cDNA cloning of chicken orexin receptor and tissue distribution: sexually dimorphic expression in chicken gonads

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Abstract

Orexin-A and -B are known to stimulate food intake in mammals. However, the critical roles of orexins in birds are not fully understood, since orexins have no stimulatory effect on food intake in the chicken. To understand the physiological role(s) of orexins in birds, we have cloned chicken orexin receptor (cOXR) cDNA by RT-PCR, and analysed the tissue distribution of OXR mRNA in the chicken. The cOXR cDNA is 1869 bp long and encodes 501 amino acids. The cloned cDNA for cOXR corresponds to the type 2 OXR in mammals, and shows approximately 80% similarity to those of mammals at the amino acid level. Expression analysis by RNase protection assay revealed OXR mRNA was distributed widely in brain regions, and expression in the cerebrum, hypothalamus and optic tectum were abundant. In peripheral tissues, OXR mRNA was expressed in the pituitary gland, adrenal gland and testis, but no mRNA expression was observed in other tissues examined. Furthermore, we found that the amount of cOXR mRNA was different between testis and ovary, while prepro-orexin mRNA is equally expressed in the gonads of both sexes in the chicken. These data indicate that the orexins have neuroendocrine actions in chickens, which are mediated through hypothalamic receptors as has been observed in mammals. In addition, orexin may have specific role(s) in the regulation of gonadal function in which sex-dependent mechanisms could be involved.

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Introduction

The neuropeptides orexin-A and -B have been identified as endogenous ligands for two orphan G-protein coupled receptors and were shown to stimulate food intake in rodents (Sakurai *et al.* 1998). The cellular localisation of the orexin-producing cells was restricted in the hypothalamus, but neurons expressing orexins project widely throughout the brain (Peyron *et al.* 1998, Date *et al.* 1999). Orexins bind and activate two subtypes of receptor called type 1 OXR (OX1R) and type 2 OXR (OX2R) (Sakurai *et al.* 1998), and these are widely distributed throughout the brain in the rat (Trivedi *et al.* 1998, Marcus *et al.* 2001). Consistent with this, physiological studies have implicated orexin peptides in the control of food and water intake, cardiovascular function, sleep–wake cycle

Table 1 Sequences of oligonucleotide primers for PCR

Orexin receptor

Primer 1	AACCACCACATGAGGACAGTCACC
Primer 2	CGCAGCACTATTGGCATATACAA
Primer 3	GTGACAAATTGCATACCATCGATCC
Primer 4	TGCTGGAAGACAAGTAATTGTGACC
Primer 5	AATGGAAGTCTCTGCAGTCCTCAGC
Primer 6	CACGTATGCTAATGGTTGTCCTCCT
Primer 7	ACAGTTTCAGTCTCTGTGTC
Primer 8	CCAGTGTTCATCACACACTG

Prepro-orexin

Primer 1	ATGGAGGTGCCCAACGCCAAGCTGC
Primer 2	CAGGTCCTTCTCAGCGTGCTCCTGG

and neuroendocrine secretion in the central nervous system in mammals (Hagan *et al.* 1999, de Lecea & Sutcliffe 1999, Sakurai 1999). In addition, peripheral localisation of OXRs was reported in

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GGCAATCACAAGTCTCAATGGAGGGGCGGGAGAGAGCTGTGGAGCGCTGAGAGGGTTCCTCCAGCCCCCCGGATCACCACCAGGGATGTC   90
                                                                                  M   S     2

CGGGACTCAGCCCGAGGATGTTTCGCCTCCGTGCAGGGACTGGACGTCCTCTCCAGAGCTGAACGAAACGCGGGAGCCCTTTTTAAACCC  180
 G   T   Q   P   E   D   V   S   P   P   C   R   D   W   T   S   S   P   E   L   N   E   T   R   E   P   F   L   N   P    32

ATCCGCCGATTACGACGATGAGGAGTTTCTGCGCTACCTCTGGAAGGAGTATTTGCATCCCAAGGGATACGAATGGGCGCTGATCGCCGG  270
 S   A   D   Y   D   D   E   E   F   L   R   Y   L   W   K   E   Y   L   H   P   K   G   Y   E   W   A   L   I   A   G    62

