ORIGINAL ARTICLE

Evaluation of orexin A activity on LH and FSH release from primary culture pituitary cells in immature and mature female rats

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Abstract

OBJECTIVES: Orexin A (OxA) is a regulatory neuropeptide which is involved in the control of various autonomic and neuroendocrine functions. It regulates sleep-wake cycle, food intake and modulates the hypothalamic and pituitary hormones secretion. Orexin A acts through two types of receptors, which proved to exist in the pituitary. This may indicate the possibility of direct action of OxA on the adenohypophysis level. The aim of this study was to evaluate the direct effect of orexin A on gonadotropin (LH and FSH) release from cultured pituitary cells of immature female rats as well as mature female rats (ovariectomized and ovariectomized and estradiol treated rats).

MATERIAL AND METHODS: The effect of 0.1 nM and 100 nM orexin A on LH and FSH release from anterior pituitary cells after 1 h of incubation was examined in immature female rats (IM) as well as mature female (ovariectomized – M/OVX; and ovariectomized and estradiol treated – M/OVX+E_2) rats. The concentration of LH and FSH in medium was determined by RIA method.

RESULTS: Orexin A at a dose of 0.1 nM and 100 nM significantly stimulated LH secretion in IM group. In M/OVX group release of LH was inhibited by OxA only in higher dose (100 nM). No effect of orexin A on FSH secretion was found.

CONCLUSIONS: OxA may directly modulate LH secretion from cultured pituitary cells and it has the contradictory effect on LH release in immature and ovariectomized mature female rats.

INTRODUCTION

Orexin A (OxA), also known as Hypocretin 1, is a peptide synthesized mainly, but not solely, by neurons which perikarya are located in the lateral hypothalamus (de Lecea *et al.* 1998; Sakurai *et al.* 1998). These neurons form an extensive system in the central nervous system (Kukkonen *et al.* 2002). Orexin A acts through two different G-protein coupled receptors, OX1R and OX2R. The binding properties of these receptors are partially different. OX1R is highly selective for OxA, whereas OX2R is a receptor that binds both orexins, OxA and orexin B (Sakurai *et al.* 1998). Orexin receptors presence has been demonstrated in various areas of the brain and peripheral tissues e.g. pituitary

and ovary (Date *et al.* 2000; Voisin *et al.* 2003; Sakurai 2005; Silveyra *et al.* 2007a; Silveyra *et al.* 2007b). Such a wide occurrence of OxA and its receptors suggests the pleiotropic effects of this peptide. The first known function of OxA was to stimulate the process of food intake and energy expenditure (Sakurai *et al.* 1998). Orexin A was also shown to participate in regulating the sleep-wake rhythm and locomotor activity (Ohno & Sakurai 2008; de Lecea & Sutcliffe 2005; Sakurai 2007). The influence of OxA on the regulation of tropic hormones secretion by influencing the level on the hypothalamus and pituitary also appears to be significant (López *et al.* 2010). Orexinergic fibres have been shown to have connections with about 80% of GnRH neurons, and that approximately 85% of GnRH neurons express both types of orexin receptors (Campbell *et al.* 2003). This may indicate the possibility of direct influence of OxA on these neurons. On the other hand, orexin expression was found in the pituitary (Date *et al.* 2000). The presence of orexin receptors in the anterior pituitary was also demonstrated (Blanco *et al.* 2001; Jöhren *et al.* 2003; Silveyra *et al.* 2007b). These data may suggest that orexin acts on the pituitary gland as a hormone, in addition to the modulatory action of OxA at the hypothalamus.

Several reports have demonstrated that OxA plays a regulatory role in the reproductive axis. It was found that intracerebroventricular administration of orexin in steroid primed ovariectomized (OVX) rats stimulated LH secretion (Pu *et al.* 1998) but suppressed LH secretion in unprimed OVX rats (Tamura *et al.* 1999). It has been reported that the activity of orexin system in the hypothalamus, pituitary and ovaries was associated with hormonal milieu of estrous cycle (Silveyra *et al.* 2007a; b).

It is also known that sexual maturation process is associated with increased pulsatile secretion of GnRH. GnRH secreted from nerve endings in the median eminence of the hypothalamus, are the main stimulator of the transcriptional activity of genes encoding gonadotropin subunits and their release from the pituitary (Gajewska 2006). Reaching sexual maturity is associated with an increased frequency of pulsatile GnRH secretion. However, the mechanism of control of its secretion during puberty is complex and remains unclear (Terasawa & Fernandez 2001; Ojeda *et al.* 2006).

An analysis of the published data indicates that orexin A may modulate the activity of GnRH neurons and gonadotropin – secreting pituitary cells (Silveyra *et al.* 2010). GnRH neuronal activity and the synthesis and secretion of LH and FSH from the pituitary depends not only on the effects of neuropeptides secreted in the CNS, but may also be associated with the degree of sexual maturity and the hormonal status. In the literature, there is no study in vitro (with the use of primary cultures of pituitary cells), analyzing the influence of orexin A on the secretion of both gonadotropic hormones (LH and FSH) depending on the degree of maturity.

