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Variation in the Expression of Orexin and Orexin Receptors in the Rat Hypothalamus During the Estrous Cycle, Pregnancy, Parturition, and Lactation

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The widespread distribution of mRNA encoding orexin-1 (OX1R) and -2 receptors (OX2R) in the central nervous system suggests that orexin may be involved in multiple functional pathways. Central administration of orexin stimulates feeding and also affects ovarian steroid-dependent luteinizing hormone secretion, suggesting involvement of orexin in the regulation of reproductive function. To investigate a possible role for orexin in reproductive function, we examined variations in prepro-OX, OX1R, and OX2R mRNA levels in the female rat hypothalamus during the estrous cycle, pregnancy, parturition, and lactation using competitive reverse transcription–polymerase chain reaction. During the estrous cycle, only OX1R mRNA expression during late proestrus was significantly higher than that at metestrus. The prepro-OX and OX1R mRNA levels on d 1 of lactation were significantly higher than that during late pregnancy and lactation. Immunohistochemistry revealed the presence of orexin-A immunoreactive cells and the OX1R subtype in the lateral hypothalamic area as well as the magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei, respectively, in pregnant and lactating rats. These results suggest a role for orexin in reproduction that may be involved in regulating physiological function in early lactation through important binding sites in hypothalamic PVN and SON.

Key Words: Orexin (hypocretin); orexin receptors; hypothalamus; lateral hypothalamic area.

Introduction

Recently, a new class of peptides was isolated from the rat hypothalamus and named orexin-A and orexin-B, with identical amino acid sequences to hypocretin 1 and hypocretin 2 *(1,2)*. They are derived by proteolytic cleavage from the same precursor, prepro-orexin (prepro-OX), bind and activate two closely related G protein–coupled receptors named orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R) *(2)*. The widespread orexin immunoreactive fibers are present throughout the rat brain and spinal cord *(3)*. Furthermore, the mRNAs encoding OX1R and OX2R are widely distributed in the central nervous system *(4)*. Multiple functional pathways of orexin have been reported, including an involvement in feeding behavior *(2,5)*, endocrine function *(6–8)*, and activity/arousal *(9–12)*.

Orexin exerts differing effects on luteinizing hormone (LH) secretion in ovarian steroid primed and unprimed ovariectomized (OVX) rats. Earlier studies reported that central injections of orexin rapidly stimulate LH secretion in a dose- and time-related fashion in steroid-pretreated OVX rats and inhibit LH release in unprimed OVX rats *(6–8)*. Intracerebroventricular (icv) administration of orexin-A significantly restores the prolactin (PRL) surge in fasted steroid-primed OVX rats *(13)* and decreased serum PRL concentrations in estrogen-primed ovariectomized rats *(14)*. These lines of evidence suggest a possible involvement of orexin in reproductive function. The hypothalamus is well known as the center regulating reproduction. We have therefore investigated whether any changes occur in the expression of prepro-OX and orexin receptors (OX1R and OX2R) mRNA in the rat hypothalamus during the estrous cycle, pregnancy, parturition, and lactation. We also evaluated the distribution of cells immunoreactive for orexin-A and orexin receptors in the rat hypothalamus during pregnancy and lactation.

Results

Prepro-OX, OX1R and OX2R mRNA Levels in Cyclic Rats

During the estrous cycle, there were no statistically significant differences in the expression of prepro-OX mRNA (Fig. 1A) or OX2R mRNA level among the animal groupings (Fig. 1A, C). However, there was a significantly ($p < 0.05$, one-way ANOVA followed by Student–Newman–Keuls test) higher expression of OX1R mRNA in the afternoon of proestrus (Ppm, 1600–1700 h) compared with that on metestrus (D1) (Fig. 3 B).

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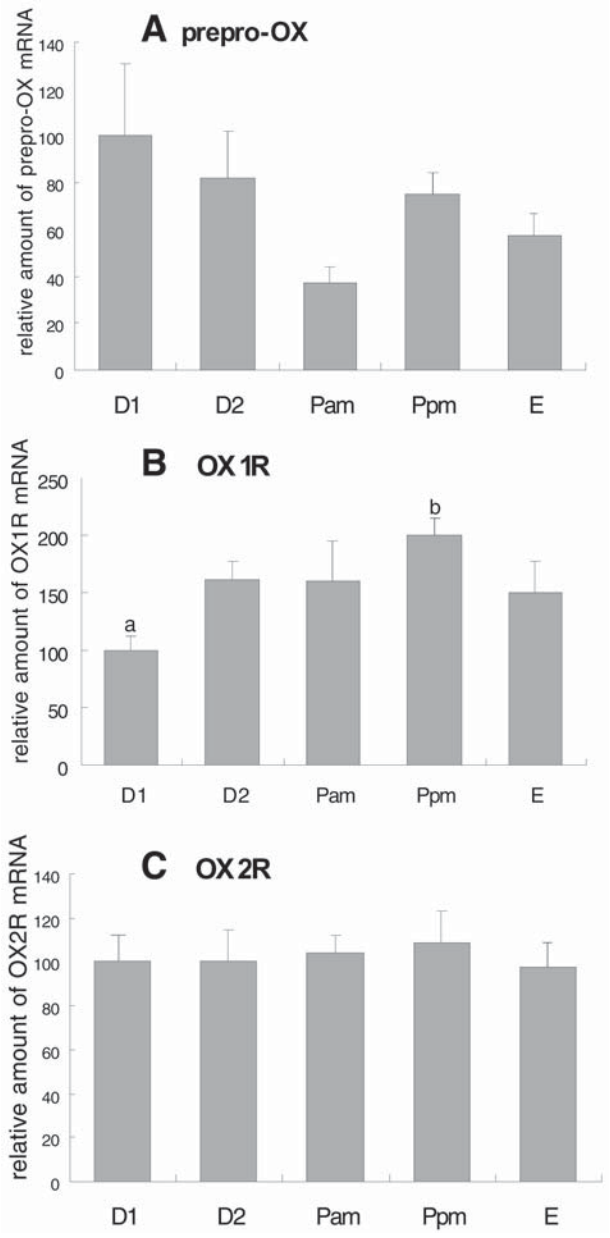