ATACATCGTCGTGTTCATCGTGGCTCTCGTCGGGAACGTGCTGGTTTGTATTGCAGTGTGGAAGAACCATCACATGCGAACAGTCACCAA  360
 Y   I   V   V   F   I   V   A   L   V   G   N   V   L   V   C   I   A   V   W   K   N   H   H   M   R   T   V   T   N    92

CTATTTCATAGTGAATCTATCTCTGGCTGACATTCTGGTCACAATTACTTGTCTTCCAGCAACTTTAGTGGTGGACATCACAGAAACTTG  450
 Y   F   I   V   N   L   S   L   A   D   I   L   V   T   I   T   C   L   P   A   T   L   V   V   D   I   T   E   T   W   122

GTTCTTTGGGCACCACCTCTGCAAAGCAATTCCCTACTTACAGACAGTTTCAGTCTCTGTGTCTGTACTAACACTTAGCTGCATTGCTTT  540
 F   F   G   H   H   L   C   K   A   I   P   Y   L   Q   T   V   S   V   S   V   S   V   L   T   L   S   C   I   A   L   152

GGATCGATGGTATGCAATTTGTCACCCTTTGATGTTTAAAAGCACAGCCAAGCGTGCAAGGAACAGCATTATAATTATCTGGATTGTGTC  630
 D   R   W   Y   A   I   C   H   P   L   M   F   K   S   T   A   K   R   A   R   N   S   I   I   I   I   W   I   V   S   182

CTGCATCATAATGATTCCTCAGGCTATTGTTATGGAGTGCAGCAGCGTGTTCCCAGGATTAGCCAATAAAACCACCTTGTTCACAGTGTG  720
 C   I   I   M   I   P   Q   A   I   V   M   E   C   S   S   V   F   P   G   L   A   N   K   T   T   L   F   T   V   C   212

TGATGAACACTGGGGAGCTGAGGTTTACCCCAAAATGTATCATACATGCTTTTTCCTGGTGACGTACATGGCACCACTGTGTCTGATGGT  810
 D   E   H   W   G   A   E   V   Y   P   K   M   Y   H   T   C   F   F   L   V   T   Y   M   A   P   L   C   L   M   V   242

GTTGGCCTATTTGCAAATATTTCGCAAGCTGTGGTGTCGCCAGATCCCTGGAACTTCTTCTGTCGTTCAGAAAAAATGGAAGTCTCTGCA  900
 L   A   Y   L   Q   I   F   R   K   L   W   C   R   Q   I   P   G   T   S   S   V   V   Q   K   K   W   K   S   L   Q   272

GTCCTCAGCACAGCAACGAGGGCTGGGACAGTCAACAAAGTCAAAGATCAGTGCTGTGGCAGCTGAAATAAAACAGATTCGTGCAAGAAG  990
 S   S   A   Q   Q   R   G   L   G   Q   S   T   K   S   I   S   A   V   A   A   E   I   K   Q   I   R   A   R   R   302

GAAAACAGCACGTATGCTAATGGTTGTCCTCCTGGTTTTTGCACTTTGCTATCTGCCAATTAGCATCCTCAATATCTTGAAAGGGTTTT  1080
 K   T   A   R   M   L   M   V   V   L   L   V   F   A   L   C   Y   L   P   I   S   I   L   N   I   L   K   R   V   F   332

TGGGATGTTTAATCATGCTGATGACAGAGAAACTGTATATGCCTGGTTCACATTCTCACACTGGCTTGTATATGCAAACAGTGCAGCCAA  1070
 G   M   F   N   H   A   D   D   R   E   T   V   Y   A   W   F   T   F   S   H   W   L   V   Y   A   N   S   A   A   N   362

TCCTATTATTTATAACTTCCTCAGTGGGAAGTTTAGAGAAGAATTTAAAGCAGCGTTTTCTTGTTGCATCTTTGGTATTCACAGTCACCA  1260
 P   I   I   Y   N   F   L   S   G   K   F   R   E   E   F   K   A   A   F   S   C   C   I   F   G   I   H   S   H   H   392

