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CLINICAL STUDY

Orexin A and B levels in the hypothalamus of female rats: the effects of the estrous cycle and age

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Abstract

Objective: Orexins have been implicated in the regulation of several physiological functions including reproduction, energy balance and vigilance state. For successful reproduction, the precisely timed hormonal secretions of the estrous cycle must be combined with appropriate nutritional and vigilance states. The steroid- and nutritional state-dependent modulation of LH release by orexins, as well as an increase of vigilance, suggest that orexins may co-ordinate these functions in the course of the estrous cycle.
Design: We studied the brain tissue levels of orexins in the course of the estrous cycle in young and middle-aged rats. Young cycling rats (3 months old) and irregularly/non-cycling (7–9 months old) female rats were inspected for vaginal smears and serum hormone levels.
Methods: Tissue concentrations of orexin A and B were measured in the hypothalamus and lateral hypothalamus on different days of the estrous cycle.
Results: Orexin A concentration in the hypothalamus of young cycling rats was higher on the day of proestrus 5–6 h after the lights were switched on than on the other days of the estrous cycle at the same circadian time. Orexin B concentration was higher on both the day of proestrus and the day of estrus as compared with the days of diestrus. The hypothalamic concentrations of both orexin A and B in the non-cycling middle-aged rats were lower than those in cycling rats on the days of proestrus and estrus.
Conclusions: We have concluded that the high hypothalamic concentration of orexins on the day of proestrus may contribute to the LH and prolactin surges. High orexin A levels may also contribute to the decreased amount of sleep on the day of proestrus.

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Introduction

Orexins (orexin A and orexin B), also known as hypocretins (hypocretin 1 and hypocretin 2 respectively), are novel hypothalamic peptides (1, 2). They are cleaved from a common precursor molecule, preopro-orexin (130 residues) forming orexin A (residues 33–66; hypocretin 1 residues 28–66) and orexin B (residues 69–96; hypocretin 2 residues 69–97) (1, 2). The cell bodies containing prepro-orexin mRNA are located in the lateral and perifornical hypothalamus (2, 3), but send wide projections to several brain areas, including the hypothalamus, which is regarded as one of the main target areas of these neurons. Since one-third of all hypothalamic neurons express orexin receptors, and orexin axon terminals are found throughout the hypothalamus, it has been suggested that orexins could influence the general level of activity in many hypothalamic systems (4). In addition to an increase in food intake (2) orexin administration decreases prolactin, growth hormone (5) and luteinizing hormone (LH) secretion in non-steroid-primed rats (6), but increases LH secretion in steroid-primed rats (7) as well as increasing cortisol secretion (5). The luteinizing hormone (LH) response to orexin A in the hypothalamus appears to be site-specific (8) and mediated through orexin 1 receptors, which are located on the gonadotropin-releasing hormone cells (9). It has recently been shown that orexin A-stimulated LH-releasing hormone release from the hypothalamic explants is dependent on the steroid milieu (10).

A role for orexins in the regulation of the vigilance state was originally suggested by anatomical evidence (3), and further by the discovery of defects in the orexin-2 receptor systems of narcoleptic dogs, humans and mice (11–13). Administration of orexin A in the locus coeruleus (5), lateral preoptic area (14) and basal forebrain (15) induces wakefulness. Moreover, the levels of orexin A in the hypothalamus are higher during waking and rapid eye movement (REM) sleep than during slow-wave sleep, further suggesting a function for orexin A in the regulation

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of vigilance states (16). These findings have established a role for orexin A as a substance that promotes wakefulness.

The vigilance state and the sleep/wake cycle are modulated by changes in the hormonal milieu: e.g. the estrous cycle and pregnancy have profound effects on both the amount and quality of sleep (17, 18). Decreases in both non-REM and REM sleep, as well as in the spectral power of sleep, have been observed in female rats during proestrus (17). Previous studies have shown modulations of orexin A tissue concentrations in several brain areas during the day of proestrus in female rats (10).

Advanced aging is associated with deterioration of several physiological functions. A multitude of hormonal changes (19), changes in energy balance (20) and changes in autonomic regulation take place (21). In elderly humans, sleep is typically disrupted, resulting in unwanted awakenings during the night time, excessive daytime sleepiness and increased napping (22). In old rats, the number of shorter sleep bouts increases and the response to prolonged wakefulness is impaired as compared with young rats (23, 24). We have previously shown that during aging both orexin A and orexin B levels decrease in the hypothalamus and other brain areas of male rats (25).