Fig. 1. The relative levels of prepro-OX (**A**), OX1R (**B**), and OX2R (**C**) mRNA during estrous cycle in rat hypothalamus. The hypothalami were dissected on various days of the estrous cycle at 1000–1030 h (estrus, E; metestrus, D1; diestrus, D2; and proestrus, Pam) and 1600–1700 h (proestrus, Ppm) ($n = 5$ each group). The levels of prepro-OX, OX1R, and OX2R mRNA were evaluated by competitive RT-PCR. The data were shown as a percentage of group D1. There was no statistically significant difference in the expression of prepro-OX mRNA (**A**) or OX2R mRNA level (**C**) among the animal groupings. There was a significantly higher expression of OX1R mRNA on Ppm in compared with that on D1 (**B**). $p < 0.05$, one-way ANOVA followed by Student–Newman–Keuls test. Bars with different letters (a and b) are significantly different from one another.

Changes in Prepro-OX, OX1R and OX2R mRNA Levels in Pregnant, Parturient, and Lactating Rats

The level of prepro-OX mRNA which was low in the morning of d 21 of pregnancy (P21am) progressively increased through day 1 of lactation (L1) and then gradually declined until d 14 of lactation (L14) (Fig. 2A). Statistically only the value on d 1 of lactation is significant compared with P20, P21am, P21pm, and L14 ($p < 0.05$, one-way ANOVA followed by the Bonferroni–Dunn test). Between P21 and L14, similar changes were observed for OX1R and prepro-OX mRNA expression (Fig. 2B). Also, OX1R mRNA was significantly higher on L1 compared with those on P21am and L14 ($p < 0.05$) and on L3 compared with that on P21am ($p < 0.05$, one-way ANOVA followed by the Bonferroni–Dunn test), but no significant change was observed in the level of OX2R mRNA (Fig. 2C).

Immunohistochemical Analyses for Orexin-A and OX1R in Pregnant and Lactating Rats

We evaluated the distribution of orexin-A, OX1R, and OX2R immunoreactive cells in pregnant (P19), lactating (L1 and L14), and male rat hypothalami. Orexin-A immunoreactive cells (irOX-A) were mainly located in the lateral hypothalamic area (LHA, typically at interaural 6.88–5.40 mm) in all the experimental rats. The most abundant expression of irOX-A cells occurred in sections corresponding to interaural 6.44–6.20 mm. No clear difference was observed in irOX-A cells in the LHA among rats on P19, L1, and L14 (Table 1). Immunoreactive OX1R (irOX1R) cells were observed in several nuclei of the hypothalamus, including the hypothalamic paraventricular nucleus (PVN), the supraoptic nucleus (SON), the ventromedial hypothalamus (VMH), the arcuate nucleus, and LHA. OX1R immunoreactivity detected in the magnocellular region of the PVN and SON was more intense than the other regions examined (Table 1). The OX1R immunoreactivity in the PVN and SON on L1 was relatively higher in comparison with those from sections on P19 and L14 (Fig. 3). Immunoreactive OX2R cells were not detected even when a higher dilution of antiserum (1:50) was used. In male rats, irOX-A cells were also present in the hypothalamic LHA and the pattern of expression was not much different from those in female rats (data not shown). The OX1R immunoreactivity observed in the PVN and SON was, however, weaker than those observed in female rats on P19 and L14 (Fig. 3 d, D).

