s) are different at $p<0.01$

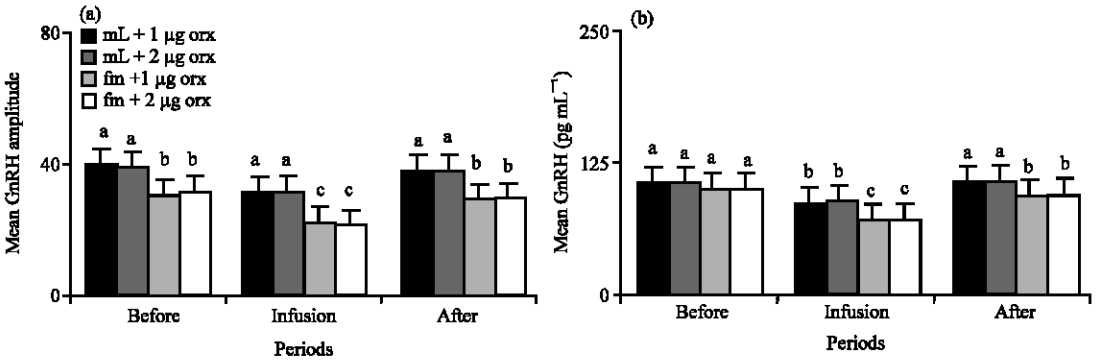

Fig. 2: (a) Mean pulse amplitude and (b) mean plasma concentrations of GnRH of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 µg orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of orexin (NE: Normal Energy; LE: Low Energy). Treatments with different letter(s) are different at $p<0.01$

0.86 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 1) . Mean portal plasma GnRH concentrations levels of the animals in group 1 and 2 were about 105, 82, 102 and 102, 81, 104 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 2). Also, mean pulse amplitudes of GnRH of the animals in group 1 and 2 were 40, 30, 39 and 38. 31, 40 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 2). Figure 4 and 5 show the changes of GnRH and LH pulsatile secretions of prepubertal female rats No. 171 and 175 as a samples. Higher dosage of orexin infusion caused more decrease in mean concentrations of GnRH and LH in rat No. 171, whereas these effects were less observed in prepubertal male rats (Fig. 6, 7).

FSH: Infusions of 1 and 2 µg orexin did not change the mean plasma concentrations of FSH in the animal of all groups. Mean plasma FSH levels of the animals in group 1-4 were about 4.1, 4.0, 4 and 4, 3.4, 3.9 ng mL^{-1} before, during and after infusion of orexin, respectively (Fig. 3).

Glucose: Orexin did not change the mean plasma glucose concentrations of the animals in group 1 and 2 that were pubertal males. Mean plasma glucose concentrations of the animals in group 1 and 2 were 47, 51, 42 and 52, 48, 47 mg dL^{-1} before, during and after infusion of orexin, respectively (Fig. 3). Infusions of 1 and 2 µg orexin significantly ($p<0.01$) decreased the glucose levels of the prepubertal female nimals. Mean plasma glucose concentrations of the animals in groups 3 and 4 were 48, 16, 22 and 52, 14, 24 mg dL^{-1} before, during and after infusion of orexin, respectively (Fig. 3).

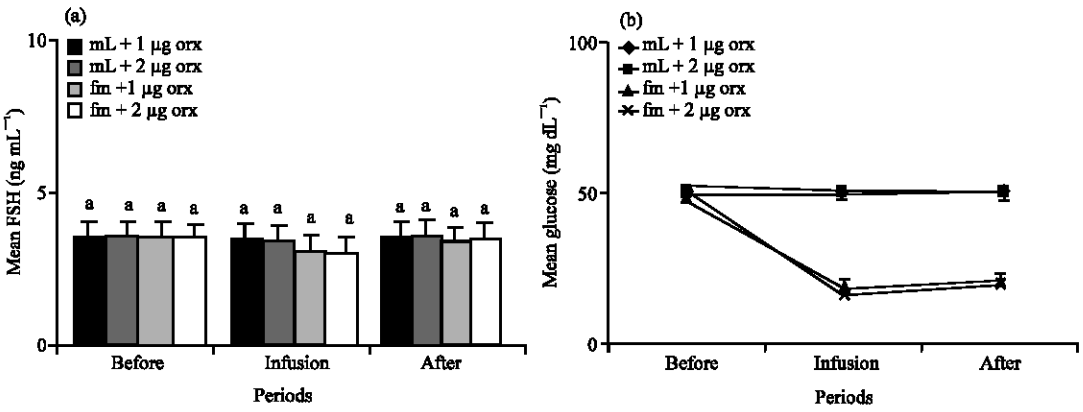

Fig. 3: (a) Mean plasma concentrations of (a) FSH and (b) glucose of the animals in the different groups of 1 (NE and 1 µg orexin), 2 (NE and 2 µg orexin), 3 (LE and 1 µg orexin) and 4 (LE and 1 µg orexin) and before, during and after infusions of orexin (NE: Normal energy; LE: Low energy). Treatments with different letter(s) are different at $p<0.01$