TGATGAACGGCTTACAAGAGGTCGTGCCAGTACAGAGAGTCGGAAATCTTTGACCACCCAGATCAGCAATTTTGACAATGTTTCAAAACA  1350
 D   E   R   L   T   R   G   R   A   S   T   E   S   R   K   S   L   T   T   Q   I   S   N   F   D   N   V   S   K   H   422

TTCAGAACATGTCCTGCTGACCAACATAAACACACTTACGGCAAATGGTATTACAGCTACATTCAGCCCTTTGAAGTCAATGGAACTGCA  1440
 S   E   H   V   L   L   T   N   I   N   T   L   T   A   N   G   I   T   A   T   F   S   P   L   K   S   M   E   L   H   452

TCTCCACATACCAGGGGTGAACGTGTCTTCAAATTTAGATGAAGCAGTGAGAATTTCTGCAGGATGTACTGGCAACACAGAGAATGCAGA  1530
 L   H   I   P   G   V   N   V   S   S   N   L   D   E   A   V   R   I   S   A   G   C   T   G   N   T   E   N   A   E   482

ATGGGATAAATTTGTCCCTAGTGTAACTAAACTTACCTCAATGGAATTACAGCAAGGATGACTTTGGCTCAATACAGCTGAGAATGGCAT  1620
 W   D   K   F   V   P   S   V   T   K   L   T   S   M   E   L   Q   Q   G   *                               501

TTGTTTTTAGCTGTGTTACAGACTAGAATAACTGGACAAAAGGATTCCTGCAAAACGACTGCCTCTTCTACCTCTTCTCAGTCTTATGGA  1710

AACCTACTTACCTCAGTATGAGCTAAACAACGTCTTTACAAGTCTTTTTAAAGAATGGATTGTATCATACTTTGTAAATACTGTAGATAT  1800

TTATTTTTATATATTTTAGATGCTGTGGAATGTTCTAAAATGTACCATATAAAGAACTCTATGTATTTG                        1869
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mammals (Jöhren *et al.* 2001, Blanco *et al.* 2002, Näslund *et al.* 2002), and peripheral actions of orexins are also discussed (Kirchgessner 2002, Smart & Jerman 2002).

On the other hand, information on the orexin pathway and its actions are very limited in non-mammalian vertebrates. In teleost fish, central injection of human orexin peptides can stimulate food intake in goldfish (Volkoff *et al.* 1999, Volkoff & Peter 2001). Contrary to results demonstrated in other species, human orexins did not stimulate food intake in neonatal chicks (Furuse *et al.* 1999). In addition, we recently cloned prepro-orexin cDNA from chicken hypothalamus and described that food deprivation for 24 h did not alter orexin mRNA expression in the chicken hypothalamus (Ohkubo *et al.* 2002). In amphibians, the *Xenopus* orexin gene has been cloned and the peptides were bioactive in a human OXR *in vitro* functional assay; however, *in vivo* actions of orexin were not addressed (Shibahara *et al.* 1999). Furthermore, there is no available report describing cDNA cloning for OXR(s) in non-mammalian species, and the tissue distribution of the receptor would be invaluable for gaining insight into the physiological actions of orexin in non-mammalian vertebrates. At least in birds, data suggest that orexin is not involved in the regulation of food intake (Furuse *et al.* 1999, Ohkubo *et al.* 2002); thus alternative functions other than orexigenic actions should be clarified. To address this issue, we aimed to clone chicken OXR (cOXR) cDNA and reveal tissue distribution of the mRNA in both central and peripheral tissues in the chicken.

Materials and methods

Animals

White Leghorn chickens were purchased from a local hatchery and maintained under long-day conditions (14 h light/day) at an ambient temperature of 20–23 °C with free access to food and water. At 20 weeks of age, the chickens were killed by cervical dislocation and tissues were immediately removed and frozen in liquid nitrogen before RNA extraction. Animal procedures were performed according to the guidelines of the Experimental Animal Care Committee of Kagawa University.