Discussion

The presence of orexin in the LHA has been demonstrated by *in situ* hybridization *(1,2)* and immunohistochemical analyses *(1,2,15,16)*. Our immunohistochemical findings support these previous reports, while the localization of OX1R, especially in the PVN, is somewhat controversial as the results appear to vary depending on the experimental approach adopted for investigation. An earlier study using *in situ* hybridization reported that OX1R and OX2R mRNA were most abundant in the VMH and PVN in male rats, respectively; OX1R mRNA was not detected in the PVN *(4)*. On the other hand, immunohistochemistry showed

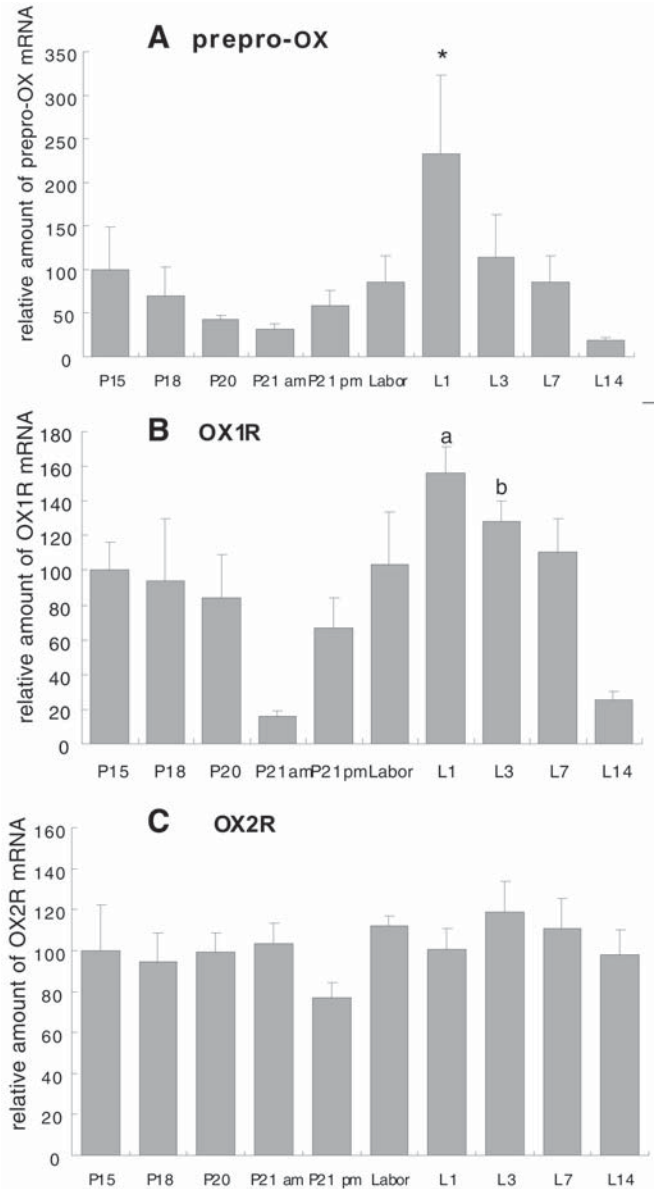

Fig. 2. The relative levels of prepro-OX (**A**), OX1R (**B**), and OX2R (**C**) mRNA during pregnancy, labor, and lactation in rat hypothalamus. The hypothalami were dissected at 1000–1030 h on d 15 (P15), 18 (P18), 20 (P20), and 21 (P21am) of pregnancy, during labor, and on d 1 (L1), 3 (L3), 7 (L7), and 14 (L14) of lactation and at 2200–2300 h on d 21 of pregnancy (P21pm) (n = 4 each group). The levels of prepro-OX, OX1R, and OX2R mRNA were evaluated by competitive RT-PCR. The data were shown as a percentage of group P15. The level of prepro-OX mRNA that was low on P21am progressively increased through and then gradually declined until L14. The value on d 1 of lactation was statistically significant compared with P20, P21am, P21pm, and L14 ($p < 0.05$, * vs P20, P21am, P21pm, and L14; one-way ANOVA followed by the Bonferroni–Dunn test) (**A**). OX1R mRNA was significantly higher on L1 compared with those on P21am and L14 and on L3 compared with that on P21am ($p < 0.05$, [a]vs P21am and L14; [b]vs P21am; one-way ANOVA followed by the Bonferroni–Dunn test) (**B**), but no significant change was observed in the level of OX2R mRNA (**C**). Prepro-OX, prepro-orexin; OX1R, orexin 1 receptor; OX2R, orexin 2 receptor.

Table 1
Relative Abundance
of the Immunoreactive Cells of Orexin-A and OX1R

	P19	L1	L14	Male
Orexin-A				
Lateral hypothalamic area	++[a]	++	++	++
OX1R				
Suprachiasmatic nucleus	±	±	±	±
Paraventricular hypothalamic nucleus	+	++	+	±
Supraoptic nucleus	+	++	+	±
Lateral hypothalamic area	–	–	–	–
Ventromedial hypothalamic nucleus	±	±	±	±
Dorsomedial hypothalamic nucleus	–	–	–	–
Arcuate hypothalamic nucleus	±	±	±	±

[a]++, highest intensity; +, moderate intensity; ±, low intensity; –, no clear staining.

wide distribution of irOX1R cells in the male rat hypothalamus, including the magnocellular oxytocin neurons of the PVN and SON *(17)*. In our study, irOX1R cells were observed in the hypothalamic PVN and SON of pregnant and lactating rats. The intensity of immunoreactivity for OX1R in the PVN and SON of pregnant and lactating rats were much higher than other areas, such as VMH and the dorsomedial hypothalamic nucleus. The staining in male rats was lower compared with other areas. This low expression of OX1R in male rats may be due, in part, to methodological differences in signal detection between *in situ* hybridization and immunostaining in male rats.

