

**OREXINS IN THE AVIAN BRAIN: COMPARISON OF
HYPOTHALAMIC mRNA CONCENTRATIONS DURING
THE DAY-NIGHT CYCLE AND AFTER FOOD
RESTRICTION IN BROILER AND LAYER CHICKS AND
IMMUNOISTOCHEMICAL LOCALIZATION IN CHICK
HYPOTHALAMUS**

by

Bernadette Miranda

Dissertation

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for the Degree of

Doctor of Philosophy

DEDICATION

To Mamma and Papa', without whom I wouldn't have reached this goal.

To Gianmaria, for always having been present.

To Cosimo, for his unconditional love.

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TECHNOLOGIES

Ph.D. RESEARCH COURSE

in

BIOLOGY, PATHOLOGY and SANITATION in
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COORDINATOR

Prof. Dr. Giuseppe Paino

Ph.D. STUDENT

SUPERVISOR

Dr. Bernadette Miranda

Prof. Dr. Vincenzo Esposito, Ph.D

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LE OREXINE NELL'ENCEFALO DEGLI UCCELLI: COMPARAZIONE
DELLE CONCENTRAZIONI DI mRNA DURANTE IL CICLO GIORNO-
NOTTE E DOPO RESTRIZIONE DELL'ALIMENTAZIONE NEI PULCINI
"BROILER" E "LAYER" E LOCALIZZAZIONE IMMUNOISTOCHIMICA
NELL'IPOTALAMO DI PULCINO

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Prof. Dr. Giuseppe Paino

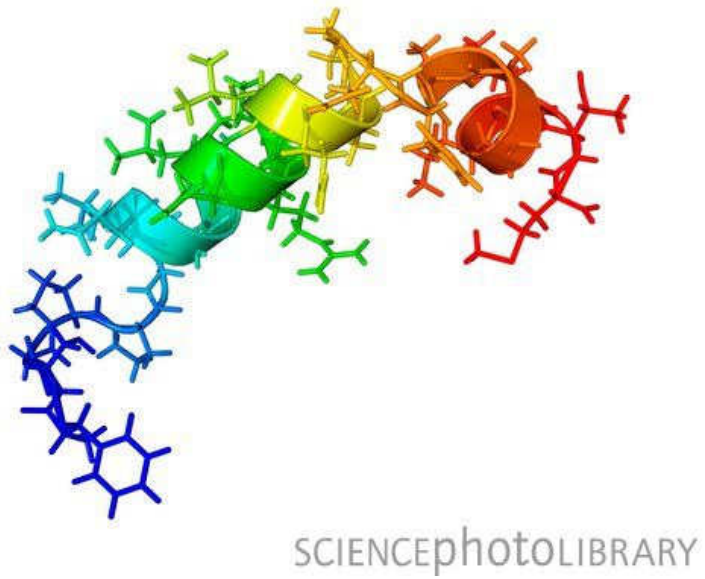
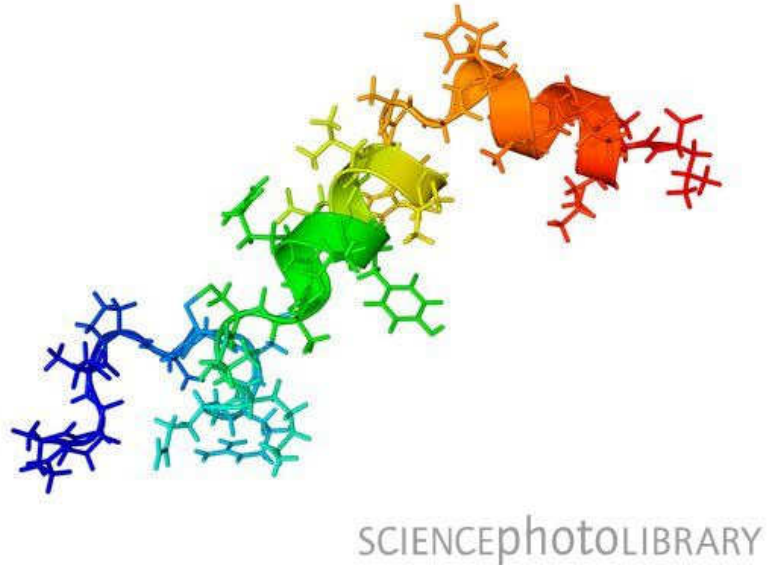
DOTTORANDA

TUTOR

Dr. Bernadette Miranda

Prof. Dr. Vincenzo Esposito, Ph.D

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Orexin-A and orexin-B molecules. Computer models showing the crystal structure of a molecule of orexin-A (upper) and orexin-B (down). The crystal structure consists of both the secondary structure, represented by alpha helices (ribbons), and primary structure (rods).

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1. INTRODUCTION.

1.1. Orexin peptides.

Orexins A and B are two neuropeptides that were originally discovered in the rat hypothalamus by two independent groups and named hypocretins because of their hypothalamic localization and their supposed similarity to secretin (de Lecea et al., 1998; Sakurai et al., 1998). After, their name was changed to orexins on the basis of their predominant expression in the hypothalamic feeding centers and their involvement in the control of food intake. Orexin A is a 33-amino acid peptide of 3562 Da with two sets of intrachain disulfide bonds. It has an N-terminal pyroglutamyl residue and C-terminal amidation (Sakurai et al., 1998). The primary structure of orexin A predicted from the cDNA sequences is conserved among several mammalian species as human (fig. 1), rat, mouse, cow, sheep, dog and pig (Sakurai et al., 1998; Shibahara et al., 1999; Dyer et al., 1999; Voisin et al., 2003).

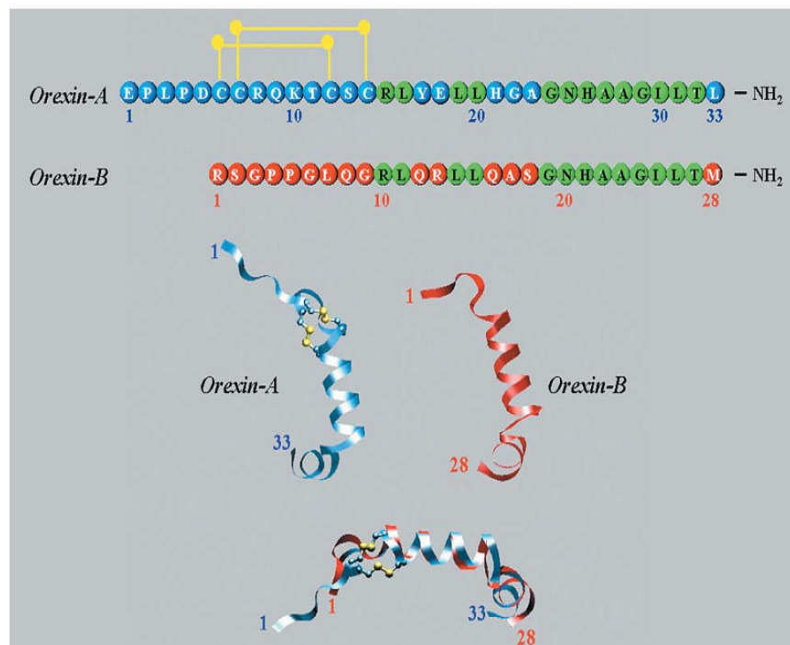


Figure 1. Comparison of human orexin-A and orexin-B structures. Upper panel: amino acid sequences of human orexin-A and orexin-B deduced from nucleic acid sequence (GenBank accession number AF041240). Green circles indicate amino acid identity. The two disulfide bonds in orexin-A are shown in yellow. Middle panel: schematic view of three-dimensional structures of human orexin-B, determined by two-dimensional $^1\text{H-NMR}$ and dynamic simulated annealing calculations [40] and orexin-A, constructed by homology using the Modeller 6.0 program (Copyright 1989–2002 Adrej Šali). Lower panel: superposition of orexin-A and orexin-B three-dimensional structures.

By T. Voisin et al., *Cell. Mol. Life Sci.* 60:72–87 (2003)

Rat orexin B is a 28-amino acid, C-terminally amidated linear peptide of 2937 Da, that is 46% identical in sequence to orexin A. The C-terminal half of orexin B is very similar to that of orexin A, while the N-terminal half is variable. Orexin B also has a high degree of sequence similarity among species. Mouse orexin B has predicted to be identical to rat orexin B. Human orexin B has two amino acid substitutions from the rodent sequence within the 28-residue stretch. Pig and dog orexin B have one amino acid substitution from the human or rodent sequence. Studies revealed that the structures of fish, *Xenopus laevis* and chicken orexin A and B have also conserved structures compared with mammalian sequences (Shibahara et al., 1999; Alvarez and Sutcliffe, 2002; Sakurai, 2005). Orexin A seems to have much higher stability than orexin B in the physiological milieu (Kastin and Akerstrom, 1999), which may explain why orexin A is more readily detected in cerebrospinal fluid than orexin B (Ripley et al., 2001). Orexin A also displays much higher lipid solubility than orexin B, probably making orexin A in contrast to orexin B blood-brain barrier permeant (Kastin and Akerstrom, 1999). Both peptides derived from a common 130 amino acid precursor peptide, prepro-orexin, by proteolytic cleavage. A single gene encodes prepro-orexin and is localised to chromosome 17Q21 in humans (Sakurai et al., 1998). The human prepro-orexin gene, consists of two exons and one intron distributed over 1432 bp (Sakurai et al., 1999). The 143-bp exon 1 includes the 50-untranslated region and the coding region that encodes the first seven residues of the secretory signal sequence. Intron 1, which is the only intron found in the human prepro-orexin gene, is 818-bp long. Exon 2 contains the remaining portion of the open reading frame and the 3-untranslated region. The human prepro-orexin gene fragment, which contains the 3149-bp 5-flanking region and 122-bp 5-non-coding region of exon 1, was reported to have the ability to express lacZ in orexin neurons without ectopic expression in transgenic mice, suggesting that this

genomic fragment contains most of the necessary elements for appropriate expression of the gene (Sakurai et al., 1999). This promoter is useful to examine the consequences of expression of exogenous molecules in orexin neurons of transgenic mice, thereby manipulating the cellular environment in vivo. For example, this promoter was used to establish several transgenic lines, including orexin neuron-ablated mice and rats (Hara et al., 2001; Beuckmann et al., 2004), mice in which orexin neurons specifically express green fluorescent protein (GFP) (Yamanaka et al., 2003b) or calcium-sensitive fluorescent protein (yellow cameleon Yc2.1) (Tsujino et al., 2005) and mice with specific expression of cre recombinase in orexin neurons. The 3.2-kb promoter region of prepro-orexin gene seems to be enough to direct expression of the downstream gene to the lateral hypothalamus in the mouse (Sakurai et al., 1999). This has been utilized to cause selective depletion of orexinergic neurons in transgenic mice expressing prepro-orexin–ataxin-3 fusion protein (Hara et al., 2001). In these mice, orexinergic neurons are progressively lost postnatally. For transcription, the most essential part has been suggested to be the 450 bp most proximal to the gene (Waleh et al., 2001).

