Orexin system is expressed in avian muscle cells and regulates mitochondrial dynamics

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Lassiter K, Greene E, Piekarski A, Faulkner OB, Hargis BM, Bottje W, Dridi S. Orexin system is expressed in avian muscle cells and regulates mitochondrial dynamics. Am J Physiol Regul Integr Comp Physiol 308: R173–R187, 2015. First published December 10, 2014; doi:10.1152/ajpregu.00394.2014.—Orexin A and B, orexigenic peptides produced primarily by the lateral hypothalamic area that signal through two G protein-coupled receptors, orexin receptors 1/2, have been implicated in the regulation of several physiological processes in mammals. In avian (nonmammalian vertebrates) species; however, the physiological roles of orexin are not well defined. Here, we provide novel evidence that not only is orexin and its related receptors 1/2 (ORXR1/2) expressed in chicken muscle tissue and quail muscle (QM7) cell line, orexin appears to be a secretory protein in QM7 cells. In vitro administration of recombinant orexin A and B (rORX-A and B) differentially regulated prepro-orexin expression in a dose-dependent manner with up-regulation for rORX-A (P < 0.05) and down-regulation for rORX-B (P < 0.05) in QM7 cells. While both peptides upregulated ORXR1 expression, only a high dose of rORX-B decreased the expression of ORXR2 (P < 0.05). The presence of orexin and its related receptors and the regulation of its own system in avian muscle cells indicate that orexin may have autocrine, paracrine, and/or endocrine roles. rORXs differentially regulated mitochondrial dynamics network. While rORX-A significantly induced the expression of mitochondrial fission-related genes (DNM1, MTPF1, MTFR1), rORX-B increased the expression of mitofusin 2, OPA1, and OMA1 genes that are involved in mitochondrial fusion. Concomitant with these changes, rORXs differentially regulated the expression of several mitochondrial metabolic genes (av-UCP, av-AKT, Ski, and NRF-1) and their related transcriptional regulators (PPARγ, PPARα, PGC-1α, PGC-1β, and FoxO-1) without affecting ATP synthesis. Taken together, our data represent the first evidence of the presence and secretion of orexin system in the muscle of nonmammalian species and its role in mitochondrial fusion and fission, probably through mitochondrial-related genes and their related transcription factors.

orexin system; muscle; mitochondrial dynamics; fusion; fission; cellular bioenergetics; gene expression

OREXINS (ORX-A AND ORX-B) (56), also referred to as hypocretin 1 and 2 (21), are two peptides proteolytically derived from a single precursor (prepro-orexin) produced mainly in the dorsal and lateral hypothalamic areas and perifornical nucleus in rat and humans (21, 56). Prepro-orexin mRNA was detected also in rat testis (56) and duodenum (47). Recently, immunoreactivity for ORX-A was detected in several human peripheral tissues, including ganglion cells of the thoracic sympathetic trunk, endocrine cells of the gastrointestinal tract, islets cells of the pancreas, and syncytiotrophoblasts and decidual cells of the placenta (46).

Orexins signal through two ubiquitously expressed G protein-coupled receptors: orexin receptor 1 (ORXR1) and orexin receptor 2 (ORXR2) (8, 25, 31, 35, 42, 56) and regulate several physiological processes in mammals. Consistent with this, central administration of orexins increases food and water intake in mammals (55). Other central effects of orexins include control of wakefulness (14, 41, 44), circadian clock (6), energy and glucose homeostasis (71–73), lipid metabolism (62, 63), heart rate and blood pressure (19, 78), and neuroendocrine response to stress (57) have been reported. As orexins and its receptors are not restricted to the hypothalamus, these aforementioned pleiotropic actions of orexins might be also mediated through a direct interaction with peripheral target tissues (20, 26, 31).

In contrast to mammals, little is known about orexin system distribution and functions in avian (nonmammalian vertebrates) species, and such studies are very limited. Ohkubo et al. (48) were the first to clone the chicken prepro-orexin, which is highly conserved among vertebrates and found that it was expressed in periventricular and lateral hypothalamic areas. In a subsequent study, the same group characterized the chicken orexin receptor and found that it corresponded to the type 2 mammalian orexin receptor (49). Both orexin and its receptor were expressed in chicken brain and gonads (testis and ovary) (49). Central administration of orexin did not affect feed intake in neonatal chickens (24), and its effects on sleep/wakefulness cycle is still controversial (33, 34, 45). The localization of orexins and its receptors in chicken peripheral tissues, such as testis, ovary, stomach, and intestine (3, 49), indicates that orexin system may have other physiological functions rather than the regulation of feeding behavior.