In the present study, orexin-A immunoreactivity in the LHA was unchanged among the various reproductive stages, although prepro-OX mRNA levels increased on L1. Several implications of this unexpected disparity in the mRNA expression are worthy of mention. The possibility that parallel changes in the irOX-A might have occurred later than L1 is unlikely because the irOX-A on L1 was not different from that on L14 when the mRNA level was very low. Then there is the fact that the release, degradation, or clearance of the orexin-A occurs equally to the increased synthesis on L1, resulting in an unchanged protein content. Indeed, some studies have reported that although hypothalamic prepro-OX mRNA expression may be up- and/or down-regulated, hypothalamic orexin-A content was unchanged *(2,18–20)*.

Oxytocin produced by the magnocellular neurons of the PVN and SON plays important physiological roles during delivery and milk ejection *(21)*. A recent report revealed the expression of OX1R immunoreactivity in oxytocin-containing magnocellular neurons of the PVN and SON *(17)*.

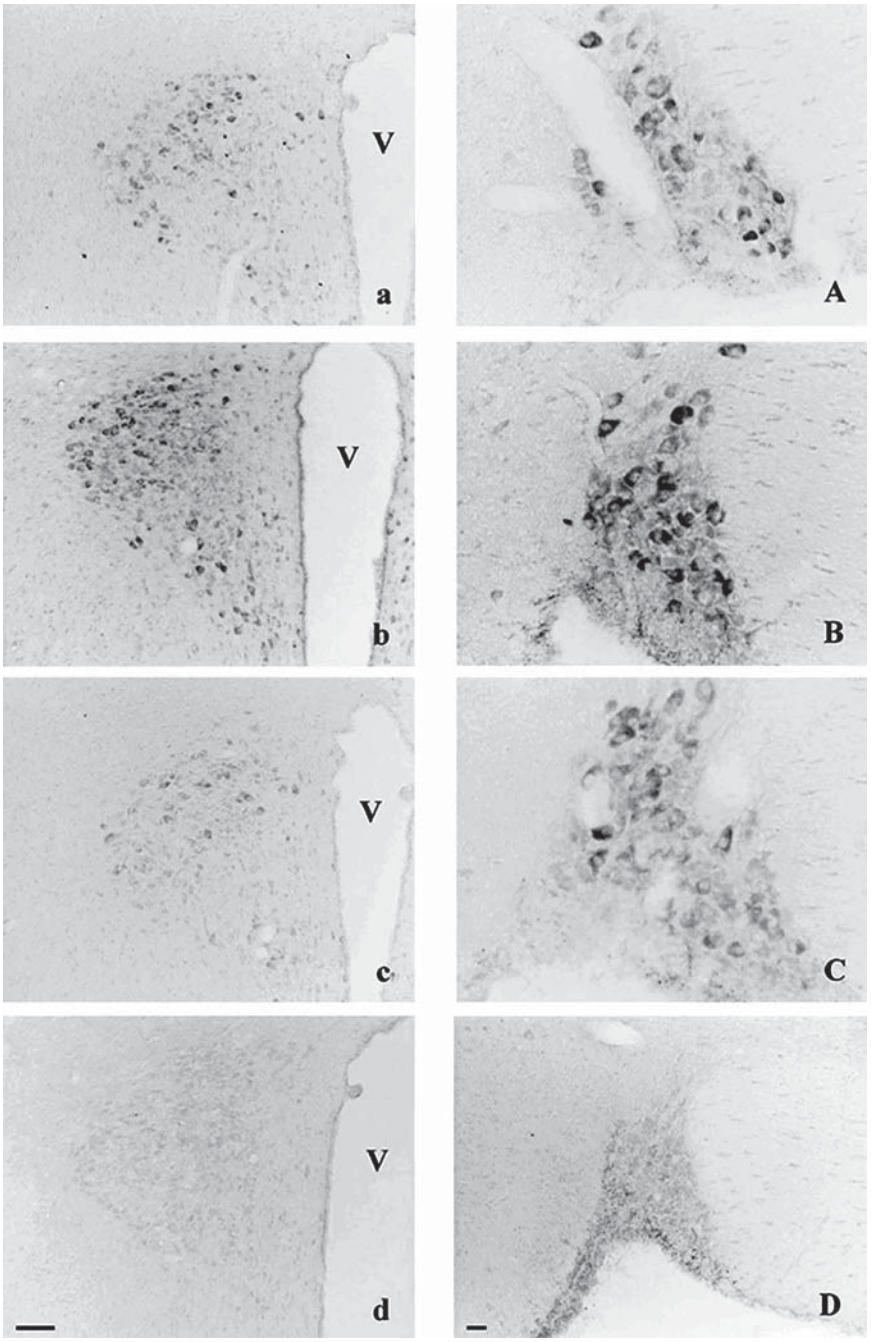

Fig. 3. The immunoreactive cells of OX1R stained in the PVN (**a,b,c,d**) and SON (**A,B,C,D**) in pregnant, lactating, and male rats. The brain tissues of rats on d 19 of pregnancy (**a,A**), d 1 (**b,B**) and d 14 (**c,C**) of lactation, and male (**d,D**) rats were perfused in Zamboni solution, sectioned at 20 μm and incubated with rabbit antisera for OX1R (1:4000). The OX1R immunoreactivity in the PVN (**a,c**) and SON (**A,C**) on d 19 of pregnancy and d 14 of lactation was moderate. The immunoreactivity in the PVN (**b**) and SON (**B**) on d 1 of lactation was relatively higher in compared with those on d 19 of pregnancy and d 14 of lactation. In male rats, the OX1R immunoreactivity in PVN (**d**) and SON (**D**) was weak. The locations of sections were in Interaural 7.40–7.30 mm. Scale bar = 100 μm (**a,b,c,d**) or 50 μm (**A,B,C,D**). V, the third ventricle.

Our study found that irOX1R cells located in the hypothalamic PVN and SON. Orexin fibers are present in the PVN and SON and *c-fos* expression can be induced in both nuclei by icv injection of orexins *(22)*. These results suggest that orexins maybe activate the PVN and SON by binding OX1R. In addition, PRL plays a key role in milk production and regulation of reproductive function *(23)*. The identification of PRL mRNA in the PVN and the presence of a 14-kDa PRL-immunoreactive protein in the magnocellular neurons of the PVN and SON have been reported *(24)*. Also,

an excitatory action of orexin-A on PRL release from the rat pituitary has been proposed following findings that icv administration of orexin-A to fasted steroid-primed OVX rat significantly restored the PRL surge *(13)*. From these results, taking our present observations, it is therefore possible that the increase in prepro-OX and OX1R mRNA levels and immunoreactivity of OX1R on L1 may be related, directly or remotely, to the initiation of milk ejection through a modulation of other intervening neuropeptides, such as oxytocin and PRL. However, details of the exact mechanism will await the outcome of further investigation.