The aim of this study was to evaluate the direct effect of orexin A on gonadotropin (LH and FSH) release from cultured pituitary cells in immature female rats as well as mature female rats (ovariectomized and ovariectomized and estradiol treated rats).

MATERIAL AND METHODS

The study was conducted on female Wistar rats from Laboratory Animal Science in Warsaw. Animals were accustomed for 7 days under controlled conditions to temperature (23 ± 1 °C) and lighting (LD 14:10), the light was switched on at 06:00. Three specimens of rats were kept in standard cages, with constant access to water and standard pelleted food (Murigran, Poland). All studies were approved by the 1st Warsaw Ethic Committee for Experiments on Animals.

The following experimental models were applied: 1) IM – sexually immature animals, with naturally low levels of endogenous estrogens (age 25 days, with body weight range from 90 to 110 g) and M – sexually mature females (age 3 months, with body weight range form 220 to 260 g): 2) M/OVX – ovariectomized females treated subcutaneously (sc.) 200 µl of oil for 3 consecutive days preceding the relevant experience; 3) M/OVX + E_2 – ovariectomized females injected sc. a suspension of 17-β estradiol in a dose of 20 µg/200 µl oil / rat for 3 consecutive days preceding the relevant experience.

Removal of the gonads is a procedure allowing the elimination of endogenous estrogens of ovarian origin. Subcutaneous injections of estradiol or oil for OVX animals allowed the unification of the estrogen level within the groups.

The procedures of pituitary tissue dissociation, cell preparation and cell culture were based on previously described methods (Baranowska *et al.* 2003). Pituitary glands were obtained from female rats that had been anesthetized by ketamine injections and decapitated. The glands were washed twice with DMEM supplemented with 2 nM glutamine, 0.3% fetal calf serum (FCS), antibiotic/antimycotic, and immediately processed for culturing. They were enzymatically dispersed by 15 min incubation at 37 °C in 0.1% trypsin in DMEM followed by 15 min incubation in DMEM supplemented with 10% FCS and antibiotic / antimycotic containing 0.1% DNase I (deoxyribonuclease I from bovine pancreas, type IV). The glands were then mechanically dispersed through a sieve (50 mesh) and the cells washed twice with culture medium DMEM-FCS, collecting them by centrifugation for 15 min at 2 000 rpm. The pituitary cells were counted in a hemocytometer and assessed for viability by trypan blue exclusion (>97%). The cell suspension was diluted to 0.5×10^6 cells/ml, placed in 24-well culture plates and incubated for up to 48 h in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

To examine effects of OxA, doses in concentrations 0.1 nM and 100 nM were added and after 1 hour the

medium was collected. The control groups were cultured without OxA. The medium was stored at –20 °C until analyses for LH and FSH were carried out.

All media and chemicals were purchased from Sigma (Sigma Aldrich, Germany) and culture dishes were from Nunc (Thermo Fisher Scientific, Denmark)

Concentrations of LH and FSH in samples of cell culture medium were measured RIA using reagents prepared by Dr A.F. Parlow and provided by the NIDDK (Bethesda, MD, USA). Values were expressed in relation to LH-RP-3 and FSH-RP-2 reference standards, respectively. The limit of detection varied for LH was 0.1 ng/ml and for FSH was 1.25 ng/ml. All measurements were made twice in one assay for each hormone. Intra-assay coefficients of variation (CV) were <7%. The data are presented as the mean ± SD.

Statistical analyses were done using Statsoft Statistica 7.1 PL for Windows. Initially all groups of data were tested for normality by the Shapiro-Wilk's test. Statistical differences between the groups were determined by nonparametric Kruskall-Wallis test followed by the Mann-Whitney U test. Differences in mean values were considered significant if $p \leq 0.05$.

RESULTS

In the control group M/OVX LH and FSH release was significantly higher as compared with IM control group ($p \leq 0.0001$ and $p \leq 0.0001$ respectively) and M/OVX+E_2 control group ($p \leq 0.001$ and $p \leq 0.01$ respectively).

In IM group adding orexin A to the medium resulted in a significant increase of LH by 110% ($p \leq 0.001$) at a dose of 0.1 nM OxA and about 98% ($p \leq 0.01$) after administration of 100 nM OxA, compared to the control group. However, no changes in FSH were observed.

In M/OVX group secretion of LH was inhibited by OxA. Orexin A in a dose of 0.1 nM resulted in a reduction of LH level by 18%, and in a dose of 100 nM by 33% ($p \leq 0.01$) compared with control. There was no significant change in the secretion of FSH after incubation with 0.1 nM of orexin A, while the tendency to inhibition of FSH secretion from the cells after 100 nM OxA was visible.

In M/OVX+E_2 group no effect of OxA on gonadotropins secretion was observed. Data were presented in Table 1.