Primers

The primers used in this study are listed in Table 1. Primers 1 and 2 for OXR were designed from the conserved nucleotide sequence in mammalian OXRs (Sakurai *et al.* 1998, Lin *et al.* 1999). Other primers were synthesised as specific primers to cOXR cDNA or chicken prepro-orexin cDNA.

cDNA cloning of cOXR by RT-PCR

Total RNA was extracted from chicken hypothalamus using a commercial kit (ISOGEN; Nippon Gene, Toyama, Japan) and poly (A)$^+$ RNA was isolated with Oligotex-dT30 (TaKaRa, Osaka, Japan). One microgram of poly (A)$^+$ RNA was used to prepare a cDNA library with a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, USA). A cDNA fragment for cOXR was amplified with primers 1 and 2 with TaKaRa Ex Taq (TaKaRa) by 30 cycles of PCR consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C and extension for 1 min at 72 °C. The 5′- and 3′-ends of the cOXR cDNA were amplified by rapid amplification of cDNA ends with the Marathon cDNA library according to the manufacturer's instruction with primers 3 (initial primer) and 4 (nested primer), and primers 5 (initial primer) and 6 (nested primer) respectively. All cDNA fragments were subcloned into pGEM-T easy vector (Promega, Madison, WI, USA) and both strands were sequenced with an Applied Biosystems 373A automated sequencer or ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Southern blot analysis for cOXR

Twenty micrograms chicken DNA were digested with BamHI, EcoRI and HindIII and electrophoresed on a 0·6% agarose gel and blotted onto a nylon membrane. The genomic DNA fragments on the membrane were hybridised to a ^{32}P-labelled

Figure 1 The nucleotide sequence of cOXR cDNA and the deduced amino acid sequence. Both strands were completely sequenced. The termination codon is marked with an asterisk. The nucleotide sequence data will appear in the EMBL/GenBank/DDBJ nucleotide databases with the accession number AB110634.

Figure 2 Multiple alignment of the amino acid sequences of OXRs from chicken, human, rat and dog. Sequences were aligned using Clustalx (Thompson *et al.* 1997). Conserved residues are shaded, and identical residues in all species are black-boxed.
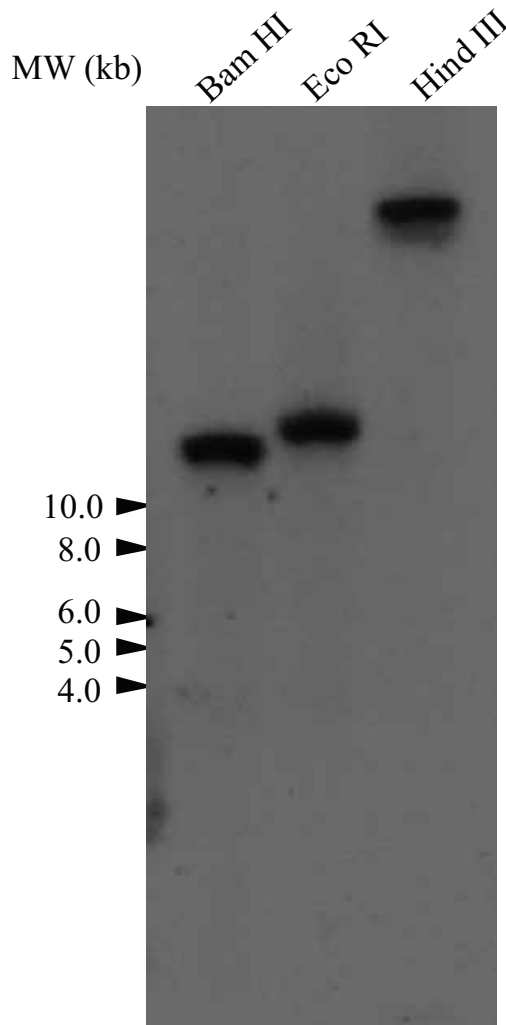
Figure 3 Southern blot analysis of the cOXR gene. DNA (20 µg) isolated from chicken testis was digested with BamHI, EcoRI and HindIII and electrophoresed on a 0·6% agarose gel. The DNA was transferred to a nylon membrane and hybridised with a ^{32}P-labelled cOXR cDNA probe, position from 494 to 565.

containing 0·1% SDS for 30 min at 37 °C, hybridised genomic DNA fragments were visualised by autoradiography.