Orexin has been recently shown to regulate mitochondrial biogenesis and induce brown adipose tissue (BAT) differentiation and thermogenesis in mammals (61, 66). Mitochondria are dynamic organelles that constantly fuse and divide, and an imbalance of these two processes dramatically alters mitochondrial morphology and function (15). The molecular mechanism that controls mitochondrial dynamics and biogenesis is a complex network requiring the participation and coordination of the nuclear and mitochondrial genomes. This network has been partially unraveled in mammals after the identification of some of the genes responsible for mitochondrial fusion [mitofusins (MFN1 and MFN2), and optic atrophy 1 (OPA1)], fission [dynamin-related protein 1 (Drp1 or DN1)], fission 1 (FIS1), and mitochondrial protein 18 kDa], and biogenesis [peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) and nuclear respiratory factor 1 (NRF1)]. Such mitochondrial network and its integration are still unknown in avian species.
As chickens do not have functional BAT, we undertook the present study to investigate whether orexin system is expressed in the avian muscle, the main site for thermogenesis, and its potential role in the regulation of muscle mitochondrial dynamics, biogenesis, and function.

MATERIALS AND METHODS

**Animals.** One-day-old male and female Cobb-500 broiler chickens were reared on floor pen in a controlled environment room with ad libitum access to food (12.6 MJ/kg, 22% protein) and clean water until 3 wk of age. The ambient temperature was reduced gradually from 32

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*Accession numbers refer to GenBank (National Center for Biotechnology Information).
to 26°C at 21 days of age and the relative humidity was 55 ± 5%. Birds (n = 4 for each sex) were killed by cervical dislocation, and leg muscles, whole brain, hypothalamus, liver, ovary and testis were quickly removed and snap frozen in liquid nitrogen and stored at −80°C until use. Animal care and housing and all procedures were approved by the University of Arkansas Animal Care and Use Committee under protocol (no. 13039).

Quail muscle (QM7) cell culture and treatments. QM7 cells were grown in M199 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 10% tryptophosphate (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Biobasic, Amherst, NY) at 37°C under a humidified atmosphere of 5% CO2 and 95% air. At 80–90% confluence, cells were synchronized overnight in serum-free medium and treated with human recombinant orexin A or B (0, 10 and 100 nM) (Interchem, Montluçon, France) for 24 h. Untreated cells were used as control. The dose and duration of treatments were chosen on the basis of pilot and previous published experiments (12, 62, 63). QM7 cells were washed twice with phosphate-buffered saline (PBS 1×) and incubated for 1 min, with a final extension at 72°C for 10 min. The amplified fragments were separated on a low-melting point agarose gel (1%), and the appropriate bands were cut out, purified by using spin-column DNA gel extraction kit (Biobasic, Amherst, NY), and stored at −20°C.

Reverse transcription and quantitative real-time PCR. Nuclear, cytoplasmic, and total RNA were isolated, as previously described (30), with some modifications. Briefly, cells were rinsed twice and harvested in 5 ml of ice-cold PBS and centrifuged [1,000 relative centrifugal force (RCF), 4°C, 5 min]. Cells were resuspended in lysis buffer A [10 mM Tris (pH 8.0), 140 mM NaCl, 1.5 mM MgCl2, 0.5% NP-40, 2 mM vanadyl ribonucleoside complex (VRC; Sigma-Aldrich)] and incubated on ice for 5 min. One-fifth of the lysate was used for total RNA isolation. The rest was centrifuged (1,000 RCF, 4°C, 3 min) to pellet the nuclei. The nuclear pellet was washed twice and then resuspended in the lysis buffer A. All RNA fractions (total, cytoplasmic, and nuclear) were extracted by TRIzol reagent (Life Technologies) according to the manufacturer’s recommendations, DNase-treated, and reverse-transcribed (Quanta Biosciences, Gaithersburg, MD). RNA integrity and quality were assessed using 1% agarose gel electrophoresis, and RNA concentrations and purity were determined for each sample by Take 3 microvolume plate using Synergy HT multimode microplate reader (BioTek, Winooski, VT). The RT products (cDNAs) were amplified by real-time quantitative PCR (Applied Biosystems, 7500 real-time PCR system) with Power SYBR Green Master Mix. Oligonucleotide primers used for chicken prepro-orexin (ORX), ORXR1, ORXR2, uncoupling protein (av-UCP), adenosine nucleotide translocator 1(ANT1), the nuclear sarcoma viral oncogene homolog (Ski), NRF-1, peroxisome proliferator-activated receptor alpha and gamma (PPARα and PPARγ), forkhead box protein O1 (FoxO-1), peroxisome proliferator-activated receptor gamma coactivator 1α and β (PGC-1α and PGC-1β), OxPhos Complex IV subunit I, Cox 5α, mitochondrial single-stranded DNA binding protein 1 (mtSSBP1), mitochondrial transcription factor A (TFAM), MFN1 and MFN2, dynamin-related protein 1 (DNML or Drp1), OPA1, OMA1 zinc metallopeptidase (OMA1), mitochondrial fission process 1 (MTPF1), mitochondrial fission regulator 1 (MITFR1), mitochondrial fission factor 1, and the housekeeping gene ribosomal 18S are summarized in Table 1. The quantitative PCR (qPCR) cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by agarose gel and showed only one specific band of the predicted size. For negative controls, no RT products were used as templates in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were determined by the 2−ΔΔCt method (60).