We hypothesized that the tissue concentrations of orexins A and B in the hypothalamus may be modulated in the course of the estrous cycle and aging in female rats.

Materials and methods

Forty-two young (3 months old, weight 200–260 g) and 27 middle-aged (7–9 months old, weight 295–400 g) female Hannover–Wistar rats were used in the experiments. Before the experiments the animals lived in the animal quarters of the department (Dept. of Physiology, University of Helsinki). Food and drink were available *ad libitum* throughout the experimental period and the lights were on from 0800 to 2000 h. Vaginal smears were inspected daily for at least 2 weeks (2 weeks for the young rats and 5–6 weeks for the middle-aged animals) and inspected for cytology to establish regular/irregular cycling. Rats were decapitated in the afternoon at 1300–1400 h. Trunk blood was collected and serum prepared as described previously (26). Brains were rapidly removed and hypothalamus and lateral hypothalamus were dissected from a brain slice between bregma co-ordinates of 1.0–4.8 mm. The slice was first cut at 2 mm from the bottom of the hypothalamus, then trimmed 3 mm lateral from the midline to each side, and then a 2 mm (1 mm lateral from the midline to each side) piece (= hypothalamus) was separated, leaving two 2 mm pieces, which contained the lateral hypothalamus (27). The weights of the tissue blocks were hypothalamus 66.5 ± 3.5 mg and lateral hypothalamus 63.0 ± 2.2 mg. Tissue was frozen on dry ice and stored at $-80\,^{\circ}C$ until RIA. The experimental protocol was accepted by the provincial administrative board in accordance with the laws of Finland and the European Convention. All efforts were made to minimize animal suffering and the number of animals used in the experiments.

RIAs for orexin A and B

The assays were performed using commercial RIA kits for orexin A and orexin B (Peninsula Laboratories Inc., San Carlos, CA, USA) according to the manufacturer's instructions and as previously described (25). In short, the tissues were homogenized in 0.5–0.8 ml 1% trifluoroacetic acid and centrifuged at 12 000 *g* for 20 min at $+4\,^{\circ}C$. The supernatant was lyophilized and dissolved in 0.5 ml RIA buffer. In each assay, 50 μl samples were measured in duplicate. The protein content of the tissue was measured using the method of Lowry (28). Results are expressed as pg orexin/mg protein. The samples were measured in two separate RIAs, each containing samples from all groups (diestrus, estrus, proestrus and old). The interassay variability for 2 years in our laboratory for orexin A RIA has been 21.2% and that for orexin B 30.3%. The intra-assay variability in the orexin A RIA was 1.21% and in the orexin B RIA 7.73% calculated by the method of Abraham *et al.* (29).

RIAs for estrogen, LH, follicle-stimulating hormone (FSH), progesterone and prolactin

Estradiol levels were measured by immunofluorometric assay (IFMA) after diethylether extraction, using the human estradiol Delfia kit (Perkin-Elmer-Wallac OY, Turku, Finland) adapted for rodent samples (30). Progesterone (31) and prolactin (32) levels were measured using RIAs as described previously. LH and FSH were measured using IFMA assays as described previously (33, 34). The interassay coefficients of variation (C.V.) of the assays were $<15\%$ and intra-assay C.V. values were $<10\%$ at the concentrations measured. To eliminate the influence of intra-assay variability, all samples to be compared were analyzed in the same assay runs.

Statistics

The statistical comparisons were made using one-way ANOVA with Student–Newman–Keul as a post hoc test. Kruskal–Wallis one-way ANOVA on ranks was used in cases of unequal variance or non-normal distribution in groups, followed by Dunn's post hoc test. In comparing two groups, Student's *t*-test was applied. Differences between groups was regarded to be significant if $P < 0.05$. Values are given as means±S.E.M. throughout.

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Results

Estrous cycle

Of the 42 young rats, three had irregular estrous cycles and were excluded from the calculations. Of the remaining 39, 22 were collected during diestrus, seven during proestrus and ten during estrus. Of the 27 middle-aged rats, five had regular cycles. Those with either irregular cycles or constant estrus ($n = 3$) formed the group named 'OLD' in the calculations ($n = 22$). Of the five regularly cycling middle-aged rats two were decapitated in diestrus, two in estrus and one in proestrus.