1.2. Orexin receptors.

The actions of orexins are mediated by two G protein-coupled receptors termed orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R) (Sakurai et al., 1998; Scammell and Winrow, 2011) (Fig. 2). Among various classes of G protein-coupled receptors, OX1R is structurally similar to certain neuropeptide receptors, most notably to the Y2 neuropeptide Y (NPY) receptor (26% similarity), followed by the thyrotropin-releasing hormone (TRH) receptor, cholecystokinin type-A receptor and NK2 neurokinin receptor (25%, 23% and 20% similarity, respectively). There is a 25.1% and 31.2% respectively between human neuropeptide FF receptor 1 with OX1R and OX2R (Bonini et al., 2000; Kukkonen et al., 2002). Taken together, this information suggests that the two orexin

receptors are likely products of a more recent gene duplication event and this hypothesis is supported by chromosome synteny analysis. OX2R's gene environment is shown to be conserved with the nearest neighboring genes (FAM83B and GFRAL) located in close proximity in all tested genomes. In contrast, only the short-range environments for the OX1R gene are conserved in mammals, for example tubulointerstitial nephritis antigen-like 1 (TINAGL1 V) and penta-EF-hand domain containing 1 (PEF1) genes are located in close proximity to mammalian OX1R but are not found in paralogous regions in non-mammalian genomes. Taking into the account the failure to identify OX1R-like genes in non-mammalian species, OX2R is likely to be evolutionary more ancient and is present in most vertebrate lineages, while OX1R evolved by gene duplication only after the divergence that gave rise to mammals. OX1 receptor (chromosome) is a 425 aa long and OX2R receptor (chromosome) is a 444 aa long in humans and there is 64% amino acid identity between the deduced full-length human OX1R and OX2R sequences. Thus, these receptors are much more similar to each other than they are to other GPCRs. The amino acid identity between the human and rat homologues of each of these receptors is 94% for OX1R and 95% for OX2R, indicating that both receptor genes are highly conserved between the species. Competitive radioligand binding assays using Chinese hamster ovary (CHO) cells expressing OX1R suggested that orexin A is a high-affinity agonist for OX1R. The concentration of cold orexin A required to displace 50% of specific radioligand binding (IC₅₀) was 20 nM. Human orexin B also acted as a specific agonist on CHO cells expressing OX1R. However, human orexin B has significantly lower affinity compared to human OX1R: the calculated IC₅₀ in competitive binding assay was 250 nM for human orexin B, indicating two orders of magnitude lower affinity as compared with orexin A. On the other hand, binding experiments using CHO cells expressing the human OX2R cDNA demonstrated that OX2R is a high-affinity receptor for human orexinB with IC₅₀ of 20 nM.

Orexin A also had high affinity for this receptor with IC₅₀ of 20 nM, which is similar to the value for orexin B, suggesting that OX2B is a non-selective receptor for both orexin A and orexin B.

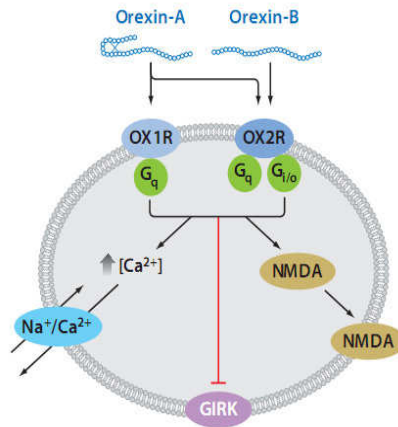


Figure 2

Orexin signaling mechanisms. Orexin-A signals through both OX1R and OX2R, whereas orexin-B signals mainly through OX2R. Intracellular cascades mediated by G proteins increase intracellular calcium and activate the sodium/calcium exchanger, which depolarizes target neurons. These cascades also inactivate G protein-regulated inward rectifier (GIRK) channels. Increased expression of *N*-methyl-*D*-aspartate (NMDA) receptors on the cell surface produces long-lasting increases in neuronal excitability.

by T. E. Scammell and C. J. Winrow - *Annu. Rev. Pharmacol. Toxicol.* 51:243–66 (2011)

1.3. Distribution of orexins and orexin receptors in the vertebrate brain.

Immunohistochemical and in situ hybridization studies have shown that in the central nervous system (CNS), orexin-producing cells are located in a few nuclei in the hypothalamus, including the perifornical nucleus, the lateral hypothalamic area (LHA), and the dorsomedial hypothalamic nucleus (DMN) (Sakurai et al., 1998; Date et al., 1999; Elias et al., 1998; Nambu et al., 1999; de Lecea et al., 1998; Peyron et al., 1998). Orexin neurons are organized bilaterally and symmetrically and have been observed in many species, included bovine, guinea-pig, hamster, human, monkey, mouse, rat, and frog (Sakurai et al., 1998; de Lecea et al., 1998; Peyron et al., 1998; Date et al., 1999; Elias et al., 1998; Nambu et al., 1999; Broberger et al., 1998; Horvath et al., 1999; Galas et al., 2001; Mintz et al., 2001). Despite their highly restricted origin, orexin nerve fibers are located in many regions in the brain, with

particularly abundant projections in the olfactory bulb, cerebral cortex, thalamus, hypothalamus, brainstem, and all levels of the spinal cord (Peyron et al., 1998; Date et al., 1999; Mondal et al., 1999; Sutcliffe and de Lecea, 2000; Broberger et al., 1998; Horvath et al., 1999; Galas et al., 2001; Mintz et al., 2001; van den Pol, 1999; Willie et al., 2001) (Fig. 3). The widespread projections of the orexin neurons throughout the neuroaxis suggest the probably role of orexin in a variety of functions (Sutcliffe and de Lecea, 2000; Willie et al., 2001). The fact that orexins can increase the release of either excitatory or inhibitory neurotransmitters, by acting directly on axon terminals suggests that the peptides could increase or decrease the activity of innervated brain circuits (de Lecea et al., 1998; van den Pol et al., 1998). Within the hypothalamus, orexin neurons project to the arcuate nucleus (Peyron et al., 1998; Date et al., 1999) and specifically innervate NPY-containing cell bodies (Horvath et al., 1999). Reciprocal connections from NPY neurons to orexin neurons in the LHA have also been studied (Elias et al., 1998; Broberger et al., 1998; Horvath et al., 1999). In addition, orexin-containing nerve fibers terminate in close apposition to NPY-immunoreactive nerve terminals in the paraventricular nucleus (PVN) (Broberger et al., 1998; Horvath et al., 1999). NPY is a potent orexigenic peptide that is released in the PVN and surrounding sites to stimulate feeding (Kalra et al., 1999 a,b). Since arcuate NPY neurons are excited by orexins (Willie et al., 2001; van den Pol et al., 1998), probably through the activation of an OX1R Gq-coupled pathway (Willie et al., 2001), this suggests that orexin-stimulated feeding might occur through NPY pathways (Date et al., 1999; Yamanaka et al., 2000). Both NPY-Y1 and -Y5 receptor antagonists reduce orexin-stimulated feeding (Jain et al., 2000). Thus, activation of NPY-containing feeding pathways is at least partially responsible for the effects of orexin on food intake. In the hindbrain, orexin-ir fibers are found in the dorsal vagal complex, comprising the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the

vagus (DMV), and the area postrema (Peyron et al., 1998; Willie et al., 2001). The NTS relays vagally transmitted afferent signals from the gut that are

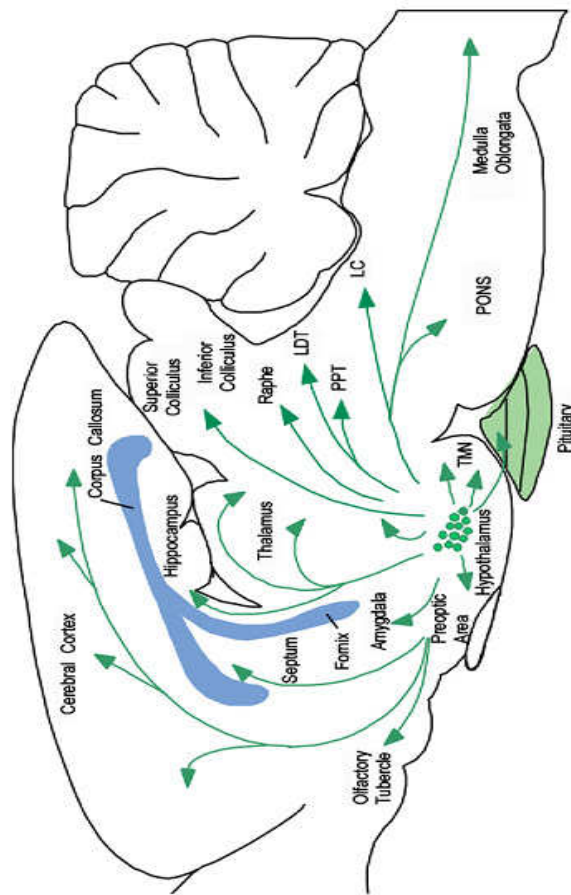


Fig. 3. Schematic drawing of sagittal section of rat brain, summarizing the organization of the orexin neuronal system. Orexin neurons are found only in the lateral hypothalamic area and project to the entire central nervous system. Abbreviations: 3V, third ventricle; TMN, tuberomammillary nucleus; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus.

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related to feeding (Bray, 2000; Schwartz, 2000). The NTS also contains glucosensitive neurons that respond with altered electrical activity to changes in blood glucose and the presence of food in the gut (Mizuno and Oomura, 1984; Yettefti et al., 1997). The DMV consists of motor neurons that control gut motility and the secretory responses that are important for digestion. The DMV is also responsible for the initiation of cephalic-phase responses that prepare the gut for the arrival and subsequent digestion of nutrients (Powley, 2000). The area postrema, which is interconnected with the dorsal vagal complex, is an important circumventricular organ through which circulating systemic factors, such as gut peptides and glucose, can gain access to the brain (Whitcomb and Taylor, 1992). Although the types of neurons in the dorsal vagal complex that are innervated by orexin-containing nerve terminals have not been identified, it is likely that orexins alter the activity of vagal motor neurons and/or modulate the response of NTS neurons to gastrointestinal stimuli. It has been demonstrated that stimulation of the LHA excites neurons in the DMV (Nishimura and Oomura, 1987) and increases the activity of vagal efferents (Yoshimatsu et al., 1988). Thus, it is reasonable to expect that modulation of vagal activity by orexins could influence cephalic phase reflexes and/or affect gastrointestinal motility and secretion. Furthermore, since neurons in the NTS project back to the LHA (Horst et al., 1989), orexin neurons may be regulated by satiety signals relayed through the NTS. Similar in the chicken, *in situ* hybridization and immunohistochemistry studies have revealed the hypothalamus (PVN and lateral hypothalamus) to be exclusive brain locality in which orexin neurons are found (Ohkubo et al., 2002). From the PVN, orexin fibers are extended in the caudal preoptic area (Singletary et al., 2006). In the reptiles, orexins neurons are located in the hypothalamus in the PVN, in the turtle *Pseudemys* and