Reverse transcription and conventional PCR. Total RNA was reverse transcribed as described in the previous section. Long fragments of ORX (411 bp), ORXR1 (832 bp), and 18S (515 bp) were amplified by PCR using oligonucleotide primers specific for chicken ORX, ORXR1, and 18S (Table 1). PCR was performed in 50 µl containing 5 µl of the RT reaction, 1 µl of forward and reverse primer, and 43 µl of platinum PCR SuperMix (Life Technologies). Thermal cycling parameters were as follows: 1 cycle of 94°C for 4 min, followed by 39 cycles of 94°C for 30 s, 50–55°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified fragments were separated on a low-melting point agarose gel (1%), and the appropriate bands were cut out, purified by using spin-column DNA gel extraction kit (Biobasic, Amherst, NY), and stored at −20°C.
Vacuum Blotting System, GE Healthcare Bio-Sciences). The RNAs were crosslinked to the membranes by ultraviolet irradiation and baked at 80°C for 20–30 min. Membranes were hybridized with biotin-labeled DNA probes (prepro-orexin or 18S) at 42°C overnight. On the following day, the membranes were rinsed twice with 1×SSC, 0.1% SDS at 55°C. Each wash was for 20 min, and then the signals were detected by using the chemiluminescent nucleic acid detection kit (Pierce Thermo Scientific, Rockford, IL) and the FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA).

**Western blot analysis.** Muscle tissues and QM7 cells were homogenized in lysis buffer (10 mM Tris base, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 0.5% NP-40, protease, and...
phosphatase inhibitor cocktail). Protein concentrations were determined using Synergy HT multimode microplate reader (BioTek, Winooski, VT) and a Bradford assay kit (Bio-Rad, Hercules, CA) with BSA as a standard. Proteins (70 μg) were run on 4–12% Novex Bis-Tris gels (Life Technologies). The transferred membranes were blocked for 1 h at room temperature, and incubated with primary antibodies (diluted 1:500–1:1,000) at 4°C overnight. The polyclonal antibodies used were as follows: rabbit anti-mouse ORX, rabbit anti-rat ORXR1 and ORXR2, rabbit anti-ANT1, rabbit anti-PGC-1α, rabbit anti-MFN1, rabbit anti-MFN2, and rabbit anti-OPA1. Protein loading was assessed by immunoblotting using rabbit anti-β actin or rabbit anti-vinculin. Prestained molecular weight marker (precision plus protein Dual color) was used as a standard (Bio-Rad, Hercules, CA). All of the primary antibodies were purchased from (Interchim, Montlucon, France) except for anti-β actin from Cell Signaling Technology (Danvers, MA), anti-vinculin from Sigma-Aldrich and anti-ANT1 from Pierce Thermo Scientific (Rockford, IL). Anti-PGC-1α, anti-MFN1, anti-MFN2, and anti-OPA1 were from Dr. Nicholas Greene (University of Arkansas). The secondary antibodies were used (1:5,000) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence (ECL plus) (GE Healthcare Bio-Sciences) and captured by FluorChem M MultiFluor System (Protein-Simple). Image acquisition and analysis were performed by AlphaView software (version 3.4.0, 1993–2011; Proteinsimple).

Immunofluorescence. Immunofluorescence was performed as previously described (23). Briefly, cells were grown to 50–60% confluence in chamber slides (Lab-Tek, Hatfield, PA) and fixed in methanol for 10 min at −20°C. Cells were blocked with protein block serum-free blocking buffer (Dako, Carpinteria, CA), and incubated with rabbit anti-ORX, anti-ORXR1, or anti-ORXR2 antibody (1:200; Interchim, Montlucon France) overnight at 4°C and visualized with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Life Technologies). After DAPI counterstaining, slides were cover-slipped in Vectashield (Vector Laboratories, Burlingame, CA). Images were obtained using the Zeiss Imager M2 with a 20× Plan-APoCHROMAT 20×/0.8 objective and a 100× EC PLAN-NEOFLUOR 100×/1.3 oil objective. The Alexa Fluor 488 fluorophore was observed through filter set 38 1031–346 with an excitation of BP 470/40, beamsplitter of FT 495, and emission spectrum of BP 525/50. Differential interference contrast images were collected using DIC M27 condensers. The Alexa Fluor 488 fluorophore was excited for 500 ms prior to capturing each image using an Axio Cam MR3 camera. All analysis was performed using AxioVision SE64 4.9.1 SP1 software (Carl Zeiss Microscopy 2006–2013).