Hormone levels

In the course of the estrous cycle of young animals Serum estradiol concentrations were higher on the day of proestrus as compared with the days of diestrus and proestrus (diestrus 0.094±0.010, estrus 0.054± 0.012, proestrus 0.49±0.29 nmol/l; Kruskal–Wallis one-way ANOVA on ranks $H(2) = 19.95$; post hoc Dunn proestrus vs diestrus and proestrus vs estrus, $P < 0.05$; diestrus vs estrus, not significant (ns)).

There was no statistically significant difference in serum LH, FSH, progesterone or prolactin concentrations between the days of the estrous cycle (Table 1).

In the course of aging The serum estradiol concentrations were lower in non-cycling middle-aged animals as compared with proestrous day concentrations in young animals (proestrus 0.49±0.29, OLD 0.11±0.01 nmol/l; Kruskal–Wallis one-way ANOVA on ranks $H(3) = 22.00$, $P < 0.001$; post hoc Dunn proestrus vs OLD, $P < 0.05$).

Serum prolactin levels were higher in non-cycling middle-aged animals as compared with rats in diestrus or estrus (Kruskal–Wallis one-way ANOVA on ranks $H(3) = 18.82$, $P < 0.001$; post hoc Dunn $P < 0.05$ for both diestrus vs OLD and estrus vs OLD).

There was no statistically significant difference in serum LH, FSH or progesterone concentrations between young and middle-aged rats (Table 1).

The effect of the estrous cycle on orexin A and B hypothalamic and lateral hypothalamic tissue concentrations

Hypothalamic orexin A concentration was higher on the day of proestrus in young cycling animals as compared with both the days of diestrus and estrus (diestrus 885±113, proestrus 2071±256 and estrus 1378±265 pg/mg tissue; one-way ANOVA degreees of freedom $(2,38) = 9.37$, $P < 0.001$; post hoc Newman–Keul's proestrus vs diestrus and proestrus vs estrus, $P < 0.05$; estrus vs diestrus, ns) (Fig. 1a).

Hypothamic orexin B concentration was higher on both the days of proestrus and estrus in young cycling animals as compared with diestrus (diestrus 937±305, proestrus 3307±1235, estrus 3693±963 pg/mg tissue; Kruskal–Wallis one-way ANOVA on ranks $H(2) = 13.40$, $P = 0.001$; post hoc Dunn diestrus vs estrus and diestrus vs proestrus, $P < 0.05$) (Fig. 1b).

There was no significant difference in the lateral hypothalamic tissue concentrations of orexin A between the days of diestrus and proestrus (diestrus 621±113 ($n = 14$), proestrus 866±400 ($n = 6$) ng/mg tissue; *t*-test $t(18) = 0.80$, $P =$ ns; for estrus the mean±S.E.M. was 338±142 ($n = 4$)). For orexin B, the number of samples in the proestrous and estrous groups and orexin A in the estrous group was insufficient ($n < 5$) to allow proper statistical analysis. In the diestrous group, orexin B tissue concentration was 1908±522 pg/mg tissue ($n = 8$).

The effect of age on orexin A and orexin B tissue concentration in the hypothalamus and lateral hypothalamus

Hypothalamic orexin A tissue concentration in young animals was higher on the days of proestrus and estrus than in the non-cycling middle-aged animals, but there was no difference when the levels on the day of diestrus in young rats were compared with those of middle-aged rats (diestrus 885±113, proestrus 2071±256, estrus 1377±264, OLD 531±86 pg/mg tissue; Kruskal–Wallis one-way ANOVA on ranks $H(3) = 22.52$, $P < 0.001$; post hoc Dunn proestrus vs OLD and estrus vs OLD, $P < 0.05$; diestrus vs OLD, ns) (Fig. 1a).

Hypothalamic orexin B tissue concentration was higher on all days of estrus in young animals as

Table 1 Serum concentrations of hormones during the estrous cycle in rats. Animals were decapitated at 1300–1400 h. Values are means±S.E.M.