lizard *Anolis*, and in dorsolateral nuclei in the lizard *Gekko*. (Dominiguez et al., 2010; Farrell et al., 2003). The fibers are found in the septal region, preoptic area and hypothalamus. In contrast to mammalian, avian and reptilian species, in the amphians the orexin neurons are found also outside the hypothalamus and are located in the suprachiasmatic nucleus, whereas cells in the preoptic area and in the hypothalamic tuberal region are less numerous (Galas et al., 2001; Lopez et al., 2009a; Singletary et al., 2006; Suzuki et al., 2008). About the distribution of fibers, they are located in the septal region, the basal ganglia, the striatal region, in the preoptic area, suprachiasmatic nucleus, the tuberal region, dorsal thalamus, opticum tectum, and cerebellum (Lopez et al., 2009a). In fish, the distribution of neurons is different, in fact orexin neurons are located in the preoptic area and suprachiasmatic nucleus in the lungfish and zebrafish; nucleus posterioris periventricularis (NPPv) in medaka; and NPPv and nucleus lateralis tuberis (NLT) in the goldfish. The fibers innervate the aminergic nuclei, raphe, locus coeruleus (LC), the mesopontine-like area, dopaminergic clusters and histaminergic neurons (Amiya et al., 2007; Huesa et al., 2005; Kaslin et al., 2004; Kojima et al., 2009; Nakamachi et al., 2006; Yokogawa et al., 2007). In parallel to the diffuse orexin-containing projection from the LHA, in situ hybridization studies with orexin receptor riboprobes demonstrate that orexin receptors are expressed in a pattern consistent with orexin nerve fibers (Trivedi et al., 1998; Marcus et al., 2001). However, the expression patterns for OX1R and OX2R are strikingly different. Within the hypothalamus, OX1R mRNA is most abundant in the dorsomedial portion of the ventromedial hypothalamic nucleus (VMH). Dense expression of OX1R is also found in the anterior hypothalamic area just dorsal to the suprachiasmatic nucleus. In contrast, OX2R mRNA is expressed in many hypothalamic nuclei such as the tuberomammillary nucleus, the LHA, the arcuate nucleus, and PVN (Lu et al., 2000; Marcus et al., 2001). The tuberomammillary nucleus is the only source of histamine in the CNS. Since

histamine is important for the maintenance of wakefulness (Lin et al., 1998), OX2R in this region has been postulated to play a role in the regulation of sleep/wake states (Marcus et al., 2001). The expression of orexin receptors in the VMH, LHA, arcuate nucleus, and PVN is consistent with a role of hypothalamic orexin systems in regulating food intake. The VMH is implicated in the regulation of food intake since its destruction causes obesity (Bray, 1984). The PVN is the site of action of many orexigenic agents including NPY (Kalra et al., 1999b) and galanin (Tempel et al., 1988). The PVN is also involved in the regulation of gut functions via its projection to the dorsal vagal complex (Zhang et al., 1999). For example, stimulation of the PVN evokes an increase in gastric acid secretion (Rogers and Hermann, 1986) and a transient increase in motility (Rogers and Hermann, 1987). Little orexin receptor mRNA has been detected in the NTS and DMV (Marcus et al., 2001) although these regions appear to receive a moderately dense orexin innervation (Peyron et al., 1998; Willie et al., 2001). Other brain areas that display relatively dense expression of OX1R include the CA1 and CA2 regions of the hippocampus, raphe nuclei, and the locus coeruleus (Trivedi et al., 1998; Marcus et al., 2001). The locus coeruleus and dorsal/median raphe nuclei are major centers for the noradrenergic and serotonergic neurons, respectively. High levels of OX1R expression in these nuclei suggest a regulatory role of orexins on the monoaminergic systems. On the other hand, OX2R mRNA is also present in basal forebrain structures (amygdala and bed nucleus of the stria terminalis), linked to such functions as memory storage and attention, and the nucleus accumbens (NAc) (Trivedi et al., 1998; Marcus et al., 2001). The NAc is the major recipient of the mesolimbic dopaminergic projection and serves a key role in brain reward mechanisms, which may mediate the positive reinforcing effect of food (Smith, 1997).

1.4. Expression of the orexin system in peripheral and endocrine tissues.

Orexin peptides and receptors are also located outside the central nervous system, especially in endocrine tissues. Orexins are expressed in the rat (Date et al., 2000) and human (Blanco et al., 2003) anterior pituitary. In the human adenohypophysis orexin A is found in lactotropes, thyrotropes, somatotropes and gonadotropes (both in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) expressing cells) but not in corticotropes. On the other hand, orexin B is found in all corticotropes of the anterior pituitary (Blanco et al., 2003). This pattern of expression of orexins seems to have been conserved through evolution, since orexin A and orexin B immunoreactivity has been found in the anterior pituitary of fish species, such as Nile tilapia (*Oreochromis niloticus*) (Suzuki et al., 2008) and amphibians, such as African clawed frog (*Xenopus laevis*) (Suzuki et al., 2007). OXRs have also been shown to be present in anterior pituitary of rat (Date et al., 2000; Johren et al., 2001, 2003; Silveyra et al., 2007 a,b) and human (Blanco et al., 2001). Both OX1R and OX2R mRNAs are expressed in the intermediate lobe of rat pituitary, whereas in the anterior lobe, OX1R is more abundant than the OX2R. The two receptors mRNAs are also found in the posterior lobe of rat pituitary (Date et al., 2000). In human anterior pituitary, OX1R is expressed in somatotrope cells and OX2R in corticotrope cells (Blanco et al., 2001). These data may suggest actions of orexins on the pituitary gland, in addition to their possible neuromodulatory role within the hypothalamus. OX1R is abundantly detected in the anterior pituitary of *Xenopus*, which also indicates a highly conserved function (Suzuki et al., 2007). While neither RNA nor protein expression of prepro-orexin has been detected in the rat adrenal gland (Johren et al., 2001, 2003; Karteris et al., 2005; Lopez et al., 1999) immunoreactivity for prepro-orexin and orexin A has been described in human adrenal gland (Karteris et al., 2001; Nakabayashi et al., 2003; Randeva et al., 2001) and human adrenocortical adenomas (Spinazzi et al., 2005). OXRs have been described in the adrenal cortex and/or in the adrenal medulla of the

rat (Johren et al., 2001, 2003; Karteris et al., 2005; Lopez et al., 1999; Malendowicz et al., 2001 a,b; Nanmoku et al., 2000; Spinazzi et al., 2005), pig (Nanmoku et al., 2002), and human (normal adrenals, adrenocortical adenomas and pheochromocytomas) (Blanco et al., 2002; Karteris et al., 2001; Mazzocchi et al., 2001 a,b; Randeva et al., 2001; Spinazzi et al., 2005; Ziolkowska et al., 2005). Orexin and OX1R mRNA expression, is present in the rat testis (Barriero et al., 2004; Blouin et al., 2005; Lopez et al., 1999; Sakurai et al., 1998) with presence of prepro-orexin and orexin A signal in interstitial Leydig cells and seminiferous tubules (Barriero et al., 2004) and predominant location of OX1R mRNA in the tubular compartment of the testis (Barriero et al., 2005). In contrast, in humans prepro-orexin mRNA is only expressed in the epididymis and penis whereas both OXRs mRNAs are present in testis (Leydig cells, testicular peritubular myoid cells and some Sertoli cells), epididymis, seminal vesicle and penis (Karteris et al., 2004). Orexins are located also in the gastrointestinal tract, in fact orexin A immunoreactivity is detected in enterochromaffin (EC) cells of the gastric and intestinal mucosa as well as in the α and β cells of the pancreas of rat mouse and human (de Miguel and Burrell, 2002; Ehrstrom et al., 2005; Kirchgessner and Liu, 1999, Kirchgessner, 2002; Nakabayashi et al., 2003; Naslund et al., 2002; Ouedraogo et al., 2003). Both OXRs are also found in endocrine cells in the gut and in the pancreas of several species, such as rat and human, suggesting a role in the regulation of gut and pancreatic function (Ehrstrom et al., 2005; Kirchgessner and Liu, 1999, Kirchgessner, 2002; Naslund et al., 2002). Low levels of orexins and OXRs mRNA and protein have been detected in other peripheral tissues. In humans, prepro-orexin is found in the stomach, kidney, colon, colorectal epithelial cells, white adipose tissue and placenta (Digby et al., 2006; Nakabayashi et al., 2003). In the rat, OX1R mRNA has been detected in the kidney, thyroid, ovary and placenta (Johren et al., 2001; Silveyra et al., 2007b). In addition, OX2R mRNA has been

found in rat lung, placenta (Johren et al., 2001), ovary (Silveyra et al., 2007b) and pineal gland (Mikkelsen et al., 2001). Orexin A immunoreactivity has been detected in human (Adam et al., 2002; Arihara et al., 2001; Dalal et al., 2001; Higuchi et al., 2002; Kok et al., 2002; Komaki et al., 2001; Matsumura et al., 2002; Sugimoto et al., 2002) and rat (Ouedraogo et al., 2003) plasma. However, the source of these circulating orexins is not clear. Despite the fact that orexin neurons project to the median eminence (ME), where both orexins are detected, (Acuna-Goycolea and van den Pool, 2009; Campbell et al., 2003; Chen et al., 1999; Date et al., 2000; Martynska et al., 2006; Spinazzi et al., 2006), peripheral plasma levels are too high to be explained by unique hypothalamic origin. Moreover, no differences in plasma orexin A concentrations are found between men and women, which suggest that a testicular origin for plasma orexins is unlikely (Arihara et al., 2001). A gut origin has also been proposed for circulating orexins (Arihara et al., 2001; Kirchgessner and Liu, 1999; Kirchgessner, 2002). However, the fact that orexin A plasma levels are elevated after fasting in both humans (Arihara et al., 2001; Komaki et al., 2001), and rats (Ouedraogo et al., 2003), and that glucose levels regulate secretion from the pancreas suggest that the major source of circulating orexin A could be the pancreas (Ouedraogo et al., 2003). Whatever the case, the presence of orexin A in the blood, as well as the widespread distribution of both OXRs, indicate an endocrine role for this peptide.