Analysis of cellular bioenergetics. Analysis of cellular bioenergetics was conducted using the XF24 extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). Cells were plated into each well of a 24-well Seahorse cell culture plate and allowed to attach. Once the cells were attached, the growth media listed in the previous section were exchanged for fasting (no FBS) media for overnight incubation. Cells were treated then with rORX-A or rORX-B (10 and 100 nM, Interchim, Montlucon, France) for 24 h. Untreated cells were treated with 24-well Seahorse cell culture plate and allowed to attach. Once the cells were attached, the growth media listed in the previous section were exchanged for fasting (no FBS) media for overnight incubation. Cells were treated then with rORX-A or rORX-B (10 and 100 nM, Interchim, Montlucon, France) for 24 h. Untreated cells were

Fig. 3. Secretion of orexin by QM7 cells. A: cell monolayers were incubated in serum-free medium with or without rORX-B (100 nM) for 24 h. B: QM7 cells were incubated in serum-free medium for different time periods. C: cells were incubated in serum-free medium with or without brefeldin A (0.3 μg/ml) for 12 h. Medium and/or cell lysates were subjected to immunoblot analysis using anti-orexin antibody, as described in MATERIALS AND METHODS.
used as a control. The following day, the cell culture media were changed to XF Assay Media (unbuffered DMEM containing 25 mM glucose and 10 mM sodium pyruvate; Seahorse Biosciences, North Billerica, MA) for the XF bioenergetics analysis. Cellular bioenergetics analysis was conducted by first measuring the baseline oxygen consumption rate (OCR), followed by measuring the OCR after sequential addition of chemical mitochondrial effectors; oligomycin (inhibitor of ATP synthesis by blocking the proton channel of the F0 portion ATP synthase, complex V), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, FCCP (uncoupler), and antimycin A (blocker of O2 consumption and inhibitor of complex III). All three chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Pilot studies were conducted to optimize QM7 cell seeding density (25,000–100,000 cells/well) and concentrations of oligomycin (1.5 μM), FCCP (250 nM), and antimycin A (10 μM) prior to assessing bioenergetics response to orexin treatments. Nonmitochondrial OCR values for each well were subtracted from basal OCR, OCR following oligomycin, and OCR following FCCP in the same well and bioenergetics components, including oxygen consumption due to ATP synthesis, mitochondrial oxygen reserve capacity, and proton leak were determined, as previously described (28, 39).

Mitochondrial biogenesis and DNA quantitation. After orexin treatments for 24 h, mitochondrial content of QM7 cells was determined by measuring mitochondrial DNA levels and mitochondrial mass with MitoTracker Red CMXRos (Life Technologies). MitoTracker Red CMXRos is a cell-permeable mitochondrion-selective dye that has been used for mitochondrial mass measurement (61). QM7 cells were washed twice with PBS and stained with 75 nM MitoTracker Red CMXRos for 15 min at 37°C, and fluorescence intensity was detected following the protocol provided by the manufacturer. DNA was extracted using the EZ-10 spin column genomic DNA kit (Biobasic, Amherst, NY), and the expression of the chicken mtDNA was measured by real-time qPCR in the presence of the oligonucleotide primers summarized in Table 1. The qPCR conditions and parameters are described above.

RESULTS

Both orexin and its related receptors are expressed in avian muscle tissue and cells. Using RT-PCR, a 411 bp and 832 bp partial ORX and ORXR1 cDNA corresponding to nucleotides 51–462 (GenBank accession no. AB056748) and 335–1145 (GenBank accession no. NM_001024584), respectively, were detected in the total RNA extracted from 3 wk-old male broiler chicken leg muscle (Fig. 1A) and quail muscle (QM7) cell lines (Fig. 2A). The same bands (size and sequences) were observed in the positive controls (brain, testis and ovary) (Figs. 1A and 2A). The use of negative control (water instead of cDNA or RNA that was not reverse transcribed) did not produce any PCR product confirming the absence of genomic DNA contamination (Figs. 1A and 2A). Northern blot analysis revealed a single prepro-orexin mRNA signal (~3.1 kb) in both brain and QM7 cell (Fig. 2C). Next, using a rabbit anti-mouse prepro-orexin and a rabbit anti-rat orexin receptor 1 and 2 antibodies, Western blot analyses showed bands of 16, 48, and 51 kDa in chicken muscle and QM7 cells corresponding to prepro-orexin, ORXR1, and ORXR2, respectively (Figs. 1B and 2B). These molecular weights were observed in the positive controls (whole brain and hypothalamus) and correspond to those of prepro-orexin and ORXR1/2 found in mammalian brain and chicken gastroenteric tract. Immunofluorescence staining demonstrated abundant immunoreactivity for orexin

Statistical analyses. Data were analyzed by one-factor ANOVA. Significant differences among individual group means were determined with Student-Newman-Keuls (SNK)’s multiple range test using the GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla, CA). Significance was set at $P < 0.05$. Data are expressed as the means ± SE.