RNase protection assay for cOXR mRNA

For RNA probe preparation, cDNA fragments for cOXR, position from 330 to 565, and chicken S17 cDNA (Trüeb *et al.* 1988), position from 273 to 387, were amplified by RT-PCR and cloned into pGEM-T easy vector. The antisense probes for cOXR and chicken S17 from the linealised plasmids were labelled with [α-^{32}P]CTP (Amersham Bioscience, Piscataway, NJ, USA) using T7 RNA polymerase (Promega).

Total RNA (20 µg) extracted from each chicken tissue was incubated with ^{32}P-labelled cOXR and S17 complementary RNA probes for 16 h at 50 °C in 30 µl hybridisation buffer containing 80% formamide, 400 mM NaCl, 1 mM EDTA (pH 8·0) and 40 mM PIPES–NaOH (pH 6·7, piperazine-N,N-bis-2-ethanesulphonic acid monosodium salt). Following digestion with RNases A (40 µg/ml) (Boehringer Mannheim) and T1 (2 µg/ml) (Boehringer Mannheim) for 30 min at 30 °C, the reaction mixture was treated with proteinase K (50 µg/ml) (Boehringer Mannheim, Tokyo, Japan) for 15 min at 37 °C. The samples were electrophoresed on a 6% polyacrylamide gel containing 7 M urea to separate the protected RNA fragments. The specifically protected bands by cOXR and S17 RNA probes were determined by a Fuji BAS 2000 imaging analyser (Fuji Photo Film Co, Tokyo, Japan).

Determination of prepro-orexin and OXR mRNAs in chicken gonads by RT-PCR

The RT-PCR conditions for OXR expression in chicken gonads were the same as described above, except using primers 7 and 8. For prepro-orexin amplification by RT-PCR, a cDNA fragment for chicken orexin was amplified with primers 1 and 2, and FastStart Taq DNA polymerase (Roche Diagnostics, Penzberg, Germany). After sample denaturation and activation of the DNA polymerase at 95 °C for 4 min, 35 cycles of PCR were carried out as follows: denaturation for 30 s at 95 °C, annealing for 30 s at 65 °C and extension for 1 min at 72 °C. Amplification was terminated by a 7 min final extension step at 72 °C. Amplified

cOXR cDNA probe, position from 494 to 565, that corresponds to the highly conserved sequence between OX1R and OX2R in mammals (Sakurai *et al.* 1998, Peyron *et al.* 2000) for 16 h at 37 °C in a buffer consisting of 50% formamide, 5 × Denhardt's solution (1 × Denhardt's solution; 0·2% BSA, 0·2% Ficoll 400 and 0·2% polyvinylpyrrolidone), 5 × SSPE (1 × SSPE; 0·15 M NaCl, 0·01 M NaH$_2$PO$_4$ and 1 mM EDTA), 0·2 mg/ml salmon sperm DNA and 0·1% SDS. After low-stringency washing with 2 × SSC (1 × SSC; 0·15 M NaCl and 0·015 M sodium citrate)
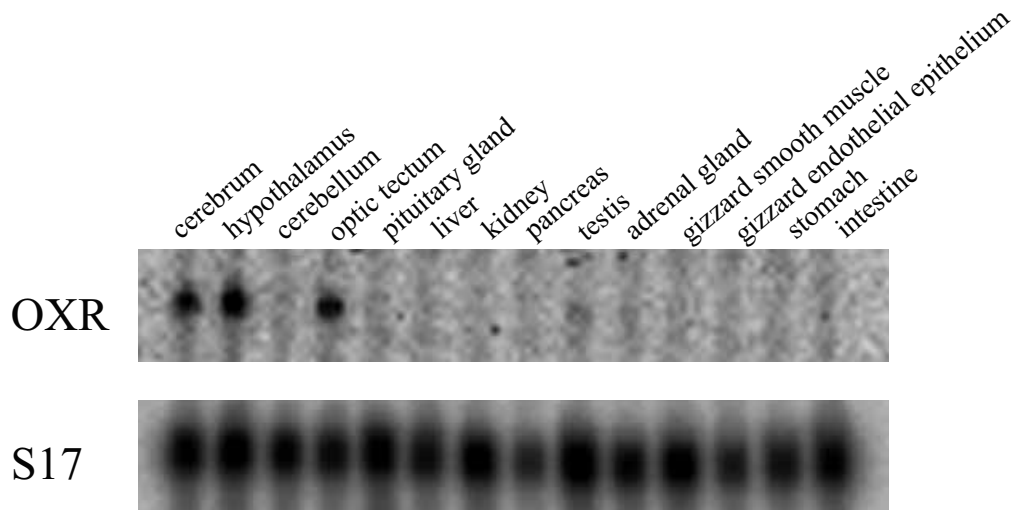
Figure 4 Tissue expression profiles of cOXR mRNA measured by RNase protection assay. Twenty micrograms total RNA from chicken tissues were subjected to hybridisation with antisense RNA probes for cOXR and S17. The data represent an autoradiogram from three independent experiments.