The only significant changes in OX1R mRNA levels during the estrous cycle were observed in this study. There was a higher OX1R mRNA expression at late proestrus compared with that on metestrus. Orexin-A stimulates luteinizing hormone-releasing hormone (LHRH) release from rat hypothalamic explants harvested on proestrus, but not on estrus or metestrus *(25)*. Thus, orexin may stimulate LHRH release from the hypothalamus by increasing the sensitivity of LHRH neurons to orexin followed by an increase in OX1R synthesis. In addition, orexin-A plays an important role in regulating the preovulatory LH surge based on a report that central administration of orexin-A restored the LH surge in fasted steroid-primed OVX rats *(13)*. And, icv injection of orexin stimulates LH secretion in a dose- and time-related fashion in estradiol benzoate- and progesterone-pretreated OVX rats *(7)*. These results suggest that increased OX1R mRNA expression presumably facilitates orexin-A binding to OX1R, thereby exerting the actions of stimulating LHRH and LH release, and inducing the onset of preovulatory LHRH and LH surge at late proestrus. Consequently, ovulation occurs at estrus.

Orexin is known to stimulate feeding *(2,5)*, and irOX-A in LHA was significantly higher in the middle lactating rats (on d 11–12 of lactation) compared with that in non-lactating rats *(26)*. So, orexin appears to be partly involved in food intake. In the rat, food intake increases gradually during pregnancy until in late pregnancy when it decreases *(27)*. In our finding, the prepro-OX and OX1R mRNA levels gradually decreased until P21. This seemed to parallel the decrease in food intake during late pregnancy, but the prepro-OX and OX1R mRNA levels began to increase on P21pm with a surge on L1 before gradually decreasing again. It is obvious that these changes during lactation cannot totally account for the increased food intake to meet the great energy demand for milk production. Therefore, orexin action in early lactation may relate to activities other than the regulation of feeding, such as milk ejection, arousal/sleep state, or sympathetic activation *(10,12,28,29)*.

Although further studies will be necessary to determine the physiological relevance of orexin in reproductive function, the present study has clearly demonstrated an alteration in prepro-OX and OX1R mRNA expression in late pregnancy and early lactation. The hypothalamic PVN and SON may be important binding sites of orexin in these changes.

Materials and Methods

Animals

Adult male (used in immunohistochemical experiment) and female Wistar rats (body weight 200–250 g) were obtained from Japan SLC (Hamamatsu, Japan). They were housed in an environmentally controlled room (temperature 23 ± 3ºC; lights on 0600–1800 h) and were allowed free access to standard laboratory pellets of rat chow (NMF; Oriental Yeast Co. Ltd, Tokyo, Japan) and tap water. Estrous cycles were monitored by vaginal smears taken daily in the morning (0930–1030 h). Proestrus females were allowed to mate with conspecific males housed under similar conditions. The following day when spermatozoa were present in the vaginal smear and the day when pups were delivered were designated d 1 of pregnancy and d 0 of lactation, respectively. Some of the rats were decapitated on various days of the estrous cycle at 1000–1030 h (estrus, metestrus, diestrus, and proestrus) and 1600–1700 h (proestrus) (n = 5 each group). Some rats were also killed at 1000–1030 h on d 15, 18, 20, and 21 of pregnancy, during labor, and on d 1, 3, 7, and 14 of lactation and at 2200–2230 h on d 21 of pregnancy (n = 4 each group). Animal care, maintenance, and surgery were approved by Animal Care and Use Committee and were conducted for according to Guidelines for Animal Experiment, Fukui Medical University.