Fig. 4. Effect of orexin treatment on orexin system expression in QM7 cells. Cells were treated with 0 (control), 10, or 100 nM of recombinant orexin A and B for 24 h. Total RNA and protein were isolated as described in MATERIALS AND METHODS. Relative expression of ORX and ORXR1/2 was determined by qPCR using 2^{-ΔΔCt} method (A, C). Protein levels were measured by Western blot analysis (B, D). Data are expressed as means ± SE ($n = 6$). *Significant difference between orexin-treated and control cells ($P < 0.05$).
and its receptors in the cytoplasmic compartment of QM7 cells, which has been confirmed by Western blot using cell fractions (Fig. 2, D–F). When the primary antibodies were replaced by isotype antibodies or nonimmune PBS solution, no reactivity could be observed (data not shown).

**Secretion of orexin in QM7 cells.** The first 33 amino acids of prepro-orexin exhibit characteristics of a secretory signal sequence (hydrophobic core followed by residues with small polar side chains) in combination with its presence in the circulation indicate that orexin is a secretory protein (4). To ascertain whether orexin is, in fact, secreted, QM7 cell monolayers were incubated in serum-free medium for 24 h, after which orexin levels in lysates (Fig. 2B) and medium (Fig. 3A) were assessed by SDS-PAGE and immunoblot analysis with antibody against prepro-orexin. Orexin was detected in both the cell lysate (Fig. 2B) and the medium from untreated or orexin-treated cells (positive control) (Fig. 3A). Consistent with the predicted molecular weight of orexin, the immunoreactive protein exhibited a mobility by SDS-PAGE corresponding to molecular mass of ~16 KDa (Fig. 3A). It is, however, noteworthy that a second band with higher molecular weight (~230 kDa) was detected in the medium and not in the cell lysate. Furthermore, the time course study of orexin secretion in QM7 cells showed that orexin steadily accumulated in the medium over a 48 h period (Fig. 3B). Treatment of cells with brefeldin A, an inhibitor of translocation of secretory proteins from the endoplasmic reticulum to the Golgi apparatus (36), for 12 h blocked secretion of orexin into the medium leading to increased intracellular expression (Fig. 3C).

**Orexins regulate the expression of its own system in QM7 cells.** The effects of 10 and 100 nM of recombinant human orexin A (rORX-A) or B (rORX-B) on the expression (mRNA and protein) of ORX and its related receptors (ORXR1 and ORXR2) in QM7 cells are shown in Fig. 4. Treatment of QM7 cells for 24 h with either 10 or 100 nM rORX-A upregulated ORX and ORXR1, but not ORXR2, gene expression (P < 0.05; Fig. 4A). Treating cells with rORX-B downregulated ORX and ORXR2 and increased ORXR1 mRNA levels (P < 0.05 Fig. 4C). ORX, ORXR1, and ORXR2 proteins showed the same expression patterns as their corresponding genes (Fig. 4, B and D). These effects seemed to be dose-dependent.

**Orexins differentially regulate mitochondrial-related genes and their transcriptional regulators in QM7 cells.** The rORX-A and rORX-B treatments had differential effects on av-UCP, av-ANT, Ski, and NRF-1 expression, as shown in Fig. 5. Whereas rORX-A had no effect on av-UCP mRNA abundance (Fig. 5A), it downregulated the expression of av-ANT (mRNA and protein levels), Ski, and NRF-1 in a dose-dependent manner (Fig. 5, B–D). rORX-B, however, downregulated the expression of av-UCP and increased the expression of Ski and NRF-1 without altering the expression of av-ANT (Fig. 5, A–D). These changes coincided with a significant downregulation of PPARγ, PPARα, and FoxO-1 expression by both doses of rORX-A (Fig. 6, A, B, and E). A high dose of rORX-A

![Fig. 5. Effect of orexin treatment on mitochondrial-related genes in QM7 cells.](http://ajpregu.physiology.org/)
significantly downregulated the expression of PGC-1β, and both doses did not alter PGC-1α mRNA abundance (Fig. 6, C and D). rORX-B, however, induced the expression of these transcription factors in a dose-dependent manner, but the effects were statistically significant only for PGC-1β, PGC-1α, and FoxO-1 with the high dose (Fig. 6, C, D–E).