cDNAs for chicken prepro-orexin and OXR were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide to visualise the amplified bands.

Results

Structure of cOXR cDNA

The nucleotide and deduced amino acid sequences of cOXR are shown in Fig. 1. The cOXR cDNA is 1869 bp long and encodes 501 amino acids. Since cOXR showed approximately 80% similarity to OX2R, and approximately 70% identity with OX1R at the amino acid level (Fig. 2), the cOXR is predicted as a counterpart of OX2R reported in mammals.

Southern blot analysis of cOXR gene

Using a low-stringency condition for Southern blot analysis, a single fragment was hybridised with the radiolabelled cOXR cDNA probe in all restriction enzyme treatments (Fig. 3).

Determination of OXR mRNA expression in chicken tissues by RNase protection assay

OXR mRNA was widely distributed in chicken brain, and abundant expression was observed in the cerebrum, hypothalamus and optic tectum. In peripheral tissues, mRNA for OXR was detected in the pituitary gland, adrenal gland and testis in the chicken (Fig. 4).

Determination of prepro-orexin and OXR mRNAs in chicken gonads by RT-PCR

OXR mRNA was detected in chicken gonads of both sexes, but the amount of OXR mRNA in the ovary is lower than that in testis (Fig. 5B). In addition, prepro-orexin mRNA was also expressed in chicken gonads, and the expression level was equivalent in the testis and ovary (Fig. 5A).

Discussion

This is the first report of the structure of the OXR in non-mammalian vertebrates. The cOXR showed high similarity with OX2R in mammals (Fig. 2), and phylogenetic analysis placed cOXR in the cluster of OX2R (Fig. 6). Thus, the cloned cOXR is likely to be the analogue of OX2R in mammals. However, low-stringency Southern blot analysis did not provide any evidence that a gene encoding OX1R exists in the chicken genome (Fig. 3). This result raises two possibilities: that there is no OX1R in the chicken or chicken OX1R is sufficiently different from the cOXR and failed to
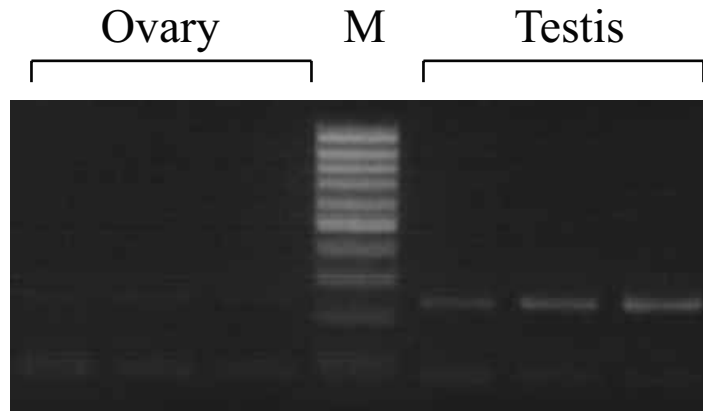
(A) Orexin
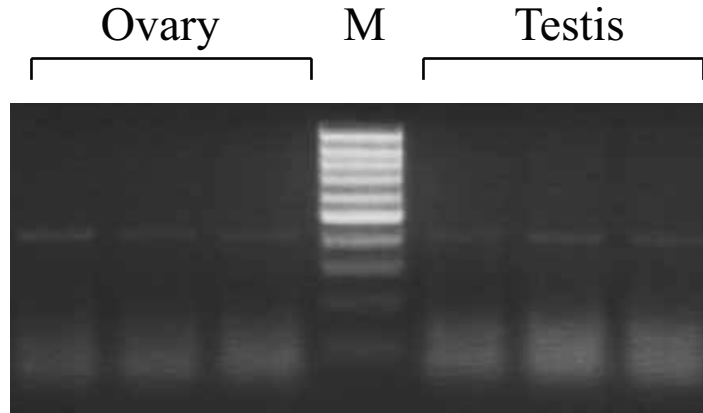
(B) OXR