Hypothalamic Dissection

To isolate the hypothalamus, animals were decapitated and their brains were removed rapidly. Hypothalamic fragments were dissected with the following limits: anterior border of the optic chiasm, anterior border of the mamillary bodies, and the lateral hypothalamic sulci, and the depth of dissection was 3 mm. The tissues were immediately frozen in liquid nitrogen and stored at −80ºC until RNA extraction.

Construction of Normal and Mutant DNAs of Prepro-OX, OX1R, OXR and β-Actin

The oligonucleotide primers for prepro-OX, OX1R, OX2R, and β-actin (prepared as previously described in ref. *30*) are shown in Table 2. Each PCR product was subcloned into pGEMT easy vector (Promega, Madison, WI, USA) and each sequence was confirmed by BigDye Sequencing Kit (Applied Biosystems, CA, USA). Mutated DNAs, as competitors for each assay, were prepared by deleting 30–40% nucleotides in length from normal DNA. Normal DNA in prepro-OX, OX1R, and OX2R were deleted by amplifying each plasmid with lower primer and mutant upper primer shown in Table 2. The cDNA sequence of each PCR product for prepro-OX, OX1R, and OX2R was identified and no band was detected when the PCR reaction was carried out with total RNA sample without the reverse transcription to check contamination with genomic DNA or nonspecific reaction in each experiment (shown in Fig. 4). Mutant primers were designed to amplify shorter DNA than primary (normal) DNA amplified by RT-PCR and were linked with

Table 2
Sequences of Oligonucleotides Used for Competitive RT-PCR

Transcript primer	Sequence	Position (Accession)	Size
prepro (normal)	upper 5'-CGGATTGCCTCTCCCTGAGC-3'	61-80	470 bp
-OX	lower 5'-GCGAGGAGAGGGGAAAGTTA-3'	511-530	
(mutant)	upper[a] 5'-CGGATTGCCTCTCCCTGAGCTCTCTACGAA-3'	61-80/**231–240** (AF041241)	320 bp
OX1R (normal)	upper 5'-TGGGCTGTGTCGCTGGCTG-3'	1070-1088	479 bp
	lower 5'-CGAAACATCCCAAACACTCT-3'	1529-1548	
(mutant)	upper[a] 5'-TGGGCTGTGTCGCTGGCTGTGGCCCCGCT-3'	1070-1088/**1239–1248** (AF041244)	329 bp
OX2R (normal)	upper 5'-TTGGGGTTCATCGTCGTCAA-3'	1207-1227	742 bp
	lower 5'-TCGGTCAATGTCCAATGTTC-3'	1929-1948	
(mutant)	upper[a] 5'- TTGGGGTTCATCGTCGTCAATCACTCCTGT-3'	1207-1227/**1413–1422** (AF041246)	557 bp

[a]Mutant upper primer was used only for competitor DNA construction.

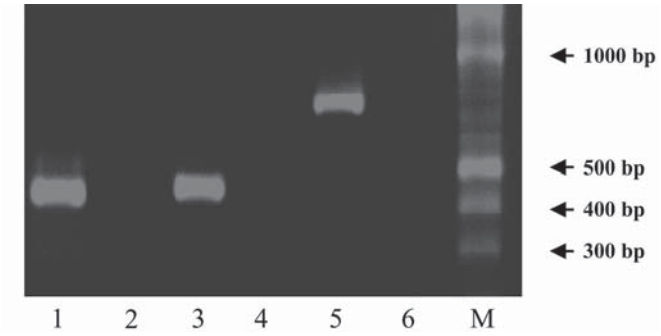

Fig. 4. Detection of prepro-OX, OX1R and OX2R mRNA in female rat hypothalamus by RT-PCR. Total RNA was extracted from rat hypothalamus and treated with (lanes 1, 3, and 5) and without (lanes 2, 4, and 6) reverse transcriptase. Then samples were amplified with primers for prepro-OX (lanes 1 and 2), OX1R (lanes 3 and 4), and OX2R (lanes 5 and 6). The products were separated on a 2% agarose gel and visualized with ethidium bromide. Expected sizes are 470 bp (prepro-OX), 479 bp (OX1R), and 742 bp (OX2R). The cDNA sequence of each product was identical to the corresponding region of prepro-OX, OX1R, or OX2R *(2)*.

normal upper primer at 5' end. Mutant DNAs for prepro-OX, OX1R, and OX2R amplified with mutant upper primer and lower primer were subcloned into pGEMT vectors. For deletion in β-actin, the inserted vector was digested with Eco0190I and AvaI, reacted with T4 DNA polymerase (Invitrogen, Carlsbad, CA, USA) to make both restriction sides blunt and ligated with T4 ligase (Invitrogen). Normal and mutant DNAs were amplified with M13 primer or gene-specific primers and purified with rapid PCR Clean-up system (Invitrogen). Concentrations of amplified DNAs were assessed with a spectrophotometer at a wavelength of 260 nm. Normal and mutant DNAs were used as standard DNA and competitor, respectively.

