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Chapter

Orexin System and Avian Muscle Mitochondria

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Abstract

In mammals, orexin A and B (also known as hypocretin 1 and 2) are two orexigenic peptides produced primarily by the lateral hypothalamus that signal through two G-protein-coupled receptors, orexin receptors 1/2, and have been implicated in the regulation of several physiological processes. However, the physiological roles of orexin are not well defined in avian (non-mammalian vertebrate) species. Recently, we made a breakthrough by identifying that orexin and its related receptors 1/2 (ORXR1/2) are expressed in avian muscle tissue and cell line, and appears to be a secretory protein. Functional in vitro studies showed that orexin A and B differentially regulated expression of the orexin system, suggesting that orexins might have autocrine, paracrine, and/or endocrine roles. Administration of recombinant orexin modulated mitochondrial biogenesis, dynamics, function, and bioenergetics. In this chapter, we include a brief overview of the (patho) physiological role of orexin, comparative findings between mammalian and avian orexin, and in-depth analysis of orexin's action on avian muscle mitochondria.

Keywords: orexin system, muscle, avian species, mitochondria, gene expression

1. Introduction

Orexin/hypocretin was originally identified by two different groups and reported in 1998 as an orexigenic feeding-related neuropeptide mainly produced in the rat hypothalamus [1, 2]. Numerous subsequent studies conducted in mammals have shown that orexin and its receptors regulate several physiological processes including food and water intake [3], control of wakefulness [4], circadian clock [5], energy and glucose homeostasis [6–8], lipid metabolism [9, 10], heart rate and blood pressure [11, 12], and neuroendocrine response to stress [13]. Despite these advancements in understanding the orexin system, studies investigating its distribution and function in avian (non-mammalian vertebrate) species are very limited and merit more consideration and further in-depth explorations. Understanding orexin distribution and unraveling its function in avian tissues, particularly in broiler (meat-type chickens) skeletal muscle is of particular importance not only to the poultry industry, but also to the biomedical field.

The poultry (meat and egg) industry supports the livelihoods and food security of billions of people worldwide with an average annual production of 118 million metric tons of meat and 1360 billion eggs in 2017 [14]. This success was mainly achieved by intensive genetic selection for important economic and agricultural traits such as high growth rate, improved feed efficiency (conversion of feed to muscle mass), and increased muscle mass [15]. Today, indeed, modern broilers are
marketed in about half the time and at about twice the body weight compared to
50–60 years ago [16]. This fast growth is largely allocated to the beast muscle [17].

Chickens are naturally hyperglycemic compared with mammals [18], insulin-
resistant [19], lack the glucose transporter GLUT4 [20], lack functional brown
adipose tissue [21], and are prone to obesity [22], and thereby they represent a
highly relevant animal model for biomedical researches.

The proper function of mitochondria is invariably related to muscle growth
since these organelles produce a vast majority (~90%) of the ATP needed for tissue
growth and maintenance of energy metabolism homeostasis. Previous studies in
broiler chickens have shown that mitochondrial perturbations are directly related
to a decrease in feed efficiency. Additionally, the absence of orexin's effect on feed
intake in chickens [23] suggest that this neuropeptide may be more involved in
other peripheral metabolic and physiological processes rather than food/water
intake and wakefulness that is seen in mammals. Therefore, if orexin and its
receptors are shown to be present in skeletal muscle tissue of avian species, they
may be exerting control of energy metabolism and muscle growth (i.e., myogen-
esis, insulin sensitivity, and glucose transport) in the cell in part by regulating
mitochondrial dynamics. The role of orexin in skeletal muscle function could be of
interest not only in improving health and feed efficiency of agricultural animals,
but also in molecular medicine for pathophysiological understanding and thera-
peutic perspectives.