Figure 5 Semiquantitative analysis of chicken prepro-orexin (A) and OXR (B) mRNAs in chicken gonads. Each fragment was amplified with appropriate PCR conditions and primers as described under Materials and methods. Amplified cDNAs for chicken orexin and OXR were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide to visualise the amplified bands. M, DNA size marker (100 bp DNA ladder, SIGMA Genosys, Tokyo, Japan).

cross-hybridise to the cOXR probe used for Southern analysis in this study. It is reported that OX2R binds orexin-A and -B with an equal affinity, while OX1R binds orexin-A with an affinity 10 times higher than for orexin-B (Sakurai *et al.* 1998). Furthermore, orexin-A is recognised as a more potent orexigenic peptide than orexin-B in mammals (Dube *et al.* 1999, Ida *et al.* 1999, Yamada *et al.* 2000). Although, strong evolutionary conservation of amino acid sequences of mature

orexin-A and -B are observed in different vertebrate classes including chicken (Sakurai *et al.* 1998, Dyer *et al.* 1999, Shibahara *et al.* 1999, Ohkubo *et al.* 2002), it is still difficult to explain why orexins do not stimulate food intake in birds. The absence of a functional OX1R may provide an explanation as to why orexins do not stimulate food intake in the chicken.

cOXR was distributed widely in chicken brain and abundant expression was observed in the
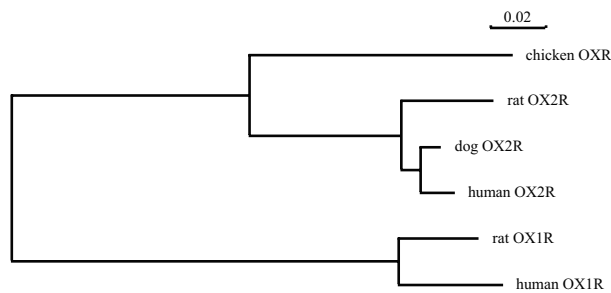
Figure 6 Phylogenetic analysis tree of vertebrate OXRs. The tree was constructed by the neighbour-joining method using a distance matrix based on amino acid sequences. Horizontal lines indicate genetic distance. The sequences were obtained from EMBL/GenBank Data Bank: human OX1R and OX2R (AF041243 and AF041245 respectively), rat OX1R and OX2R (AF041244 and AF041246 respectively) and dog OX2R (AF164626).

cerebrum, hypothalamus and optic tectum (Fig. 4). Localisation of OXRs in the central nervous system has been investigated with molecular-biological and immunological methods in the rat. OXRs are found at the projection sites of orexinergic neuron and in the many hypothalamic nuclei (Trivedi *et al.* 1998, Date *et al.* 2000, Marcus *et al.* 2001). In addition, our previous study showed that localisation of orexin-producing cells was restricted to the hypothalamus in the chicken (Ohkubo *et al.* 2002), in common with observations in mammals and amphibians (de Lecea & Sutcliffe 1999, Sakurai 1999, Shibahara *et al.* 1999, Galas *et al.* 2001). Consequently, orexins exert multiple functions via hypothalamic and other brain receptors in the chicken. It is noteworthy that the optic tectum expresses high levels of OXR in the chicken. In the chicken, it has been reported that light stimulation modulates the γ-aminobutyric acid (GABA) system (Nistico *et al.* 1979) and experimental lighting circumstances affect the postnatal development of GABA receptor sites in the optic tectum (Rios *et al.* 1987). In the rat, orexins are suggested to excite GABAergic neurons via OX2R and that leads to maintenance of the arousal state (Martin *et al.* 2002, Wu *et al.* 2002, Korotkova *et al.* 2003). Taken together, chicken orexins may be involved in the visual processing mechanism, which might relate to the control of the sleep–wake cycle in the chicken.