Orexins regulate mitochondrial bioenergetics in QM7 cells. Bioenergetics in QM7 cells treated with 10 and 100 nM of rORX-A and rORX-B were assessed by monitoring basal oxygen consumption rate (OCR) followed by sequential treatment of cells with oligomycin, FCCP, and antimycin A as shown in Fig. 7A. As described previously (27), the decrease in OCR following oligomycin (which blocks ATP synthase) reveals OCR attributed to ATP synthesis activity. Maximal OCR is revealed in response to the uncoupling compound FCCP, and the difference between maximal OCR and basal OCR (prior to oligomycin) represents mitochondrial oxygen reserve capacity that cells can draw upon when increased energy production is needed. Oxygen consumption that remains following treatment with antimycin A is attributed to nonmitochondrial OCR (i.e., OCR due to activities other than nonmitochondrial c oxidase activity, such as mitochondrial reactive oxygen species production, oxidase activities, etc.). The amount of OCR attributed to proton leak is determined by the difference between oligomycin and antimycin A-inhibited OCR. When the nonmitochondrial component of cellular OCR was subtracted and by setting maximal OCR following FCCP at 100%, the effects of ORX-A and ORX-B on ATP synthesis, reserve capacity, and proton leak were determined and are presented in Fig. 7, B–D. ATP synthesis was slightly elevated by both orexins, but the effect was not statistically discernable (Fig. 7B). Analysis of reserve capacity indicated no effect of both doses of rORX-A and rORX-B (Fig. 7C); however, proton leak was decreased by 10 nM of rORX-A, and by 100 nM of rORX-B (P < 0.05, Fig. 7D).
Orexins differentially regulate mitochondrial biogenesis in QM7 cells. Mitochondrial DNA (mtDNA) replication and quantitation are a necessary component of mitochondrial biogenesis. MtDNA and mtSSBP1 expressions were measured in QM7 cells treated with orexins as shown in Fig. 9. In contrast to rORX-A, in which both doses significantly downregulated mtDNA and upregulated mtSSBP1 expression, rORX-B (high dose) significantly increased mtDNA expression without affecting mtSSBP1 levels (Fig. 9, A and B). Consistent with these observations and in contrast to rORX-A, rORX-B increased mitochondrial content as visualized with MitoTracker Red probe (Fig. 8). Neither rORX-A nor rORX-B affected the expression of the mitochondrial transcription factor TFAM (data not shown). The expression of Cox IV and Cox 5a genes, commonly used markers for mitochondrial mass and biogenesis, was determined. The high dose (100 nM) of rORX-A decreased Cox IV gene expression; however, the high-dose of rORX-B increased Cox IV and Cox 5a mRNA levels compared with untreated cells (P < 0.05, Fig. 9, C and D).

Orexins differentially regulate mitochondrial dynamics in QM7 cells. The expression of four genes related to mitochondrial fusion and three genes related to mitochondrial fission were measured as shown in Fig. 10. Recombinant ORX-B at high dose significantly induced the expression of MFN2, OPA1, and OMA1, but decreased the mRNA levels of MFN1 (Fig. 10, A–D). The same effect was observed at the protein levels (Fig. 10E). However, rORX-A significantly downregulated the expression of MFN1 and OMA1 with both doses, and OPA1 with the high dose, but did not affect that of MNF2 (Fig. 10, A–D). Interestingly, and in contrast to rORX-B, where no significant effects were observed, rORX-A upregulated the expression of mitofission-related genes MTFP1, DNMI, and MTFR1 (P < 0.05, Fig. 10, F–H).

DISCUSSION

The growing obesity epidemic has sparked numerous studies on the identification and the roles of feeding-related hypothalamic neuropeptides in the regulation of energy homeostasis. Orexins (A and B) or hypocretins (1 and 2) are multifunctional neuropeptides that bind to two receptors, ORXR1 and ORXR2, to regulate feeding behavior (56), sleep-wake cycle (41), circadian clock (6), and glucose and lipid metabolism in mammals (62). Such physiological roles in avian (nonmammalian vertebrate) species are not well defined yet, although orexins are expressed in several tissues, including hypothalamus (48), testis, ovary (49), and gastroenteric tract (3). Intracerebroventricular injection of orexin did not affect feeding behavior in neonatal chickens (24). Katayama et al. (33) reported that intracerebroventricular administration of orexin A induced arousal in layer-type chickens; however, Miranda et al. (45) could not find evidence that hypothalamic orexin plays a similar role. This suggests that orexins may have other physiological roles in avian species.

Because birds lack functional BAT, and avian muscle is the main site for thermogenesis (43), the recently discovered function of orexin in mammalian BAT development, differentiation, and thermogenesis (61) prompted our group to investigate whether orexin system is expressed in chicken muscle.