Competitive RT-PCR Analysis

Complementary DNA synthesis, PCR amplification, and analysis of PCR products were performed as described previously *(30,31)*. Briefly, the hypothalamus was homogenized using TRIzol Reagent (Invitrogen). Total RNA samples were prepared according to the acid guanidinium thiocyanate-phenol-chloroform extraction method *(32)* using TRIzol reagent and treated with RNase-free DNaseI (Invitrogen) to exclude genomic DNA. The quantity of total RNA was assessed with a spectrophotometer at a wavelength of 260 nm. Total RNA (1 μg) of sample was reverse transcribed with 200 U SuperScript II reverse transcriptase according to the supplier's manual (Invitrogen) and 10 pmol 9-mer random primer. All the samples and the standards in each experiment were assayed in a single run. Constant amounts of competitor DNA for prepro-OX (0.94 f*M*), OX1R (0.94 f*M*), OX2R (30 f*M*), and β-actin (5.5 p*M*) were added to all the samples. The PCR amplification was carried out in PCR buffer (50 m*M* KCl, 2 m*M* $MgCl_2$, and 20 m*M* Tris-HCl, pH 8.4), with 0.2 μmol primers and 1 U Taq polymerase (Applied Biosystems, CA, USA) in a total volume of 20 μL for 40 cycles consisting of denaturation at 94ºC for 1 min, annealing at 62ºC (prepro-OX, OX1R, and OX2R) or 55ºC (β-actin) for 1 min and extension at 72ºC for 1 min after the initial denaturation at 94ºC for 10 min. Amplification was completed with an additional extension step at 72ºC for 10 min. The PCR products were separated on 2% agarose gel, and then stained with ethidium bromide (0.5 μg/mL) and fluorescence density was analyzed using NIH Image software. The ratio of native prepro-OX, OX1R and OX2R mRNA-derived DNA to competitor DNA-derived DNA of each sample was obtained and calibrated from a standard