2. Identification of orexin and its receptors

Orexin, which regulates wakefulness, energy homeostasis, and appetite/feeding
behavior based on nutritional status, is a neuropeptide that was originally
discovered in the hypothalamus of rats by two different research groups in 1998.
De Lecea's group isolated cDNAs selectively expressed within the hypothalamus
and found that two peptides showed high amino acid sequence homology with
secretin (the gut peptide hormone), therefore they named the two peptides
hypocretin 1 and 2 [1]. Sakurai's group, on the other hand, used reverse phar-
macology to identify ligands of orphan G-protein-coupled receptors. Orphan
receptors are those whose ligand and physiological actions are unknown [24].
They identified a novel family of neuropeptides that induced feeding behavior, so
they named them orexin A and B [2]. The term orexin originates from the Greek
word “orexis,” meaning appetite. Both orexin A and B are formed by proteolytic
cleavage of the precursor prepro-orexin [2]. When initially discovered in rats,
the precursor peptide prepro-orexin was shown to be a 130-residue polypeptide
from which the mature peptides ORX-A and ORX-B were formed, with ORX-A
containing 33 amino acids and a molecular weight 3,562 kDa and ORX-B contain-
ing 28 amino acids and a molecular weight of 2,937 kDa [2]. When comparing the
two peptides, ORX-B was shown to be 56% identical in amino acid sequence to
ORX-A. However, when comparing mammalian species (human, rat, mouse, pig,
and cow), the sequence and structure of both peptides is highly conserved [2]. A
number of studies have also shown that the structures of ORX-A and ORX-B in
chicken and certain types of fish are conserved when compared to their mamma-
lian counterparts [25–28].

ORX-A and ORX-B signal through the G-protein-coupled receptors orexin
receptor 1 (ORXRx1) and orexin receptor 2 (ORXRx2). These two ubiquitously
expressed receptors were first identified in human brain tissue through expressed
sequence tags combined with database searching using tBLASTn [2, 29]. In humans
it has been shown that the amino acid sequence for ORXRx1 and ORXRx2 is more than
60% identical, making them more similar to each other than to other G-protein-coupled receptors [2]. The same study also showed that both receptors are highly conserved between humans and rats, with the sequence identity being greater than 90% for both. The two orexin peptides have different binding affinities for the two orexin receptors. ORX-A is able to bind to both receptors but has a higher affinity for ORXR1, while ORX-B binds to ORXR2 with the same affinity as ORX-A [28]. Several studies have indicated that the binding of orexins to orexin receptors activates multiple G-proteins. In studies conducted using humans [30, 31] and rats [32], it was shown that the binding of ORXR2 activates Gi, Gs, Go, and Gq proteins in adrenal cortical tissue. It appears that the responses to orexin receptor signaling are highly diverse. The activation of the various G-proteins can lead to a variety of cellular responses such as the regulation of protein/lipid kinases [33, 34]. In the case of orexin stimulation, activation of G-proteins can lead to the excitation of neurons that affect the regulation of ion channels, the activation of signaling cascades that regulate the activity of adenylyl cyclase and phospholipases, and activation of cell death pathways [35, 36].

3. Orexin system in avian species

Significantly fewer studies concerning the orexin system have been conducted in avian species when compared to mammals. Chicken prepro-orexin was first cloned, sequenced, and characterized in 2002 [37]. In that study, chicken orexin cDNA was shown to be expressed in the periventricular and lateral hypothalamic areas and consisting of 658 bp that encode 148 amino acids. Also, chicken ORX-A and ORX-B are evolutionarily conserved with their mammalian counterparts, showing ~85 and 65% similarity at the amino acid level [37]. Characterization of the chicken orexin receptor shows that its cDNA has a length of 1869 bp that encode 501 amino acids, which corresponds to mammalian ORXR2 with an 80% homology [37]. Studies looking at tissue distribution of orexin and orexin receptors in chickens show that the peptides are expressed in the brain [37–40], pituitary gland, adrenal gland, testis and ovary [38], and the stomach and intestine [41].

Orexin does not appear to elicit the same responses in birds as it does in mammals. One of the most noted actions that centrally administered orexin has in mammals is that it stimulates feeding/food intake [2, 3, 28, 42]. However, central administration of ORX-A or ORX-B did not stimulate feed intake in neonatal broiler and layer chicks [23, 43] or adult pigeons [44]. Studies examining mRNA expression of prepro-orexin in the hypothalamus of chicken [37] and quail [45] following 24 h fasting showed no increase in expression, providing further evidence for the lack of a stimulatory effect on feeding behavior in birds. Another feeding behavior study did show an increase in prepro-orexin mRNA [46], but this was measured after 48 h fasting, which would be an extreme fasting condition for broiler chickens.