The present study is the first to report the presence of orexin system in chicken skeletal muscle and quail QM7 cells. We provided novel evidence that avian skeletal muscle expresses
both prepro-orexin and its related receptors (ORXR1 and ORXR2). In birds, one type of orexin receptor (ORXR1) has been previously identified (49). In the present study, we used specific primers for the predicted 2,170 nucleotide chicken ORXR2 that has 43% homology with the previous identified 1,869 nucleotide chicken ORXR1 and amplified only one band corresponding to the expected size and sequence by real-time PCR. Additionally, by using orexin and orexin receptor polyclonal antisera raised against the well-conserved NH2 terminal region of mouse ORX and the COOH and NH2 terminus of the rat ORXR1/2, we detected immunoreactivity to ORX, ORXR1, and ORXR2 in chicken muscle tissue and QM7 cells by Western blot and immunofluorescence staining, corroborating previous data in chicken intestine, stomach, and pancreas (3). Orexin receptor ORXR1 and two isoforms of ORXR2 (ORX2aR and ORX2bR) have been also shown to be expressed in mouse muscle (17).

The expression of orexin and its related receptors (ORXR1 and ORXR2) in avian muscle and the detection of orexin in culture media suggest that avian muscle might be a source for orexin production and secretion and indicate possible autocrine, paracrine, and/or endocrine roles. In support of this, rORX-A and rORX-B differentially regulate its own gene and protein expression. Although the underlying mechanism is still unknown, the divergent effects of orexin A and orexin B on orexin expression might be related to their structure (presence of disulfide bonds in orexin A and not in orexin B) and their different binding affinity to ORXR2, since they had similar effects on ORXR1 expression. In mammals, indeed, the ORXR1 preferentially binds orexin-A, whereas ORXR2 binds both peptides (orexin A and B), apparently with similar affinity (56). Interestingly, it is likely that ORXR2 and not ORXR1 mediated the activation of the dorsomedial and lateral hypothalamic neurons leading to the adaptive response (thermogenesis and physical activity) to diet restriction in rat (59). As we used here recombinant human orexins and chicken orexin A and B that showed approximately only 85% and 65% identity with the corresponding mammalian sequences (48), further binding studies are warranted. Moreover, the potential involvement of the structure, the function of the cytoplasmic tails, and the desensitization of ORXR1/2 in the divergent effects of orexins are not ruled out.

In line with the aforementioned divergent effects, orexins differentially regulated the expression of mitochondrial-related genes (av-UCP, av-ANT, Ski, NRF-1) and their related transcription factors and coactivators (PPARα, PPARγ, PGC-1β, FOXO1) with downregulation for orexin A and upregulation for orexin B. Such divergent effects of orexins on
gastric acid secretion and arousal have been previously reported in rats and chickens, respectively (33, 52). In contrast to the well-known role of UCP1 in BAT thermogenesis, the function of av-UCP that is homologous to mammalian UCP2 and UCP3 is still under debate. We previously hypothesized that av-UCP might be involved in avian muscle adaptive thermogenesis and energy dissipation as heat based on its upregulation during cold exposure and after chronic treatment with glucagon (53). Teulier et al. (69), however, suggested that av-UCP might not be involved in heat production through mitochondrial uncoupling. Two other independent groups reported a possible role for av-UCP in mitochondrial reactive oxygen specie (ROS) production (1, 54). As in mammals, the avian mitochondrial anion carrier ANT (also known as avATP/ADP carrier) has been reported to be involved in thermogenesis and mitochondrial ROS control (11, 67, 70).

Mitochondria are responsible for producing over 90% of the ATP for the cell by oxidative phosphorylation associated with the electron transport chain (37), and mitochondrial dysfunction in skeletal muscle is closely associated with insulin resistance in mammals (50) and feed inefficiency in chickens (10). The alteration of mitochondrial-related genes and their transcriptional regulators indicated that orexin might control avian muscle mitochondrial dynamics and respiratory function (11, 67, 70).

Mitochondria are constantly undergoing both fusion (biogenesis) and fission (15). Fusion involves complete mtDNA replication; however, fission occurs when the existing copies of mtDNA are simply divided between the new fissioned mitochondria (15). In support of our above-mentioned hypothesis, orexins differently regulated mitochondrial dynamics-related genes. While rORX-B increased mitofusion, as indicated by the upregulation of MFN2, OPA1, and OMA1 gene expression, rORX-A promoted mitofission as reflected in the increased expression of MFIP1, DNMI, and MTFRL1 genes. Despite the unchanged expression of TFAM (the direct regulator of mtDNA replication/transcription), the increased levels of mtDNA and CoxIV and Cox5a mRNA indicated that RORX-B might induce mitochondrial content and mass through other TFAM isoforms (22), TFAM posttranslational modifications (58), and/or other transcription factors (9). An increase in mitochondrial content following rORX-B treatment is also supported by the increased MitoTracker staining.