1.5 Physiological roles of vertebrate orexins.

1.5.1. Food intake.

The regulation of feeding behavior is an important function played by the hypothalamus. In mammals, orexin neurons are found in the LHA and dorsomedial hypothalamic nucleus, otherwise known as the classical “feeding center”. In fact, it is well known that the lateral hypothalamus is involved in food intake and energy homeostasis, and intracerebroventricular injection (icv) of orexins

during the light period induces feeding behavior in rats (Sakurai et al., 1998). The effect of orexin A is significantly more effective than orexin B, possibly due to activation of both OX1R and OX2R subtypes (Sakurai et al., 1998). Based on these findings, the orexins were named after the Greek word orexis, which means appetite (Sakurai et al., 1998). Subsequently, orexin A has been reported to increase food intake in many species, (Edwards et al., 1999; Ida et al., 1999; Sweet et al., 1999; Volkoff et al., 1999; Willie et al., 2001; Rodgers et al., 2000), and after microinjections in several hypothalamic nuclei, including the PVN, dorsomedial hypothalamic nucleus, LHA, and perifornical area (Dube et al., 1999; Sutcliffe and de Lecea, 2000; Willie et al., 2001). In contrast to orexin A, the results obtained with orexin B have been more variable; therefore, orexin B has been concluded to have a little, if any, effect on feeding (Edwards et al., 1999; Sweet et al., 1999; Dube et al., 1999; Haynes et al., 1999). Changes in extracellular glucose concentration produces electrophysiological changes in orexin-neurons (Yamanaka et al., 2003a). It has been suggested that LHA contains glucose-sensitive neurons that are activated by glucopenia and thus implicated in the positive short-term regulation of feeding and energy expenditure (Oomura et al., 1974). These studies suggested that orexin neurons are glucose-sensitive neurons and might play an important role in feeding and energy expenditure. Prepro-orexin mRNA levels is also increased in hypoglycaemic conditions, suggesting that expression of the gene is also regulated by plasma glucose level (Griffond et al., 1999; Moriguchi et al., 1999). Recent reports also showed that infusion of orexin A into the shell of the NAc increased feeding behaviour (Thorpe and Kotz., 2005). In addition, infusion of the gamma-aminobutyric acid (GABA) receptor agonist muscimol into the NAc shell strongly induced food intake and increased Fos expression specifically in orexin-neurons (Baldo et al., 2004). These findings indicate that the orexin and limbic system have a role in the regulation of feeding. Orexin is suggested to be

necessary for NPY and ghrelin to exert their effects on feeding behavior. Injections of orexin anti-serums attenuate NPY and ghrelin-induced feeding (Niimi et al., 2001; Toshinai et al., 2003). In addition orexin-deficient mice show lowered ghrelin-induced food intake compared to their wild-type littermates (Toshinai et al., 2003). ICV-ghrelin was able to induce neuronal activity and gene expression of NPY and orexin (Nakazato et al., 2001; Toshinai et al., 2003). Clearly, orexins, NPY and ghrelin play independent roles in regulating feeding. Similarly in birds, orexin neurons and fibers are present in the PVN and LHA, but both mammalian orexins not induce feeding in the chicken and pigeon (da Silva et al., 2008; Furuse et al., 1999; Katayama et al., 2010). It is possible that orexins are either not involved or play a minor role in controlling feeding behavior in birds. In amphibians, the presence of abundant orexin fibers in the amphibian LHA (Lopez et al., 2009a; Suzuki et al., 2008), suggests an important role of orexin in the regulation of feeding. In fish, icv-injection of human orexins induces feeding in goldfish, whereas intraperitoneal (ip)-injection of glucose or anti-orexin serum reduces food consumption (Nakamachi et al., 2006; Volkoff et al., 1999). Like in mammals, orexin A also induces a more potent stimulatory effect in feeding than orexin B in goldfish and fasting increases prepro-orexin brain expression and physical activities in zebrafish (Novak et al., 2005). Furthermore, orexins are suggested to work interdependently with other peptides involved in food intake control. This was demonstrated by the colocalization of NPY and orexin A neurons in the NPPv and the close contact of their respective nerve endings (Kojima et al., 2009). In addition, a mutual relationship of orexins with ghrelin regarding food intake has also been shown; icv of ghrelin at a dose sufficient to stimulate food intake is able to simulate expression of orexin A mRNA in the goldfish diencephalon and the opposite is also found to be true (Miura et al., 2007). Both NPY- and ghrelin-induced food intake were completely inhibited by application of an orexin receptor

antagonist (Miura et al., 2007).

1.5.2 Sleep/wake cycle

Regulation of sleep/wakefulness is an important function of orexins. Sleep and wakefulness are regulated to occur at appropriate times that are in accordance with our internal and external environments. Avoiding danger and finding food, which are life-essential activities that are regulated by emotion, reward and energy balance, require vigilance and therefore, by definition, wakefulness. The orexin (hypocretin) system regulates sleep and wakefulness through interactions with systems that regulate emotion, reward and energy homeostasis (Sakurai, 2007). At the anatomic level, orexinergic neurons are present and project to a multitude of nuclei at different levels of CNS involved in sleep/arousal (Sakurai, 2007). Endogenous orexin A levels display diurnal variation, with increased concentrations in the pons during arousal (Taheri et al., 2000). Orexins are proposed to maintain the balance between the sleep and wake stages. Orexin neurons are active during the wake stage but are in low activity during the sleep stage. During the wake stage, orexin neurons send stimulating signals to depolarize the “arousal center” (Saper et al., 2001; Yoshida et al., 2006). This has been demonstrated by *in vitro* experiment where orexin A application to the rat brain slices induces dose-dependent depolarization of serotonergic and cholinergic neurons (Brown et al., 2001; Takahashi et al., 2002). *In vivo* experiments, icv of orexins can induce hyper-locomotion and stereotypy (Nakamura et al., 2000). During the sleep stage, the ventrolateral preoptic nucleus (VLPO) is suggested to send inhibitory signals to both orexin neurons and the “arousal center” (Saper et al., 2001; Yoshida et al., 2006). In birds, icv injection of mammalian orexin A induces potent and dose-dependent arousal-promoting effects. It also increased alertness of the chicken (Katayama et al., 2010). In the pigeon, these changes are

accompanied by a substantial increase in head and neck exploratory movements as well as in wing-flapping behavior (da Silva et al., 2008). In amphibians, orexin fibers are found in areas similar to the mammalian “arousal center”. In fact, orexin fibers are present in the raphe nuclei, cholinergic nuclei in the isthmus and upper rhombencephalon and also in histaminergic cells similar to those in the mammalian tuberomammillary nucleus (Lopez et al., 2009a; Suzuki et al., 2008). Furthermore, amphibian orexin fibers also innervate cholinergic and non-cholinergic region of the pedunculopontine tegmental nucleus which has been described to be important in regulation atonia during REM sleep in mammals (Gonzalez et al., 2002; Lopez et al., 2009a; Marin et al., 1997; Takahashi et al., 2005; Takakusaki et al., 2004). From the immunostaining pattern of orexins in amphibians, it is suggested that orexins also play a basic role in the sleep–wake cycle, acting as arousal agents. In fish, the effects of orexins differ in larva and adult fish. Overexpression of orexin in zebrafish larva leads to reduced arousal threshold and hypersensitivity to arousing stimuli (Prober et al., 2006). ICV of orexin A in normal adult zebrafish reduced activities and promoted sleep. However, OX2R knockout adult fish showed sleep fragmentation and decreased sleep at night (Yokogawa et al., 2007). Based on experiments performed on larva and adult fish, it is difficult to conclude whether orexin acts as an arousal agent or sleep inducer. This is complicated by the expression pattern of orexin in fish brain which has been shown present in both “arousal” and “sleeping” centers. Orexinergic fibers are found in a single cluster of histaminergic neurons in ventrocaudal hypothalamus, raphe nucleus, isthmus and upper rhombencephalic tegmentum resembling the mammalian “arousal center” (Kaslin and Panula, 2001; Kaslin et al., 2004; Lopez et al., 2009b). Orexinergic fibers and OX2R are found in melatonin-releasing pineal gland, and orexins are shown to stimulate pineal gland explants to release melatonin (Appelbaum et al., 2009; Yokogawa et al., 2007; Zhdanova et al., 2001). As melatonin is the

main sleep-promoting agent in fish, it is proposed that fish orexins have dual effects on the sleep–wake cycle similar to those in mammals; however the regulatory mechanisms is not clear. In mammals and fish, there are at least two separate centers for controlling sleep and wake, and future usage of orexin/receptor cell-specific knockouts should provide the needed information to characterize the function of this ligand-receptor axis in regulating sleep/wake cycle in vertebrates.

1.5.3. Reproduction

In mammals, orexinergic fibers are found in the lateral hypothalamus and are extended in the septal-preoptic and arcuate nucleus-ME regions. These areas are important for the the control of the hypothalamo-gonadotropic axis through the synthesis and release of the gonadotropin releasing hormone (GnRH). Orexin A stimulates GnRH expression and release in GnRH neurons, (Iqbal et al., 2001; Martynska et al., 2006 ; Pu et al., 1998 ; Sasson et al., 2006). Orexins and their receptors are found in the gonads of rat and sheep (Tafari et al., 2009; Zhang et al., 2005), and their expression is related to the sex and estrous cycle whereby young cycling females have higher expression than males or middle-aged females without cycle. The expression of orexin receptors is augmented in the ovary in proestrous, during which icv of orexin A in female rats results in luteinizing hormone (LH) and prolactin surges that are essential for ovulation (Johren et al., 2001; Porkka-Heiskanen et al., 2004; Russell et al., 2001a). It has also been suggested that orexins can regulate the male reproductive system as testicular testosterone secretion could be stimulated by intratesticular injection of orexin A (Barreiro et al., 2004). In birds, OX2R is found in the pituitary and the testis, and prepro-orexin has been detected in the gonads of both sexes, thus suggesting a important neuroendocrine role of orexin in bird reproductive function (Ohkubo et al., 2002, 2003; Russell et al., 2001a). In amphibians, orexin fibers are found in the

paraventricular organ (PVO), which suggests its involvement in sexual behavior since PVO-lesioned males display a characteristic impairment in courtship behavior (Dube et al., 1990; Suzuki et al., 2008). In fish, orexinergic fibers are found to reach the ME in lungfish, orexin A is detected in the pituitary of medaka and Japanese sea perch, whilst orexin B was detected in the pituitary LH cells of Nile tilapia (Amiya et al., 2007; Lopez et al., 2009b; Suzuki et al., 2007, 2009). However, the effect which orexins exert on the fish reproductive system may be different to from that of mammals, icv of GnRH was found to suppress food intake and decrease orexin mRNA expression in goldfish in a dose-dependent manner. Alternatively, icv of orexin A inhibited spawning behavior and lowered GnRH expression (Hoskins et al., 2008). From the data obtained, orexins seem to act as inhibitory agents in reproduction.

1.5.4. Orexins in autonomic/endocrine functions

Orexinergic efferents are present in particular areas of the brain stem and spinal cord, which suggest a direct involvement in autonomic and endocrine functions (Bingham et al., 2001; Cutler et al., 1999; Date et al., 2000; Mintz et al., 2001; van den Pol, 1999). The icv of orexin A induces a autonomic effects as increase of blood pressure, heart rate, intestinal motility, gastric acid secretion (Okumura et al., 2001; Takahashi et al., 1999) and sympathetic nerve activity (Kunii et al., 1999; Monda et al., 2001; Shirasaka et al., 1999). Orexins may affect the water balance via effects on drinking, water/salt homeostasis in fact, icv of orexin A and B increase water intake in the rat (Kunii et al., 1999; Rodgers et al., 2000). Injection of orexin A or B into particular brain stem sites increases mean arterial pressure and heart rate in the rat (Antunes et al., 2001; Chen et al., 2000; Samson et al., 1999; Shirasaka et al., 1999). Orexins may regulate hypothalamo-hypophysial hormone secretion indirectly via neuronal circuits, but also a direct effect on the hypophysis is possible (Lopez et al., 2010) (Fig. 4).