Contrary to the situation in the brain, OXR expression in peripheral tissues was selective. Three out of nine tissues examined expressed OXR

(Fig. 4). It has been shown that i.c.v.-injected orexins increase plasma adrenocorticotrophin and corticosterone levels and reduce plasma prolactin and growth hormone levels (Hagan *et al.* 1999, Ida *et al.* 2000, Kuru *et al.* 2000). Also there is evidence that OX2R produced in the human adrenal cortex increases cAMP and inositol trisphosphate *in vitro* (Randeva *et al.* 2001). Consequently, the occurrence of OXRs in the pituitary and adrenal glands may suggest that the orexins, at least in part, play roles in these organs that are similar to those observed in mammals. However, whereas cOXR is likely to be a counterpart of OX2R in mammals (Figs 2 and 6), the expression profile of cOXR was slightly different from that of rat OX2R. For example, high levels of OX2R are expressed in rat adrenal glands, whereas cOXR expression was very faint and is similar to levels of rat OX1R in the adrenal glands (Jöhren *et al.* 2001). In the testis, OXR was abundantly expressed in the chicken, whereas in the rat testis only OX1R was expressed (Jöhren *et al.* 2001). This may indicate that the orexin–OXR pathway in the chicken is different from that of the rat or other mammals. Moreover, we have carried out semiquantitative RT-PCR analysis for orexin and OXR in the gonads of both sexes (Fig. 5). Interestingly, we have found much higher levels of OXR mRNA in the testis when compared with that in the ovary (Fig. 5A). This result suggests that sex steroid hormones may differentially regulate gonadal OXR expression in the chicken. This view is supported by the observation that the sexually dimorphic expression of OXRs observed in rat pituitary and adrenal glands was critically regulated by gonadal steroids (Jöhren *et al.* 2003). For instance, the mRNA levels for OX1R in the pituitary gland and OX2R in the adrenal gland were higher in male rats compared with female rats. These receptors were down-regulated by oestrogen in the female rat, while testosterone down- and up-regulates pituitary OX1R and adrenal OX2R expression in male rats respectively (Jöhren *et al.* 2003). Although we described a gender difference in OXR expression in chicken gonads and prepro-orexin mRNA is expressed in the testis and ovary, a prominent role of orexin in testicular or ovarian function is not clarified in any species. Consistent with our observation in this study, orexin mRNA is found in rat testis (Sakurai *et al.* 1998, Jöhren *et al.* 2001), and furthermore, at least in the rat, the mRNA is

translated (Mitsuma *et al.* 2000*a,b*). It is notable that OXR and prepro-orexin are expressed in the ovary as well as in the testis of the chicken (Fig. 5). However, there are no reports of the functional relationship between orexin and the ovary in mammals. Ovarian OXRs have not been confirmed in any mammal, and no prepro-orexin mRNA was expressed in rat (Jöhren *et al.* 2001). In relation to orexin and gonadal actions, it is reported that orexins modulate luteinising hormone (LH) release from rat pituitary gland in an ovarian steroid-dependent manner (Pu *et al.* 1998). Orexin A suppresses LH-releasing hormone (LHRH)-stimulated LH release in dispersed anterior pituitaries from males and proestrous females, and hypothalamic responsiveness to orexin A on LHRH release is different in the stages of the oestrous cycle (Russell *et al.* 2001). These results strongly indicate that the steroid hormone state is responsible for orexin actions on the hypothalamo–pituitary gonadal axis. Therefore, it would be possible that orexin directly regulates gonadal functions. In the present study, we found expression of prepro-orexin and OXR mRNAs in the gonads of both sexes in chickens, which may suggest that orexins are involved in the control of gonadal function by a paracrine or endocrine manner in the chicken. Therefore, further studies of the functional relevance of gonadal orexin(s) are required. In addition, evidence as to whether or not other subtypes of OXR exist, and the localisation of OXR(s) in the central and peripheral tissues, will help us to understand neuroendocrine and endocrine actions of orexins in the chicken.

Acknowledgements

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