As stated previously, another hallmark of orexin function in mammals is its effects on the regulation of sleep/wakefulness, where a dysfunction in the orexin system is associated with the sleep condition narcolepsy [4]. Studies investigating the effects of orexin on arousal in birds have been conducted with mixed results. It has been concluded that either hypothalamic orexin does not play a role in arousal of the sleep/wake cycle [39], or that only ORX-A in conjunction with the enzyme monoamine oxidase-A (MAO-A) increases arousal in layer chicks only and not broiler chicks [43, 47]. Multiple studies investigating orexin in avian species theorize that the peptide appears to be more involved in the regulation of energy metabolism than feed intake and sleep/wake cycles [39, 40, 48].
4. Orexin system in avian muscle

Studies investigating the orexin-producing neurons in the rat/mouse brain report that central administration of the neuropeptide facilitates changes in

![Image of muscle cells and related receptors]

Figure 1.
Prepro-orexin and its related receptors are expressed in broiler chicken muscle (a, b) and QM7 cell line (c–f). (a) RT-PCR. Total RNA (1 μg) was reverse transcribed and subjected to RT-PCR. Brain, testis, and ovary were used as positive controls. (b) Western blot. 70 μg total protein extracted from each tissue were electrophoresed and blotted onto polyvinylidene difluoride membrane. Prepro-orexin (ORX) and orexin receptor 1 (ORXR1) expression was detected by immunoblot using rabbit anti-mouse ORX and rabbit anti-rat ORXR1 antibodies. Hypothalamus and brain were used as positive controls. The figure is a representative picture from one animal. (c) RT-PCR. Total RNA (1 μg) was isolated from chicken brain and QM7 cells and subjected to RT-PCR. (d) Western blot. Total protein extracted from the cells were electrophoresed and blotted onto polyvinylidene difluoride membrane. Prepro-orexin (ORX) and ORXR1/2 expression was detected by immunoblot using rabbit anti-mouse ORX and rabbit anti-rat ORXR1/2 antibodies. Brain was used as positive control. (e) Northern blot. Total RNA (10 μg) was separated by agarose gel electrophoresis and transferred to a nylon membrane and hybridized with specific biotin-labeled DNA probe toward chicken ORX, and 18S. Hybridization signals were detected by FluorChem M MultiFluor system. (f) Immunofluorescence staining. Intracellular ORX system distribution visualized by fluorescent microscope in the presence of a secondary antibody conjugated with Alexa Fluor 488 (green) and DAPI (blue).
skeletal muscle tone [49, 50] in addition to increasing glucose metabolism and insulin sensitivity of skeletal muscle [51]. This indicates that orexin signaling in the central nervous system regulates muscle glucose metabolism by activating muscle sympathetic nerves. Even though the orexin system has been identified in the peripheral tissues of a variety of vertebrate species [52], very few studies have been conducted to determine whether orexin and its related receptors are expressed in vertebrate skeletal muscle and if so, what effects it’s presence may have on vertebrate muscle function and physiology. Review of the current literature shows that to date orexin has only been identified as being expressed in the heart muscle of zebrafish [53] and skeletal muscle of goldfish, chicken, and quail [54–56].

We have conducted studies using RT-PCR and Western blot analysis to identify both orexin and its related receptors as being expressed in broiler chicken muscle (Figure 1a, b). In addition, RT-PCR, Western blot analysis, Northern blot analysis, and immunofluorescence have been used to illustrate that orexin and its receptors are also expressed in the cytoplasmic compartment of a spontaneously immortalized quail muscle (QM7) cell line (Figure 1c–f). In humans, scientific evidence indicates that orexin is a secretory peptide due to its presence in the blood circulation. Additional support for orexin being secreted is given by the first 33 amino acids of human prepro-orexin containing a hydrophobic core followed by residues with small polar side chains, which are characteristics of a secretory signal sequence [57]. Cell culture experiments using the QM7 cell line have been carried out in order to determine whether orexin is also secreted in avian muscle. When QM7 cells are incubated in serum-free growth media in the presence of recombinant human orexin B (rORX-B), an increase in the level of prepro-orexin in the growth media is evident as seen in Figure 2a. Further support for the secretion of prepro-orexin is illustrated in Figure 2b where the levels of prepro-orexin in the serum-free growth media accumulate over a 72 h period, and in Figure 2c where application of the anti-secretory compound brefeldin A causes a buildup of prepro-orexin in the QM7 cell lysate and the subsequent absence of the peptide in the growth media. The expression and secretion of prepro-orexin from avian muscle cells suggests that avian orexin could be a myokine that probably functions in autocrine, paracrine, and/or endocrine roles.