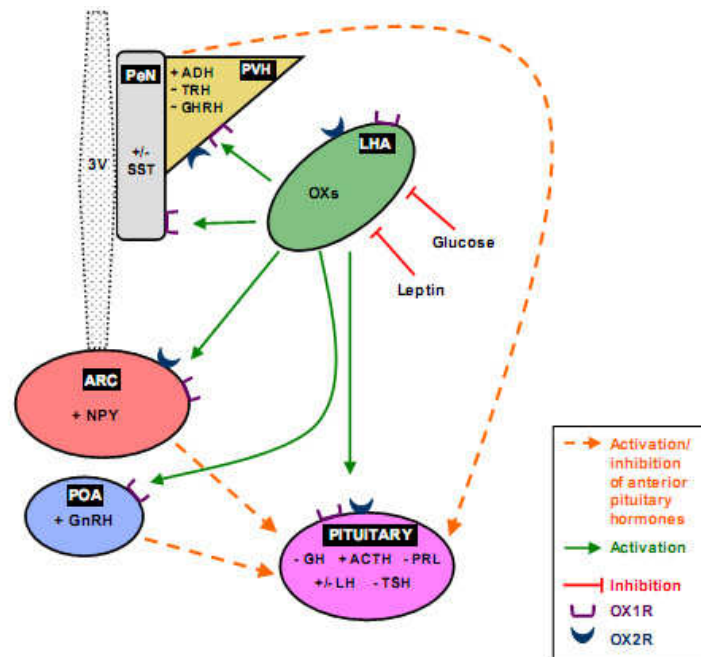


Fig. 4. Summary of neuroendocrine actions of orexins/hypocretins. Orexin/hypocretin neurons in the LHA sense the plasma levels of circulating hormones regulating energy homeostasis (such as leptin) and nutrients (such as glucose), linking metabolic state to hormonal secretion from pituitary. In order to simplify the figure the dorsomedial (DMH) and ventromedial (VMH) nuclei of the hypothalamus and the relations between hypothalamic nuclei have been omitted. ARC: arcuate nucleus of the hypothalamus; LHA: lateral hypothalamic area; POA: preoptic area; PeN: periventricular nucleus of the hypothalamus; PVH: paraventricular nucleus of the hypothalamus; 3 V: third ventricle.

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Orexins are found in ME (Date et al., 2000; Mondal et al., 1999), and mRNA for orexin receptor subtypes is found in anterior and intermediate hypophysial lobes (Date et al., 2000). There is a co-expression of growth hormone and OX1 receptors in acidophil cells of adenohypophysis and co-expression of adrenocorticotrophic hormone (ACTH) and OX2 receptors in pars intermedia and basophil cells of adenohypophysis (Blanco et al., 2001). Orexins and orexin receptors are also found at low levels in the posterior hypophysial lobe. Both OX1 and OX2 mRNA are expressed in the areas that are important in neuroendocrine regulation (Marcus et al., 2001). Orexin A injected intravenously or directly into the hypothalamic paraventricular nucleus decreases thyroid-stimulating hormone (TSH) release in the rat (Mitsuma et al., 1999; Russell et al., 2002), but no change has been seen in the plasma

levels of thyroid hormones (Russell et al., 2002; Mitsuma et al., 1999). ICV of orexin A decreases growth hormone release in the rat (Hagan et al., 1999). Effects on the release of other hormones have also been reported, but the results are equivocal. Some release effects have been shown in hypothalamic explants in vitro: orexin A stimulates release of neurotensin, NPY, VIP, somatostatin, LHRH and decreases the release of TRH but has no effect on the release of dopamine, vasopressin, or melanin-concentrating hormone (MCH) (Mitsuma et al., 1999; Russell et al., 2000, 2001b). ICV administration of orexin A increases plasma levels of corticotrophin releasing factor (CRF), ACTH, corticosterone, vasopressin, and epinephrine in the rat (Al-Barazanji et al., 2001; Hagan et al., 1999; Ida et al., 1999; Jaszbereny et al., 2000, 2001; Russell et al., 2000, 2001b). Orexin A can even stimulate CRF release in vitro (Russell et al., 2000). Orexins have also been reported to stimulate release of corticosterone/cortisol (Malendowicz et al., 1999, 2001a; Mazzocchi et al., 2001a), and aldosterone (Malendowicz et al., 2001b; Nanmoku et al., 2002), from rat, porcine, or human adrenal glands/adrenal cells in vitro. Release of epinephrine and norepinephrine from rat adrenal medulla is increased by icv injections of orexins (Shirasaka et al., 1999), and a similar but smaller effect is seen in cultured porcine adrenal medullar cells (Nanmoku et al., 2000). Both OX1 and OX2 receptor mRNA have been suggested to be expressed in the rat adrenal medulla (Lopez et al., 1999), although other studies present opposing views (Johren et al., 2001). Orexin-mediated increases in CRF, ACTH, corticosterone, aldosterone, vasopressin, and epinephrine release suggest that orexins could mediate stress responses. In the gastrointestinal tract, orexin immunoreactivity is seen both in the submucosal and myenteric plexi. Orexinergic neurons in these plexi have been suggested to be sensory or secretomotor on the basis of the co-expression of other markers such as VIP and choline acetyltransferase (Kirchgessner and Liu, 1999; Naslund et al., 2002). The neurites project to other orexin-

containing cells and to mucosa, muscle layers, and submucosal blood vessels. In the guinea pig, all the orexin-positive neurons of submucosal and myenteric plexi express leptin receptors as well (Kirchgessner et al., 1999). Fasting increases the number of orexin A immunoreactive submucosal neurons in the guinea pig, most of which also become positive for phospho-cAMP response element binding protein (pCREB) (Kirchgessner et al., 1999). This, similar to c-fos expression, indicates activation of the neurons, suggesting that fasting activates both central and peripheral orexinergic systems. Also, some endocrine cells in the gut and stomach some of which are enterochromaffin as they express serotonin display orexin immunoreactivity in mouse, rat, guinea pig, and human (Kirchgessner et al., 1999; Naslund et al., 2002). Thus orexinergic neurons in the gastrointestinal tract could affect intestinal secretion and uptake, endocrine secretion, sensory signaling, and intestinal motility. Different effects on intestinal motility patterns have indeed been measured in mouse and rat small intestine (Naslund et al., 2002; Satoh et al., 2001), and in guinea pig distal colon (Kirchgessner et al., 1999). Different orexin receptor subtypes have different distribution in the gastrointestinal tract; OX1 receptor immunoreactivity is solely expressed by the submucosal and myenteric neurons, whereas OX2 receptor immunoreactivity is only seen in the enterochromaffin cells in the rat (Naslund et al., 2002). Energy metabolism is regulated by several hormones, the most important being pancreatic glucagon and insulin, hypophysial growth hormone, glucocorticoids, (nor)epinephrine, and thyroid hormones. Insulin release is somewhat increased in the rat by both icv (Matsumura et al., 2001), and subcutaneous (Nowak et al., 2000; Switonska et al., 2002), injection of orexin A, and also in the rat pancreas in vitro (Nowak et al., 2000). Extrinsic neurons in rat and guinea pig pancreatic ganglia display orexin and OX1 and OX2 receptor immunoreactivity (Kirchgessner et al., 1999). Plasma leptin is also increased by subcutaneous injection of orexin A or B in the rat, although this has been suggested to occur via

increase insulin release (Switnoska et al., 2002).

1.6. Aims of this study

In contrast to the situation in mammals, little is known concerning the orexin system and their functional significance in birds. Therefore, in order to probe our knowledge of chick hypothalamic distribution of orexins, especially as regards the fibers system, and to clarify the possible functions of orexins in basal and experimental conditions, in this study the orexin-ir distribution in the chick hypothalamus was determined and the differences in the hypothalamic orexin mRNA concentrations during the day-night cycle and in feed and food restricted conditions was tested. For that purpose were used broiler and layer chicks, two different breeds of chickens with striking phenotypic difference that provide excellent models for studying the mechanisms regulating feeding behavior and/or sleep and wakefulness (Furuse, 2002; Furuse et al., 2007).

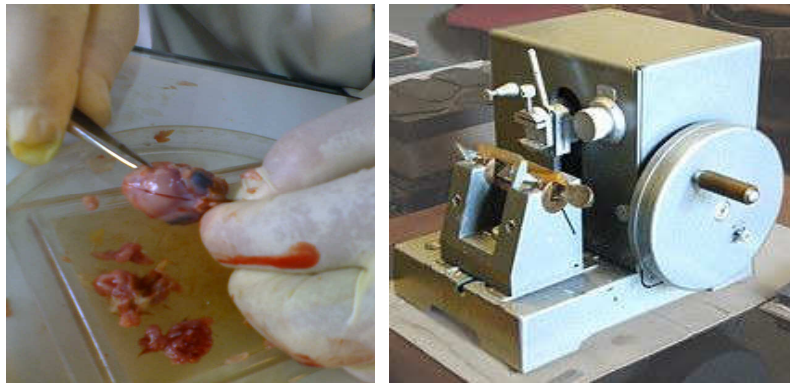
2. MATERIALS AND METHODS

2.1. *Birds*

All birds utilized in this study were reared and maintained on fourteen hours of light per day. Immunocytochemical observation were made on two week-old female (n=5) and male (n=6) broiler type birds. To test the hypothesis that chronic food restriction alters the expression of orexin in the basal hypothalamic mRNA measurements were made on female broilers (Ross 503) fed on a commercial restriction programme (~25% of ad-libitum) to 6 weeks of age. These were reared and maintained in groups of 4/5 in floor pens. From 6 weeks of age half of the birds were maintained on the commercial restriction (n=10) and the other half were fed ad-libitum till 12 weeks of age (n=10) when all birds were killed with an overdose of sodium pentobarbital. Additionally layer type white leghorn hens were hatched and reared with ad libitum access to food and were killed at the same day and age (n=10). To test the hypothesis that the expression of orexin in the basal hypothalamic mRNA may be related to activity basal hypothalamic samples were collected from 6 week old hens white leghorn chickens reared in three pens between 1 and 2 hours after lights off and between 1 and 2 hours after lights on (n=8).

2.2. *Immunohistochemistry*

All brains were rapidly dissected immediately after death and placed in Bouin's fixative for 48h for immunocytochemistry. Then they were processed as previously described in detail (Esposito et al., 1993), in order to have 7 μm sagittal and coronal paraffin sections.