DISCUSSION

Pituitary glands for *in vitro* studies were collected from animals with different estrogen milieu. The cells from IM, M/OVX or M/OVX+E_2 differed due to the hormonal environment from which they were collected. The results of the presented study have shown that OxA stimulates LH secretion from the pituitary cells of female immature rats. A reversed tendency of change, namely inhibition of LH secretion from pituitary cells in the presence of OxA was observed in animals M/OVX, while pituitary cells collected from animals with higher estrogen milieu (M/OVX+E_2) did not change the secretion of LH and FSH under the influence of OxA.

Data from the literature showed that orexins are expressed in the rat and human anterior pituitary (Date *et al.* 2000; Blanco *et al.* 2001). Expression of both types of orexin receptors have also been shown in the pituitary gland of mammals, which indicates the possibility of direct influence of OxA on pituitary cells (Date *et al.* 2000; Blanco *et al.* 2001). Furthermore it was found that there is more OX1R than OX2R (Blanco *et al.* 2001) in the anterior lobe of pituitary, which suggests a greater importance of OxA in the regulation of pituitary hormones secretion. It was observed changes in the orexin receptor expression which could connect with the reproductive cycle (Russell *et al.* 2001;Silveyra *et al.* 2009; Kaminski *et al.* 2010). The link between quantitative changes and the hormonal state of animals indicates an important role of estrogens as the regulators of the expression of orexin receptors. In rat hypothalamus, OX1R mRNA expression was shown to be significantly higher during late proestrus than at metaestrus (Wang *et al.* 2003). The highest level of expression of mRNA for the OxA and OX1R was observed in the proestrus phase (Silveyra *et al.* 2007b). In contrast, no differences in the mRNA levels of OxA and OX2R were observed in hypothalami of control, gonadectomized, and steroid-treated female or male rats (Johren *et al.* 2001; Johren *et al.* 2003). In ovariectomized rats the level of mRNA OX1R was significantly higher than in the sham operated animals, and the administration of estradiol inhibited the expression of mRNA for OX1R. The OX1R mRNA level in the pituitary was the highest in females OVX (Jöhren *et al.* 2003). Furuta *et al.* (2002) found that suppressive action of orexin A on pulsatile LH secre-

Tab. 1. Effects of orexin A (OxA) on LH and FSH release from primary culture of anterior pituitary cells in immature female rats (IM), in ovariectomized mature, rats (M/OVX) and in ovariectomized mature estrogen treated rats (M/OVX+E_2).

Hormones [ng/ml]	IM			M/OVX			M/OVX+E_2		
	control	0.1 nM OxA	100 nM OxA	control	0.1 nM OxA	100 nM OxA	control	0.1 nM OxA	100 nM OxA
LH	8.06±1.37	15.0±2.32 ***	14.2±2.56 **	34.7±4.09	25.1±3.49	24.1±5.01 **	15.7±4.64	14.0±4.44	15.6±3.28
FSH	2.34±0.23	2.24±0.60	2.20±0.60	6.89±1.46	5.94±0.60	5.51±0.48	3.82±1.39	2.90±0.67	3.90±1.38

5×10^5 cells/well were incubated in medium (control), with 0.1 nM or 100 nM OxA for 1 h (n = 8 wells/treatment group). Data are presented as mean ± SD. **$p \leq 0.01$; ***$p \leq 0.001$ compared to the appropriate control group.

tion is potentiated by low doses of estrogen. It has been known that estrogens play an important role in the regulation of GnRH – gonadotropin axis (Christian & Moenter 2010). However, the mechanism of inhibitory and stimulatory influence of estrogen on GnRH secretion and biosynthesis is controversial (Herbison 1998).

Ferguson & Samson (2003) in *in vitro* studies of the impact of OxA administered in doses ranging from 0.01 pM to 100 nM, on the secretion of LH in primary cultures of pituitary cells of mature cyclic female rats have shown no statistically significant changes in the secretion of the studied hormones. While the studies carried out on immature porcine pituitary cells demonstrated a dose-dependent (0.1–100 nM) stimulation of LH secretion by OxB in a primary culture (Barb & Matteri 2005).

In our study we did not show differential activity of OxA at low and high dose, although research in Chinese hamster ovary cell line (CHO) has been showed that stimulation of orexin receptors is dependent on the dose of the peptide and leads to different OxA signal transduction (Johansson *et al.* 2008). At the lowest OxA concentrations diacylglycerol (DAG) was mainly generated as phospholipase D activity. Whereas higher doses of OxA activated phospholipase C, probably hydrolyzing phosphatidylinositol (PI) or phosphatidylinositol monophosphate (PIP), but not bisphosphate phosphatidylinositol (PIP2). The highest dose OxA resulted in the hydrolysis of PIP2 and release of both DAG and 1,4,5-triphosphatidylinositol (IP3). Thus, activation of OX1R by OxA was combined by phospholipases D and C activities (Johansson *et al.* 2008).

Studies on the effects of OxA on pituitary cells should be continued with a focus on the stage of maturity, estrogen status and signal transduction pathways.

In conclusion, the results of our study revealed that OxA may directly modulate LH secretion from cultured pituitary cells and it has the contradictory effect on LH release in immature and ovariectomized mature female rats.

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