Subsequent experiments were conducted in order to determine whether the orexin system is capable of self-regulation. The effects of 10 and 100 nM of recombinant human orexin A (rORX-A) and rORX-B on mRNA and protein expression of ORX and its related receptors ORXR1 and ORXR2 in QM7 cells are shown in Figure 2d–g. A 24 h treatment with either 10 or 100 nM rORX-A upregulated ORX and ORXR1, but not ORXR2 mRNA expression (Figure 2f). Treating cells with rORX-B downregulated ORX and ORXR2 and increased ORXR1 mRNA levels (Figure 2g). Protein expression levels of ORX, ORXR1, and ORXR2 showed the same patterns as their corresponding genes (Figure 2d, e) with the effects on expression levels appearing to be dose-dependent. The ability of rORX-A and rORX-B to differentially regulate gene and protein expression of the orexin system supports the idea that orexins function in an autocrine, paracrine, and/or endocrine role in avian muscle. Although the underlying mechanisms behind the differential regulation of the orexin system is still unknown, the divergent effects of rORX-A and rORX-B on orexin system 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.
4.1 The orexin system differentially regulates mitochondrial-related genes and mitochondrial bioenergetics in avian muscle cells

In mammals, orexin has been shown to induce differentiation of brown adipose tissue (BAT), subsequently leading to thermogenesis [58, 59]. One of the effects orexin has in this process is the regulation of genes involved in mitochondrial biogenesis. The treatment of mouse preadipocytes with ORX-A revealed a number of changes in...
mitochondrial dynamics, including up-regulation of the expression of a number of genes involved in mitochondrial biogenesis (i.e., PGC-1α, PGC-1β, PPARγ1, and UCP1) [58]. These findings were further supported when subsequent immunofluorescence staining revealed an increase in mitochondrial abundance of the treated cells. Studies using other cell types treated with ORX-A have also shown effects on mitochondrial function. Human neuroblastoma cells treated with ORX-A had increased mitochondrial membrane potential [60]. Additionally, in studies using human hepatoma cells [61] and human embryonic kidney cells [62], treatment with ORX-A resulted in increased ATP production that shifted from glycolysis in the cytoplasm to oxidative phosphorylation in the mitochondria. Taken together, these studies indicate that orexin is able to enhance mitochondrial function, biogenesis, and ATP production in mammalian vertebrates.

Similar experiments were carried out by treating QM7 cells with recombinant human orexin, as previously described, in order to observe the differential effects on mitochondrial-related genes, transcriptional regulators, and bioenergetics. Figure 3 illustrates how orexin causes differential expression of mitochondrial genes. rORX-A had no effect on av-UCP mRNA abundance (Figure 3b), but it downregulated the expression of av-ANT (mRNA and protein levels), Ski, and NRF-1 in a dose-dependent manner (Figure 3a, c, 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 (Figure 3a–d). The mitochondrial transcriptional regulators related to these genes were also differentially regulated following treatment with recombinant orexins as seen in Figure 3e–h. Both doses of rORX-A caused a significant downregulation of PPARδ and FoxO-1 expression (Figure 3g, h). A high dose of rORX-A significantly downregulated the expression of PGC-1β, and both doses did not alter PGC-1α mRNA abundance (Figure 3e, f). rORX-B, however, induced the expression of these transcription factors in a dose-dependent manner, but the effects were statistically significant only for PGC-1α, PGC-1β, and FoxO-1 with the high dose (Figure 3e, f, h).

Since mitochondria are the powerhouse of the cell with central importance for producing more than 90% of the ATP needed to carry out essential cellular functions, these organelles are important for proper growth and development of skeletal muscle tissue. The changes in expression of mitochondrial-related genes and their transcriptional regulators suggest that orexin may control mitochondrial respiratory function in avian muscle. To gain better insight into the physiological roles of orexins in avian muscle, mitochondrial 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 (carbonyl cyanide-4-phenylhydrazone), and antimycin A as shown in Figure 4f. As described previously [63], 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 the electron transport inhibitor antimycin A is attributed to non-mitochondrial OCR (i.e., OCR due to activities other than non-mitochondrial 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 non-mitochondrial 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. ATP synthesis was slightly elevated by both orexins, but the effect was not statistically discernable. Analysis of reserve capacity indicated no effect of both doses of rORX-A.
and rORX-B; however, proton leak was significantly decreased by 10 nM of rORX-A, and by 100 nM of rORX-B (Figure 4g). The combined data from these experiments illustrate that orexins lead to the alteration of mitochondrial-related genes, their transcriptional regulators, and respiratory function in avian muscle cells. These changes suggest that orexin might also control mitochondrial dynamics (i.e., fusion/fission of mitochondria) in avian muscle.