Immunohistochemistry were performed on hypothalamic sections using two different methods: the peroxidase-antiperoxidase (PAP) and avidin biotin complex (ABC). Regarding the PAP method, deparaffinized and rehydrated sections were initially washed in 0.01 M phosphate-buffered saline (PBS; pH 7.4), subsequently incubated in 3% H₂O₂ for 20 min at room temperature (RT) to inhibit endogenous peroxidase activity, rinsed for 10 minutes in PBS, and then incubated in normal rabbit serum (Vector Laboratories), diluted 1:5 in PBS for 30 min at RT to reduce background staining. The sections were incubated overnight at 4°C in the primary antibody (goat anti-orexin A and goat anti-orexin B; both supplied by Santa Cruz Biotechnology, Santa Cruz, CA, USA, catalog number sc-8070 and sc-8071 respectively) diluted 1:500 and 1:1000 in PBS. Each is a polyclonal, affinity-purified antibody raised against a peptide corresponding to a 19 amino acid sequence at the C-terminus of the respective orexin peptides. After several rinses in PBS, the sections were incubated for 30 min at RT with secondary antibody anti- goat IgG (Dako) diluted 1:50 in PBS, washed in PBS, and subsequently treated for 30 min at RT with the PAP complex (Dako) diluted 1:100 in PBS. All incubations were performed in moist chambers. The peroxidase reaction was revealed with a fresh solution containing 10 mg of 3,3'-diaminobenzidine in 15 ml of 0.5 M Tris-buffer, pH 7.6, and 1.5 ml of H₂O₂ at 0.03%. Some of the sections were counterstained with cresyl violet to aid in delineation and identification of different structures. All sections were dehydrated and coverslipped,

and then observed and photographed using a Leitz Aristoplan microscope. Regarding the ABC method, the sections deparaffinized and rehydrated were washed in 0.01 M phosphate-buffered saline (PBS; pH 7.4), then incubated in 3% H₂O₂ for 20 min at RT, to inhibit endogenous peroxidase activity, rinsed in PBS for 10 min, and then incubated in normal rabbit serum (Vector Laboratories, code S.5000,) diluted 1:66 in PBS (150 µl in 10 ml PBS), for 30 min, to reduce background staining. The sections were incubated overnight at 4°C with the primary antibody (goat anti-orexin A and goat anti-orexin B; both supplied by Santa Cruz Biotechnology, Santa Cruz, CA, USA, code sc-8070 and code sc-8071 respectively), diluted 1:500 and 1:1000 in PBS; after several rinses in PBS, the sections were incubated for 30 min at RT with secondary antibody diluted 1:200 (DAKO), and then washed in PBS and after treated for 30 min at RT with the Vetastain elite ABC kit (PK 6101, Vector Laboratories). The peroxidase reaction was revealed with a fresh solution containing 10 mg of 3,3'-diaminobenzidine in 15 ml of 0.5 M Tris-buffer, pH 7.6, and 1.5 ml of H₂O₂ at 0.03%. Some sections were counterstained with cresyl violet to aid in delineation and identification of different structures. All sections were dehydrated and coverslipped, and then observed and photographed using a Leitz Aristoplan microscope. For anatomical identification of the nervous structures, some deparaffinized and rehydrated sections were stained with Luxol fast blue/cresyl violet. The specificity of the immunohistochemical reactions (both PAP and ABC methods) was tested with controls that included: 1) staining of some selected sections with preimmune goat serum 2) controls in which either the primary antibody, secondary antibody or the PAP complex was omitted 3) homologous and heterologous preabsorptions of the primary antibody with synthetic blocking peptides for orexin-A or orexin-B (both of Santa Cruz Biotechnology; codes sc-8070P and sc-8071P respectively; 0.1, 1.0, or 10 µM). In all these negative controls, the immunostaining was eliminated, even when the goat anti-orexin A

or goat anti-orexin B was preabsorbed with the blocking peptides at low concentration (0.1 μ M).

2.3. Orexin mRNA measurements

The hypothalamic dissection was carried out within 3-5 min through the base of the skull after removing the anterior pituitary gland and neural lobe, the dissected tissue was immediately frozen in liquid nitrogen and subsequently stored at -80°C for orexin mRNA measurements. The basal hypothalamus was delineated by cuts 1 mm to either side of the third ventricle and immediately along the caudal margin of the optic chiasma and immediately rostral to the roots of the oculomotor nerves. The weight of dissected basal hypothalamus was 74 \pm 3mg. For the experiment comparing expression between night and day, 2/3 chicks were sampled from each of 4 pens 60 mins after lights on in the morning (08:30) and 60 minutes after lights off (22:30) (n=9). In the second study, a comparison was made of broilers fed *ad libitum*, and broilers fed a diet restricted to 25% of *ad libitum* intake, following commercial practice. RNA was extracted from each basal hypothalamii using 1ml Ultraspec II in a lysing matrix D tube (*Bioteck, Texas, USA, product code BL1205*) following the supplier's instructions. The amount of total RNA produced was quantified using a nano drop ND 1000 spectrophotometer. Reverse transcription (RT) was completed using either 1 μ g or 5 μ g of total RNA and a first strand cDNA synthesis kit with notI-d(T) 18 primer (GE health care). Newly synthesized cDNA was stored at 20°C prior to measurement by real time PCR. PCR amplification was achieved using Sybergreen and rox with the primers orex 1F (CTT GGC CAC CTG AAG ACA C) and orex 3 R (GGG TCA CCG TAG GCT GAG T) primers. Quantitative PCR measurements were done in triplicate or duplicate in microamp optical 96 well-reaction plates. The PCR programme was 50°C (2 min); 95°C (2 min), 40 cycles 95°C for 15 sec and 60° for 30 sec, and 1 cycle of 60° for 30 sec and 95°C for 15 sec. For the control

lamin-B receptor was used LBr-F (GGT GTG GGT TCC ATT TGT CTA CA) and LBr-R (CTG CAA CCG GCC AAG AAA) primers.

3. RESULTS

3.1. Quantification of hypothalamic mRNA expression by real-time RT-PCR.

There were significant differences in abundance of orexin mRNA in the basal hypothalamus between broiler type chickens fed ad-libitum, broiler type chickens fed restricted and layer chickens (ANOVA; $P=0.019$ (fig. 5). The food restricted broiler chickens with expressed significantly higher orexin mRNA ($P < 0.01$) in the basal hypothalamus compared to broiler chicks fed ad-libitum. There was no statistical difference between restricted broiler chickens and white leghorn layers whilst the difference between ad-libitum fed broiler chickens and white leghorn layers was significant ($P < 0.05$). No significant difference was found for orexin mRNA expression in the hypothalamus of chicks when levels were contrasted between night and day ($P=0.621$) (Fig. 6).

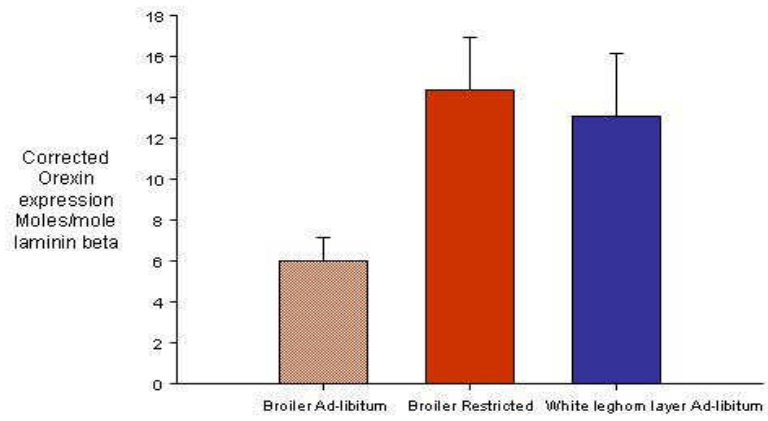


Figure 5. Compared expression of orexin mRNA in the broiler chickens feeding ad libitum and feed restricted and in the layer white leghorn chickens feeding ad libitum with RT-PCR

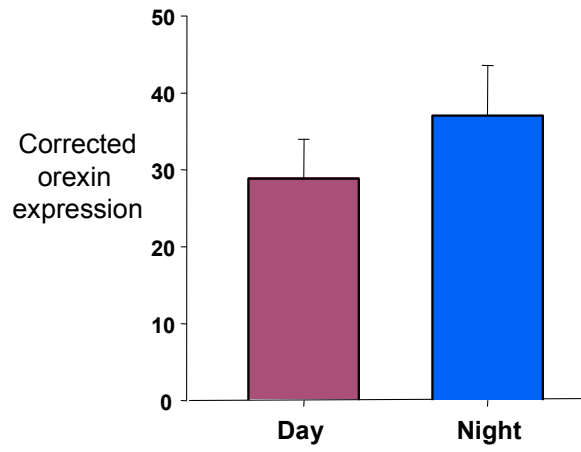
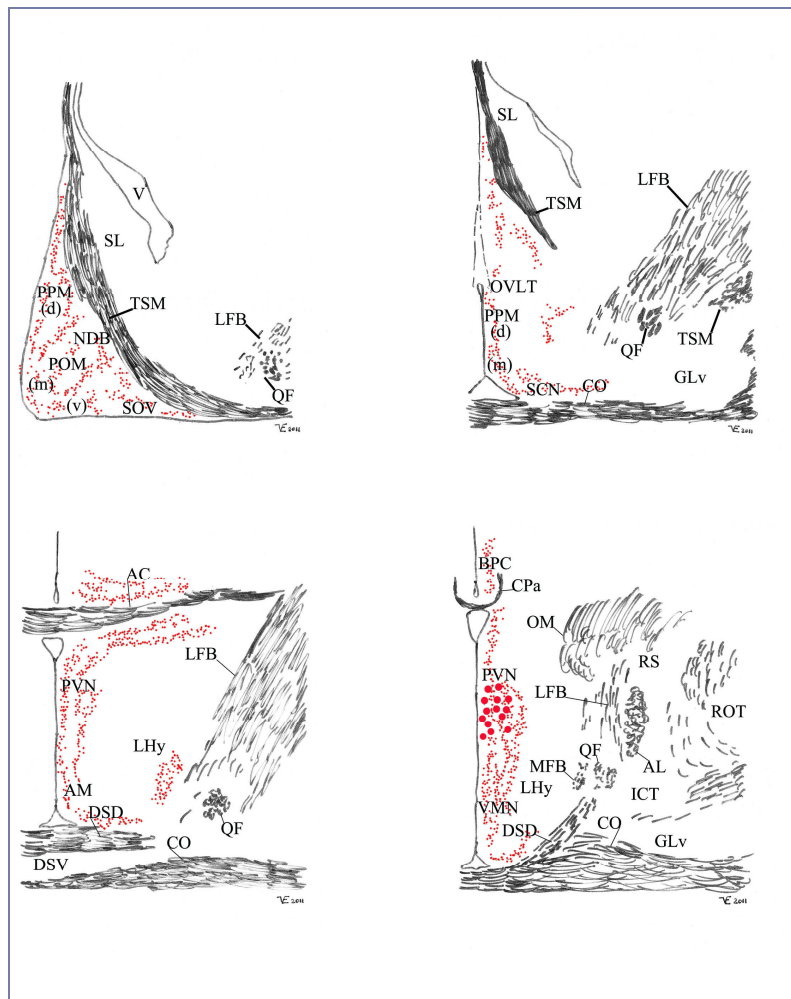


Fig. 6 Compared expression of orexin mRNA during day-night cycle in white leghorn chickens

3.2. Immunohistochemical distribution of hypothalamic orexin-ir cells and fibers.

Orexin-ir cells and fibers were observed in different areas of the chick hypothalamus (Fig. 7). Morphologically, the majority of orexin positive neurones, which were similar in size and shape, appeared typically spherical and had several primary dendrites with few secondary branching. Nomenclature of anatomical structures referred to the chick brain atlases of Kuenzel and van Tienhoven (1982), Kuenzel and Masson (1988) and Reiner et al. (2004).