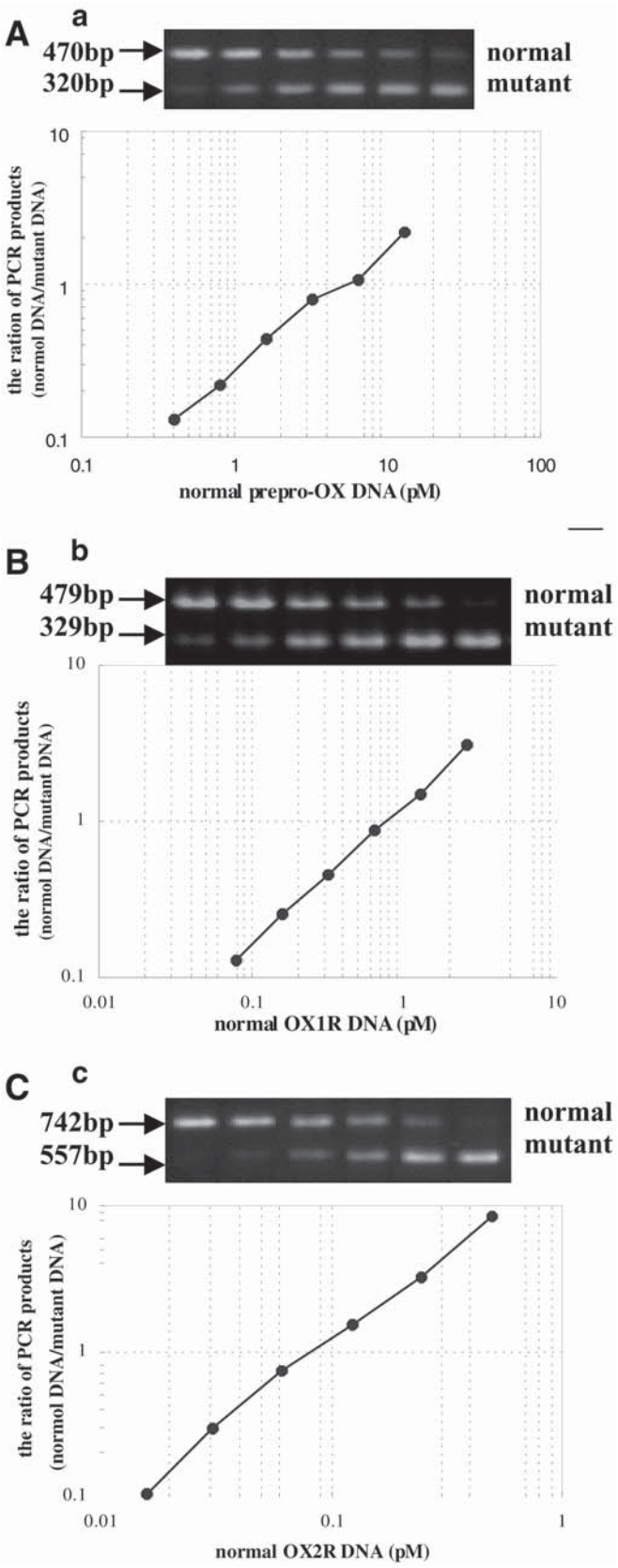

Fig. 5. Competitive PCR amplification of normal DNA and mutant DNA (a, b, c) and standard curves for prepro-OX, OX1R, and OX2R assay (A, B, C). Serially diluted normal DNAs (prepro-OX: 13–0.41 p*M*; OX1R: 2.6–0.081 p*M*; OX2R: 0.5–0.016 p*M*) were co-amplified with constant amount of competitor DNA. The PCR products derived from normal DNA and mutant DNA are 470 bp and 320 bp (prepro-OX), 479 bp and 329 bp (OX1R), and 742 bp and 557 bp (OX2R), respectively (a-c). Standard curves for prepro-OX, OX1R and OX2R. The quantitative analysis of PCR products in lanes 1-6 was performed as described in Materials and Methods. The *x* and *y* axes are the concentration of the standard normal DNA and the ratio of amplified normal DNA to mutant DNA, respectively (**A–C**).

curve as the amount of standard total mRNA (shown in Fig. 5). Dividing by the value for β-actin in the same sample standardized each value for prepro-OX, OX1R and OX2R mRNA. The content of prepro-OX mRNA and OX1R and OX2R mRNA was analyzed in each hypothalamus individually.

Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (45 mg/kg body weight, intraperitoneally) and perfused transcardially with 0.1 *M* phosphate buffer saline (PBS) (pH 7.4) followed by Zamboni fixative (4% paraformaldehyde, 0.2% picric acid in 0.1 *M* phosphate buffer, pH 7.4). The brain tissues were postfixed in Zamboni solution for 6 h at 4ºC. Following this, the tissues were incubated in 15% sucrose in 0.1 *M* PBS for 24 h and then transferred to 30% sucrose in 0.1 *M* PBS for more than 24 h at 4ºC. The specimens were frozen with liquid nitrogen and sectioned serially at 20 μm with a cryostat (Leica CM1510, Nussloch, Germany) and divided every 10 sections. One section was selected from each division including hypothalamus and the sections were used in one experiment. Stereotaxic coordinates described for the rat brain *(33)* were used to determine specific anatomical locations. Orexin-A, OX1R, and OX2R immunoreactivity were detected using a floating method. After blocking of endogenous peroxidase with 0.3% hydrogen peroxide (30 min) and skim mildly (Block Ace, Snow Brand, Japan) (30 min), the sections were incubated with primary rabbit antisera for human/rat orexin-A (1:20000; IHC9600, Peninsula Laboratories, Inc., San Carlos, CA, USA), OX1R (1:4000; AB3092, Chemicon International, Inc., Temecula, CA, USA) and OX2R (1:50; AB3094, Chemicon International, Inc.) diluted with 0.01 *M* PBS containing 0.03% Triton X-100 (PBS-T) at 4ºC overnight. Affinity-purified anti-OX1R antibody was raised against a 16-amino-acid peptides near the N-terminus and C-terminus of OX1R and recognized 50-kDa peptide in rat hypothalamus by Western blot analysis *(34)*. Anti-OX2R antibody was raised against 19-amino-acid peptides near the N-terminus and C-terminus of OX1R. No staining was observed in the section incubated without primary antibody. After washing with 0.01 *M* PBS-T, the sections were further incubated with biotinylated anti-rabbit IgG antibody (Vector Laboratories, CA, USA) diluted (1:1000) with 0.01 *M* PBS-T (60 min). This was followed by incubation with an avidin–biotin peroxidase complex reagent (Vectastain ABC kit, Vector Labs., Burlingame, CA, USA) diluted with 0.01 *M* PBS-T (45 min) after wash-

ing with 0.01 *M* PBS-T. The binding of the primary antibody in the sections was visualized by incubation with 3,3'-diaminobenzidine tetrahydrochloride in Tris buffer containing hydrogen peroxidase for 5–10 min. The sections were then mounted onto slides coated with gelatin, air dried, dehydrated in 100% ethanol, cleared with xylene, and finally coverslipped. The microscopic images of stained sections were transported to a video monitor using a digital camera (HC-2500 3CCD, fujix, Tokyo, Japan) attached to Vanox-S microscope (AH-2, Olympus, Tokyo, Japan) and Fujifilm/Photograb-2500 soft (Version 1.1). The size of images taken by digital camera was 1280 × 1000 pixels. The staining intensity of immunoreactive cells was evaluated on the basis of the average gray levels of positive neurons determined by the MacSCOPE program (Version 2.56, Mitani Corp., Fukui, Japan). The staining intensity was divided into four degrees: highest intensity (++); moderate intensity (+); low intensity (±), and no clear staining (–).

Statistical Analysis

Data for prepro-OX, OX1R and OX2R mRNA levels are expressed as the relative amounts (%) (mean ± SEM) by dividing the value of each sample with the mean value of the corresponding control group. These data were used in statistical analysis. One-way ANOVA followed by the Student–Newman–Keuls test or the Bonferroni–Dunn test was used to analyze the data. Difference was considered significant if p value was less than 0.05.

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