Figure 3.
Effect of orexin treatment on mitochondrial-related genes (a–d) and mitochondrial-transcriptional regulators (e–h) in QM7 cells. Cells were treated with recombinant orexin A or B (10 and 100 nM) for 24 h and the relative abundance of avian (av)-adenosine nucleotide translocator (ANT; a), UCP (b), Ski (c), nuclear respiratory factor 1 (NRF-1; d), PGC-1α (e), PGC-1β (f), peroxisome proliferator-activated receptor δ (PPARδ; g), and FoxO-1 (h) were determined by QPCR. Untreated cells were used as control. Protein levels of av-ANT and PGC-1α were measured by Western blot analysis. Data are expressed as means ± SE (n = 6). Significant difference between orexin-treated and control cells (P < 0.05).
4.2 Orexins differentially regulate mitochondrial biogenesis and dynamics in avian muscle cells

Since mtDNA replication and quantitation are a necessary component of mitochondrial biogenesis, the expression of mtDNA and mtSSBP1 was measured in orexin-treated QM7 cells as shown in Figure 4b, c. 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. Consistent with these observations and in contrast to rORX-A, rORX-B increased mitochondrial content as visualized with MitoTracker Red probe staining (Figure 4a). 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

Figure 5. Effect of orexin treatment on mitochondrial dynamics-related genes in QM7 cells. QM7 cells were treated with orexins (20 and 100 nM) for 24 h. The relative expression of four genes involved in mitochondrial fusion, MFN1 (a), MFN2 (b), OPA1 (c), OMA1 (d) and three genes involved in mitochondrial fission, MTPF1 (f), DNM1 (g), and MTFR1 (h) was determined by real-time PCR. The protein levels of MFN1, MFN2, and OPA1 were determined by Western blot analysis (e). The values represent the means ±SE (n = 6). Significant difference between orexin-treated and control cells (P < 0.05).
and biogenesis, was determined. The high dose (100 nM) of rORX-A decreased Cox IV gene expression; however, the high dose of rORX-B significantly increased Cox IV and Cox 5a mRNA levels compared with untreated cells (Figure 4d, e).

Mitochondria are dynamic organelles in the cell that constantly fuse and divide, forming constantly changing tubular networks, according to the needs of the organism, thus leading to alterations in their morphology and function [64]. Since orexin is shown to alter expression of mitochondrial-related genes and transcriptional regulators, it may also control avian muscle mitochondrial dynamics. The molecular mechanisms that control mitochondrial dynamics are complex and require participation and coordination of both the nuclear and mitochondrial genomes. In mammals this network has been partially unraveled after the identification of some of the genes responsible for mitochondrial fusion [mitofusins (MFN1 and MFN2), and optic atrophy 1 (OPA1)] and fission [dynamin-related protein 1 (Drp1 or DNML), fission 1 (FIS1), and mitochondrial protein 18 kDa]. Up until the current study, the integration of such a mitochondrial network is unknown in avian species. Following orexin treatment, the expression of four genes related to mitochondrial fusion and three genes related to mitochondrial fission were measured as shown in Figure 5. Recombinant ORX-B at high dose significantly induced the expression of MFN2, OPA1, and OMA1, but decreased the mRNA levels of MFN1 (Figure 5a–d). The same effect was observed at the protein levels (Figure 5e). 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 MFN2 (Figure 5a–d). Interestingly, and in contrast to rORX-B, where no significant effects were observed, rORX-A upregulated the expression of mitofission-related genes MTFP1, DNML, and MTFR1 (Figure 5f–h). These orexin-induced changes in the expression of dynamics-related genes may serve in regulating mitochondrial metabolism in muscle cells in response to the needs of the animal during stages of growth and development.

5. Conclusions

Orexins are originally identified as hypothalamic neuropeptides that have potent orexigenic effects on appetite and feeding behavior in mammals. In avian species, however, orexins seem to be myokines that regulate the expression of their own system as well as muscle mitochondrial function, biogenesis, bioenergetics, and dynamics. As intensive genetic selection for fast growth rate, driven by human nutritional needs and economic demands, have resulted in dramatic increase in chicken body weight arising mainly from increased muscle mass, these finding open new vistas on the role of orexin system in muscle development and energy metabolism. Further in depth investigations are warranted to understand the relationship between orexin system, mitochondrial network, and muscle growth and development which, in turn, will be beneficial to the poultry industry as it has the potential to lead to increased production efficiency and reduced economic costs.
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