	Estradiol (nmol/l)	**Progesterone** (pmol/l)	**LH** (ng/ml)	**FSH** (ng/ml)	**Prolactin** (ng/ml)
Diestrus (young, $n = 22$)	0.094±0.010	14 590±2300	0.528±0.078	2.90±0.62	8.60±1.14
Proestrus (young, $n = 7$)	0.490±0.287	11 150±1660	0.450±0.086	1.87±0.47	27.75±10.85
Estrus (young, $n = 10$)	0.055±0.012	11 630±1340	0.421±0.093	3.21±0.99	21.51±11.00
Middle-aged (non-cycling, $n = 22$)	0.110±0.014	15 770±1900	0.627±0.079	3.53±0.40	35.32±6.76

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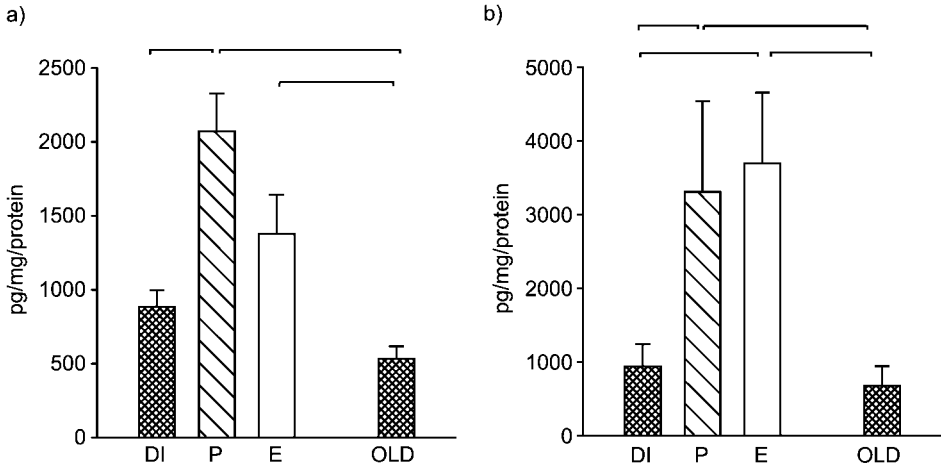

Figure 1 Tissue concentrations of orexin A and orexin B in the hypothalamus on different days of the estrous cycle in young and middle-aged rats. Young (3 months old) and middle-aged (7–9 months old) female rats were inspected for vaginal smears for at least for 2 weeks and decapitated at 1300–1400 h, and classified as diestrous (DI; $n = 22$), proestrous (P; $n = 7$), estrous (E; $n = 10$) or irregular cycling/non-cycling (OLD; $n = 22$). Tissue concentrations of orexin A and B in the hypothalamus were measured using RIAs. (a) Orexin A tissue concentration (pg/mg protein) in young animals was higher on the days of proestrus and estrus than in the middle-aged animals, but there was no difference when the levels on the day of diestrus in young rats were compared with those of middle-aged rats (Kruskall–Wallis one-way ANOVA on ranks H(3) = 22.52, $P < 0.001$, post hoc Dunn proestrus vs OLD and estrus vs OLD, $P < 0.05$; diestrus vs OLD, ns). (b) Orexin B tissue concentration was higher on all days of estrus in young animals as compared with middle-aged animals (Kruskall–Wallis one-way ANOVA on ranks H(3) = 20.72, $P < 0.001$; post hoc Dunn OLD vs diestrus, proestrus and estrus, $P < 0.05$).

compared with non-cycling middle-aged animals (diestrus 937±304, proestrus 3306±1234, estrus 3692±963, OLD 677±624 pg/mg tissue; Kruskal–Wallis one-way ANOVA on ranks $H(3) = 20.72$, $P < 0.001$; post hoc Dunn OLD vs diestrus, proestrus and estrus, $P < 0.05$) (Fig. 1b).

There was no significant difference in lateral hypothalamic tissue concentrations of orexin A between young (diestrous and proestrous groups in the statistical analysis) and non-cycling middle-aged animals (young as above, OLD 782±318 pg/mg tissue ($n = 15$); Kruskal–Wallis one-way ANOVA on ranks $H(2) = 4.26$, $P =$ ns). For orexin B, the number of samples in proestrous, estrous and OLD groups and orexin A in the estrous group was insufficient ($n < 5$) to allow proper statistical analysis.