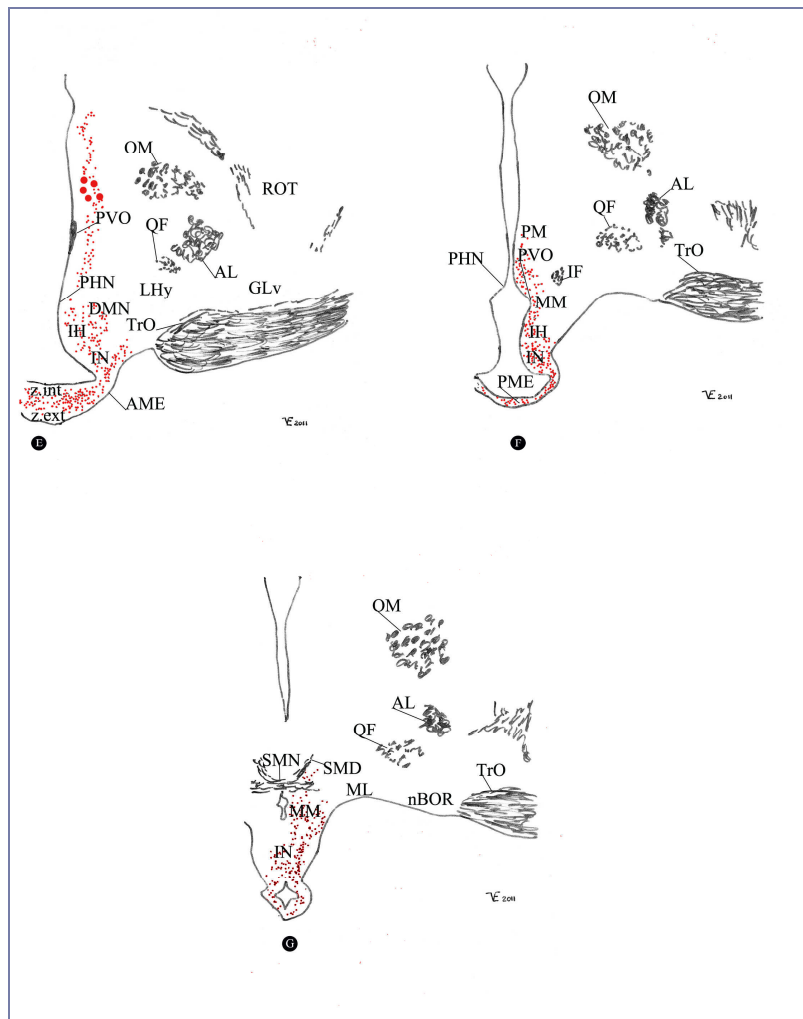


Fig. 7 Schematic drawings of cross sections of the chicken hypothalamus. Each drawing made by Prof. Vincenzo Esposito (Department of Biological Structure, Functions and Technologies, University of Naples Federico II) refers to the specific anterior-posterior hypothalamic atlas plate in Kuenzel and van Tienhoven (1982). They were suitably modified and nomenclature presented for hypothalamic structure refers to atlases of Kuenzel and van Tienhoven (1982), Kuenzel and Masson (1988) and Reiner et al. (2004). See abbreviations on page 34.

ABBREVIATIONS: AL, ansa lenticularis; AM, nucleus rostralis (anterior) hypothalami; AME, z. ext. z. int., anterior median eminence, zona externa, zona interna; BPC, bed nucleus pallial commissure; AC, anterior commissure; CO, optic chiasma; CPa, pallial commissure; DMN, nucleus dorsomedialis hypothalami; DSD, dorsal supraoptic decussation; DSV, ventral supraoptic decussation; GLv, nucleus geniculatus lateralis, pars ventralis; ICT, nucleus intercalates thalami; IF, infundibular tract; IH, nucleus inferior hypothalami; IN, nucleus infundibuli; LFB, lateral forebrain bundle; LHy, region (area) lateralis hypothalami; MFB, medial forebrain bundle; ML, nucleus mamillaris lateralis; MM, nucleus mamillaris medialis; nBOR, nucleus basal optic root (nucleus ectomamillaris); NDB, nucleus diagonalis Brocae; nCPa, nucleus commissurae pallii (Bed nucleus pallial commissure); OM, occipitomesencephalic tract; OVLT, organum vasculosum of the lamina terminalis; PHN, nucleus periventricularis hypothalami; PM, nucleus premamillaris; PME, posterior median eminence; POM, nucleus preopticus medialis; PPM (or MPO) (d, m, v), nucleus magnocellularis preopticus (dorsalis, medialis, ventralis); PVN, nucleus paraventricularis; PVO, paraventricular organ; QF, quintofrontal tract; ROT, nucleus rotundus; RS, nucleus reticularis superior; SCN, nucleus supraschiasmaticus; SL, nucleus septalis lateralis; SMD, supramamillary decussation; SMN, nucleus supramamillaris interstitialis; SOV, nucleus supraopticus ventralis; TRo, optic tract; TSM, septomesencephalic tract; V, ventricle; VMN, nucleus ventromedialis hypothalami; VIII, ventriculus tertius (third ventricle).

For our description, according to Kuenzel and van Tienhoven (1982) we divided the hypothalamus in three regions.

Anterior (preoptic) hypothalamic region

In this region that have the anterior wall of the third ventricle (VIII) as its rostral border and end at beginning of the anterior commissure (CA), only orexin-ir fiber were showed (**Fig. 7 a-c**). They were mainly located in ventral and periventricular arrangement and appeared as punctuate structures intermingled between the cells of all divisions of the nucleus magnocellularis preopticus (PPM or MPO), the nucleus supraopticus ventralis (SOV), the nucleus suprachiasmaticus (SCN) and the nucleus preopticus periventricularis (PPN) (**Fig. 8**). Some orexin-ir fibers were seen also in the organum vasculosum of the lamina terminalis (OVLT). At most posterior border of this region, orexin-ir fibers surrounded the CA (**Fig. 9**).

Midhypothalamic (tuberal) region

This region contains both orexin-ir cells and fibers (**Fig. 7 d,e**). The orexin-ir cells appeared to form a single group of neurons previously located in periventricular position and belonging to the parvocellular component of the nucleus paraventricularis (PVN) (**Fig. 10**) than more caudally located in lateral position and apparently belonging to the stratum cellulare externum (**Fig. 11**). Intermingled between these cells orexin-ir fibers were seen. In the most rostral coronal hypothalamic sections of this region, dorsally orexin-ir fibers surrounded the pallial commissure (CPa) (**Fig. 12**) while ventrolaterally they lapping on the dorsal supraoptic decussation (DSD) (**Fig. 13**). Very scarce orexin-ir fibers were seen in the region lateralis hypothalami (LHy). In the most posterior coronal sections were seen orexin-ir fibers in the area of the nucleus inferior hypothalami (IH) and the nucleus infundibuli (IN). This region is characterized ventrally by the presence of the anterior median eminence (AME). Very thin orexin-ir fibers were

observed to extend both in the zona interna and the zona externa of AME (Figs. 13,14). Some of these fibers appearing as punctate structures surround the vessels of portal vasculature (Fig. 14).

Posterior (infundibular) hypothalamic region

The rostral border of this region is marked by the medial mamillary nucleus (MM) while the supramamillary decussation (SMD) is a landmark indicating the end of the hypothalamus (Fig. 7 f,g). In this region orexin-ir fibers were observed to extend in the area occupied by the IH and the IN. Moreover, orexin-ir fibers were also founded in the posterior median eminence (PME) (Fig. 15).

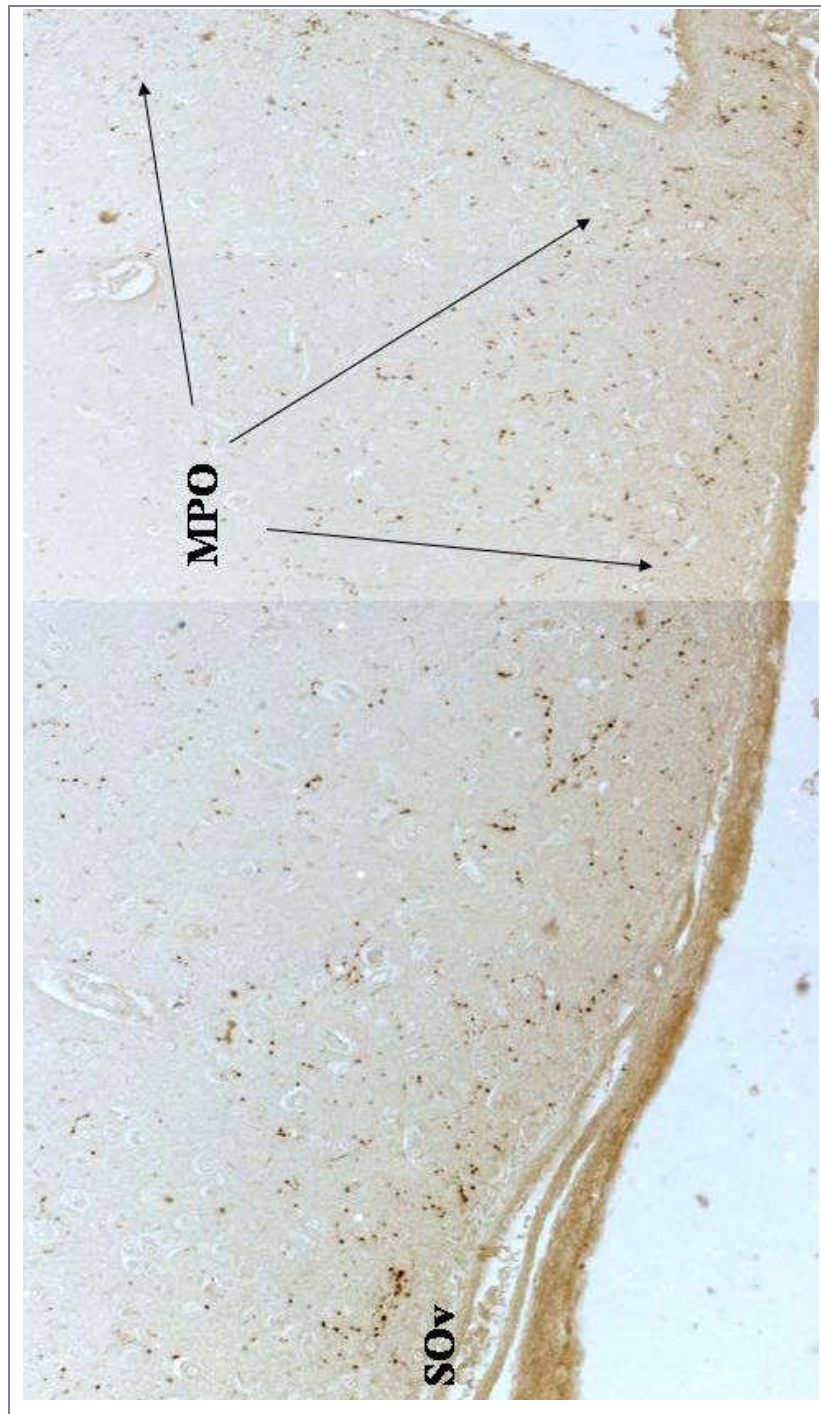


Fig. 8 Microphotographic reconstruction showing orexin-ir fibers between the cells of all divisions of the nucleus magnocellularis preopticus (MPO) and the nucleus supraopticus ventralis (SOV). PAP method. 350 X. See abbreviations on page 34.