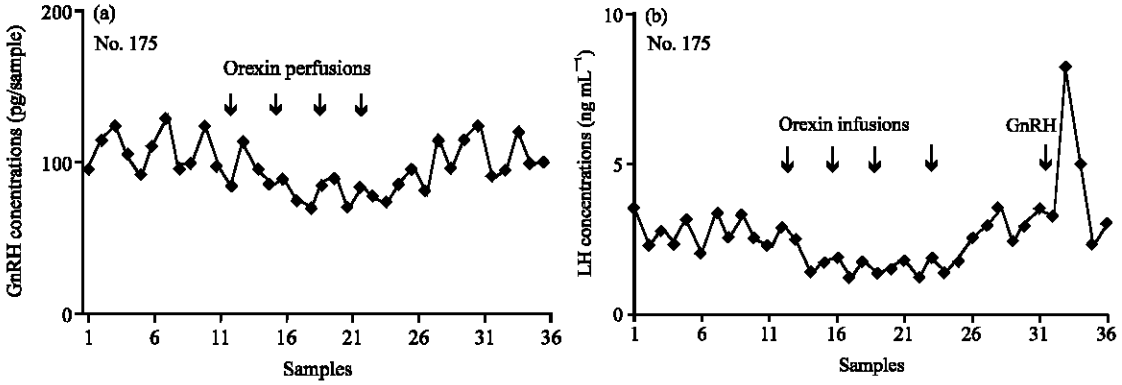

Fig. 4: (a) Mean plasma concentrations and (b) pulse amplitude of GnRH and LH of the prepubertal female rat received 1 µg orexin

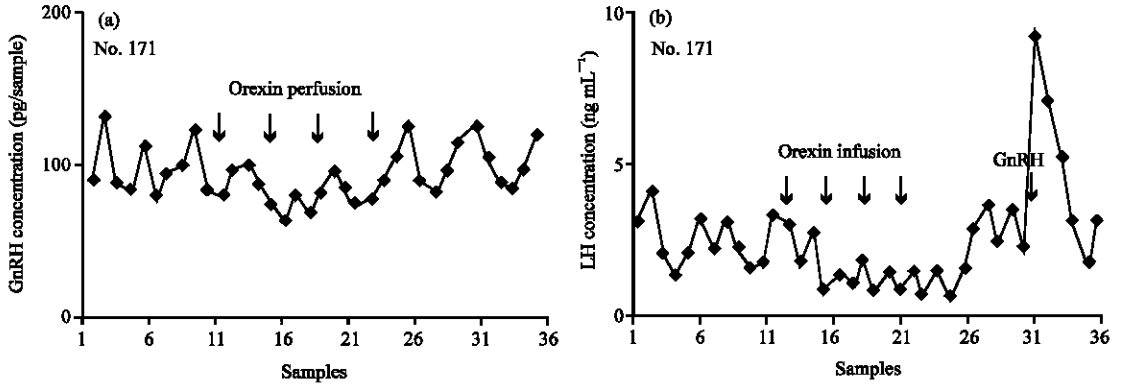

Fig. 5: (a) Mean plasma concentrations and (b) pulse amplitude of GnRH and LH of the prepubertal female rat received 2 µg orexin

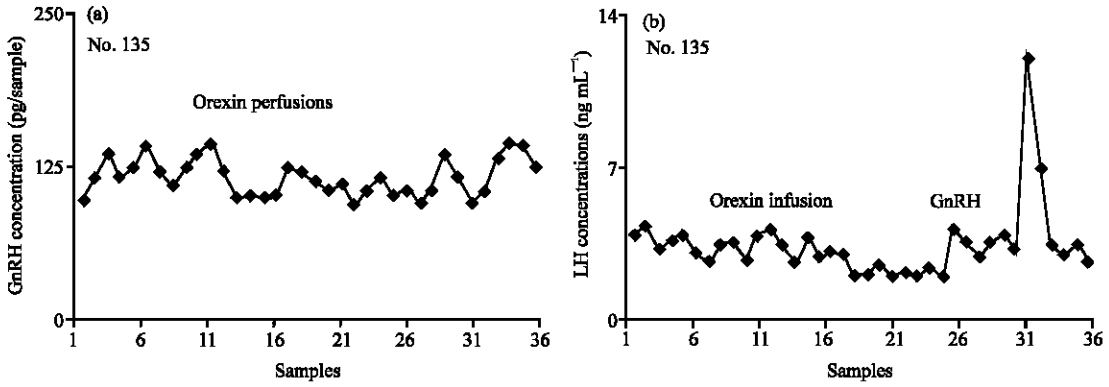

Fig. 6: (a) Mean plasma concentrations and (b) pulse amplitude of GnRH and LH of the prepubertal male rat received 1 μg orexin