Discussion

Previous studies have established a connection between the gonadal axis function and orexins: in steroid-primed, ovariectomized rats, orexin A increases LH secretion (7, 8), while in non-primed rats, LH secretion decreases (6). In the present study, the estrogen priming for LH and prolactin surges was evident on the day of proestrus, as evidenced by the elevated estrogen levels. The estrogen priming suggested that on the day of proestrus the effect of orexins on LH secretion would be stimulatory. Secretion of orexin(s) appears to be vital for the appearance of the LH and prolactin surges, as antibody to orexin A abolishes the steroid-induced LH and prolactin surges in ovariectomized rats (35). Thus the increase in orexin secretion on the day of proestrus may be an integral part of the hormonal secretion cascade which precedes ovulation. The physiological significance of this regulation remains presently speculative, though connection with food intake and energy balance has been suggested (35). This view is supported by the finding that in fasted rats orexin A partially restores the LH secretion (35).

Russell *et al.* (10) measured the hypothalamic content of orexin A at different phases of the estrous cycle and found that in the hypothalamus orexin A concentration was lowest late on the day of proestrus, while in several other brain areas the concentrations were highest at this time-point. They speculate that this may be due to the highest release of orexin A occurring at this time-point, leaving the hypothalamic orexin A content low. The tissue in the present study was collected 5–6 h after lights were turned on on the day of proestrus, which can be regarded as mid-proestrous day. The discrepancy between the orexin content profile in the two studies may be due to the difference in the time at which the tissue was collected, or there may be a difference in the size of the tissue block defined as hypothalamus – our tissue block was relatively large (more than 60 mg) and may have contained more hypothalamic nuclei than those of the previous study.

Sleep in female rats is modified by the estrous cycle: during proestrus, sleep is typically reduced and motor activity is increased during the period of darkness (= the active period for rats) (17, 18). Orexins, in

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particular orexin A (5, 14, 15), increase wakefulness. It has been suggested that their physiological role would be to consolidate the waking phases by increasing wakefulness during the natural wakefulness period. Compatible with this concept, several studies have shown diurnal variation both in the level of orexin gene expression and the concentrations of the peptide(s) (15, 36, 37), all of them showing higher levels of orexin A during the active phase of the animal, and a decline in the course of the rest phase. The increase in orexin A content on the day of proestrus, observed in the present study, and secretion followed by a decrease in the hypothalamic content later on the day of proestrus, as suggested by Russell *et al.* (10), are compatible with the findings of an increase in wakefulness starting in the later part of the rest period and extending to the active phase during the night of proestrus.

It has recently been suggested that the increased orexin levels during wakefulness could be induced by motor activity, rather than the vigilance state *per se* (38). As motor activity is increased during the day of proestrus (17, 18), it cannot be excluded that the changes in orexin A and B content in the present study could have been, at least partly, induced by an increase in motor activity.

We have previously reported a decrease in orexin A and orexin B tissue concentrations in male rats in the course of aging, which in the lateral hypothalamus was observed at the age of 12 months, while in the hypothalamus a significant decrease in orexin A was found at the age of 24 months (25). In the present study, orexin A and B levels in the hypothalamus were decreased in irregularly or non-cycling animals of the age of 7–9 months as compared with the estrous and proestrous levels of rats of 3 months of age, while in the lateral hypothalamus orexin A levels did not differ from those of young rats, probably because the animals of the present study were younger than those of the previous study, though a gender difference cannot be excluded.

Serum LH and FSH concentrations were not elevated in samples collected on the day of proestrus because the samples were collected before the rise of the LH and FSH peaks. The concentrations of FSH and LH in the middle-aged animals were not significantly elevated either, while prolactin levels were higher than in the young animals. Prolactin levels have been shown to increase with age, possibly due to decreased efficacy of the inhibitory tuberoinfundibular dopamine neurons (39).

In summary, orexin A and B tissue concentrations in the hypothalamus of young cycling rats were found to be higher on the day of proestrus than on the days of diestrus, suggesting that orexins may be part of the hormonal cascade that precedes ovulation. The elevated level of orexin A may, in addition to its effects on endocrine functions, contribute to the increased disruption of sleep that has earlier been reported to take place during the day of proestrus. Orexins may function as hypothalamic signals which co-ordinate the energy balance and vigilance state with reproduction.

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