The effects of orexin on avian muscle mitochondrial dynamics are very likely mediated through NRF-1, Ski, and their related transcription regulators (PPARγ, PGC-1α, PGC-1α, and FoxO-1). Although their roles are not well defined in birds, the NRF-1 and Ski play a key role in mammalian mitochondrial biogenesis and respiratory function (75, 76). Mammalian NRF-1 is a direct target for PPARγ and PGC-1 (75). PGC-1 stimulates induction of NRF-1 gene expression and coactivates its transcriptional function on TFAM promoter (75). MFN2 expression has been shown to be controlled by two distinct regulatory mechanisms, including PGC-1α/estrogen-related receptor (ERR) α- and PGC-1β/ERRα-dependent signaling pathways (13, 40, 64). FoxO-1 has been shown to interact with heme oxygenase 1 (HMOX1) and regulate mitochondrial biogenesis and function through sirtuin 1 (SIRT1) and PGC-1 pathways (18).

To gain better insight into the physiological roles of orexins in avian muscle and to evaluate whether the alteration of mitochondrial dynamics impairs mitochondrial respiratory function, we measured bioenergetics components in orexin-treated and control cells (P < 0.05).
treated and untreated (control) QM7 cells using extracellular XF-24 Flux analyzer. On the basis of our findings, orexin-induced mitochondrial fusion/fission seemed not to affect ATP synthesis. In mammals, the impact of mitochondrial dynamics on mitochondrial energization and function varied upon the experimental model (cell line) and conditions. For instance, inhibition of mitochondrial fusion by manipulation of MFN2/OPA1 expression caused a reduction in mitochondrial membrane potential and activity of respiratory complexes. Overexpression of a truncated form of MFN2Δ602–757 enhances mitochondrial metabolism independent of fusion activity (51); however, other MFN2 mutants did not induce metabolic alterations (68). Some OPA1 mutants showed impaired ATP synthesis driven by complex I substrates and decreased rates of mitofusion (77); however, other mutants showed normal mitochondrial activity and bioenergetics (65). One study (16) reported that overexpression of OPA1 did not modify mitochondrial metabolism in MEF cells. Emerging evidence indicated that alterations in mitofission-related proteins such as DNM1 and FIS1 produced similar effects on mitochondrial metabolism (7). Together, these data indicated that specific mutations in mitochondrial dynamics-related genes alter mitochondrial metabolism.

Although we did not see any effect on ATP synthesis in the present study, we anticipate the possibility that mitochondrial dynamics might regulate mitochondrial metabolism via differ-
ent pathways, including a direct “physical” effect of fusion/fission and/or downstream signaling cascades of dynamics-related genes that cause direct changes in the expression of oxidative phosphorylation subunits, as previously shown (51). Therefore, compensatory or feedback mechanisms between fusion-fission events or between fusion- and fission-related genes might be expected to maintain mitochondrial homeostasis and might explain the absence of metabolic alteration after orexins treatments. In addition, it is possible that some mitochondria might be only tethered or connected but not completely fissioned or fused; thus, an additional level of complexity in the regulation of mitochondrial dynamics is likely to occur (74). Finally, we measured only a few genes, and further analysis of additional genes and proteins involved in mitochondrial network is required, and a demonstration of how they are regulated in vivo conditions is mandatory.

The findings of the present study are the first evidence of orexin system expression and secretion in muscle and unveil its important role in mitochondrial network.

**Perspectives and Significance**

Orexins are originally identified as feeding-related hypothalamic neuropeptides that have effects on diverse processes, including obesity and diabetes. A major goal is to identify and unravel the mode of orexin action in these processes using different experimental models. In the present study, we used chickens as a model of choice because they are naturally hyperglycemic compared with mammals (38), insulin-resistant (2), lack of functional BAT (5), and prone to obesity (29). We provided evidence that orexin system is expressed in chicken muscle and plays a key role in mitochondrial dynamics. Because chicken muscle is the main site for thermogenesis and because mitochondrial functions are implicated in many (patho)-physiological processes, our findings open new vistas on the role of orexin in muscle energy metabolism. As genetic selection for rapid growth, driven by economic demands, have resulted in dramatic increase in body weight of broiler chickens arising from increased skeletal muscle mass, further studies investigating the role of orexin in muscle development, myogenesis, insulin sensitivity, and glucose uptake are warranted. Insights into the molecular mechanisms and physiological role of orexin in skeletal muscle are of uppermost interest not only in animal biology for health and feed efficiency improvement, but also in molecular medicine for pathophysiological under-

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**AUTHOR CONTRIBUTIONS**


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