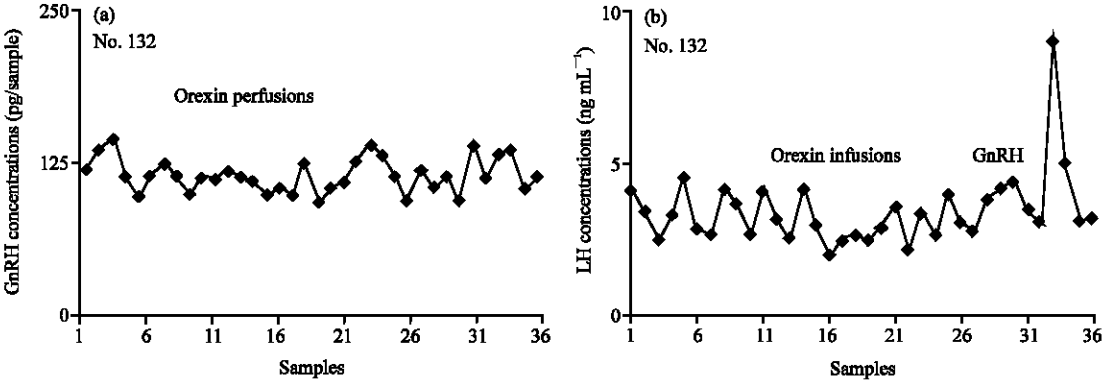

Fig. 7: (a) Mean plasma concentrations and (b) pulse amplitude of GnRH and LH of the prepubertal male rat received 2 μg orexin

Body weight: Restricted diet for 10 days significantly ($p<0.01$) decreased the mean body weight of the animals. This was similar to the earlier finding reported that negative energy balance decreases body weight (Towhidi *et al*., 2007).

DISCUSSION

The results of this study demonstrate that 1 or 2 μg of administration of orexin directly into the third ventricle significantly ($p<0.01$) decreased the mean plasma concentrations and pulse amplitudes of GnRH and LH in the pubertal male and female rats. Present results are different from earlier studies that showed a stimulatory effect of orexin on the secretory activity of the GnRH/LH axis in humans and rats (Kok *et al*., 2004; Kohsaka *et al*., 2001; Pu *et al*., 1998). This may be due to the fact that increase in orexin receptors in the cell body of GnRH neurons are observed in pubertal animal that may cause in the increasing of the secretion of GnRH and LH in the subjects. Contrastly, in this study the animals of the all groups are prepubertal and since the orexin receptors in the cell body of GnRH neurons are not formed, therefore decrease in the secretion of GnRH and LH upon infusion of orexin may happen through the ventromedial hypothalamic area and lateral hypothalamic area (Date *et al*., 1999; Dun and Chang, 2000; Ehmke and Jus, 2003). Therefore, orexin activates the lateral hypothalamic neurons that indirectly inactivate the GnRH secreting neurons (Al-Barazanji *et al*., 2001; Hagan *et al*., 1999; Horvath *et al*., 1999; Jones *et al*., 2001). The other mechanism is the inhibitory effect of orexin on the GnRH secreting neurons at the ME, as it is suggested that a subset of GnRH neurons responsible for episodic GnRH, and hence LH secretion, is located within the MBH (Boukhliq *et al*., 1999). Moreover, much of the GnRH axons have terminals in the ME, a structure located on the ventral part of the MBH.

The FSH result of this study also is first to show that orexin infusion did not change the plasma FSH level in the animals of all groups, which is an indication of negative energy balance, but not in the rats fed NE diet with normal glucose concentrations. The result of this study is different with the earlier finding of Kok *et al.* (2004) that showed plasma FSH level of hypocretin-deficient narcoleptic men is as normal as normal men. This may be due to restricted dietary intake that caused severe body weight loss.

The result of this study regarding the effect of the orexin on glucose concentration is similar to the earlier findings of Quedraogo *et al.* (2003) and Willie *et al.* (2002) that indicated a negative correlation between orexin and mean plasma level of glucose *in vitro*. This may be due to decreased level of glucagon (Meghan and Samsom, 2003) along with increase in insulin concentrations (Megahan and Samson, 2003; Nowak *et al.*, 2005). The result of this study showed that fasted animals are more susceptible to orexin physiological effect.

CONCLUSION

The results of this study indicate that orexin decrease GnRH and LH secretions in prepubetal male and female rats.

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