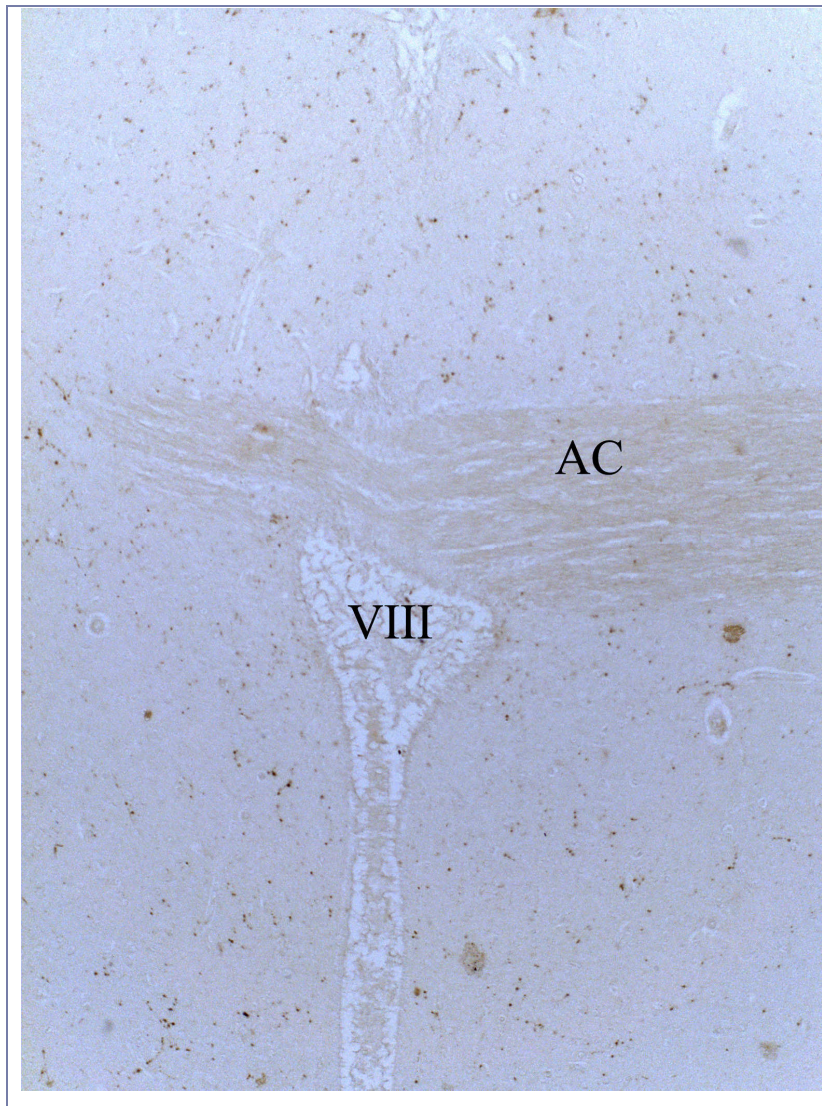


Fig. 9 Microphotograph showing orexin-ir fibers surrounding the anterior commissure (CA). ABC method. 200X. See abbreviations on page 34.

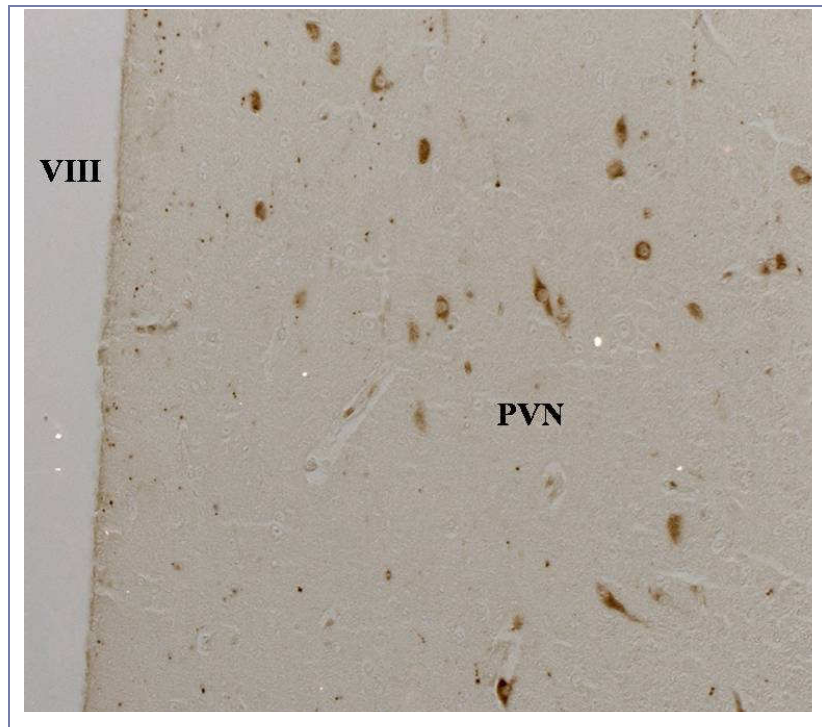


Fig. 10 Microphotograph showing orexin-ir cells and fibers in the nucleus paraventricularis (PVN). PAP method. 500X. See abbreviations on page 34.

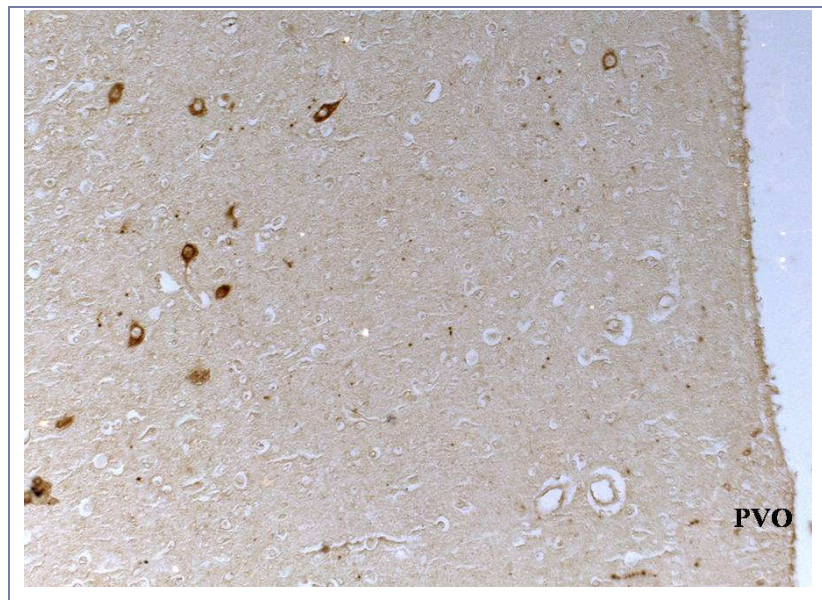


Fig. 11 Microphotograph showing orexin-ir cells dorso-laterally to the paraventricular organ (PVO). PAP method. 500X. See abbreviations on page 34.



Fig. 12 Microphotograph showing orexin-ir fibers surrounded the pallial commissure (CPa) and intermingled to cells of the nucleus of pallial commissure (nCPa). ABC method. 350X. See abbreviations on page 34.

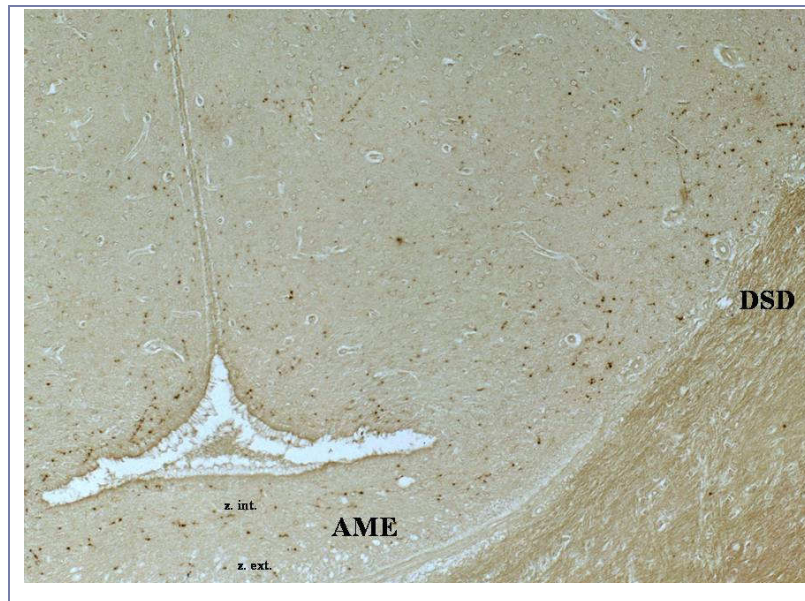


Fig. 13 Microphotograph showing orexin-ir fibers dorsally to the dorsal supraoptic decussation (DSD) and in the anterior median eminence (AME). PAP method. 250X. See abbreviations on page 34.

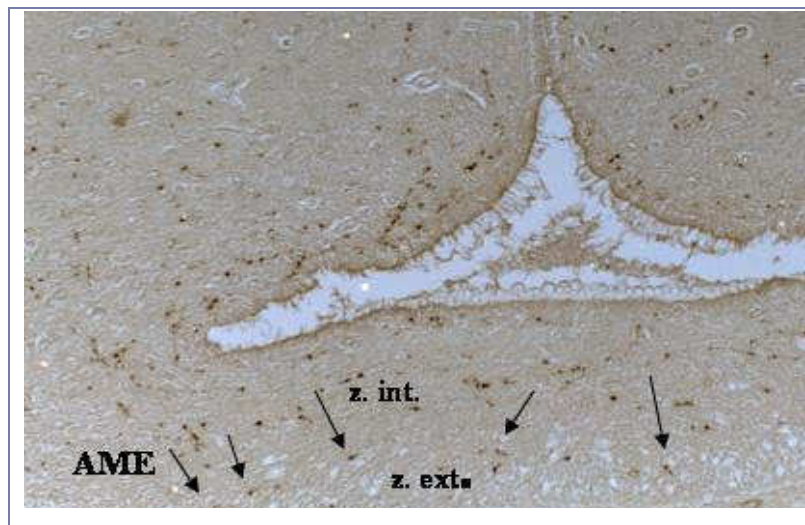


Fig. 14 Microphotograph showing orexin-ir fibers in the zona interna (z. int.) and zona externa (z. ext.) of the anterior median eminence (AME). PAP method. 350X. See abbreviations on page 34.

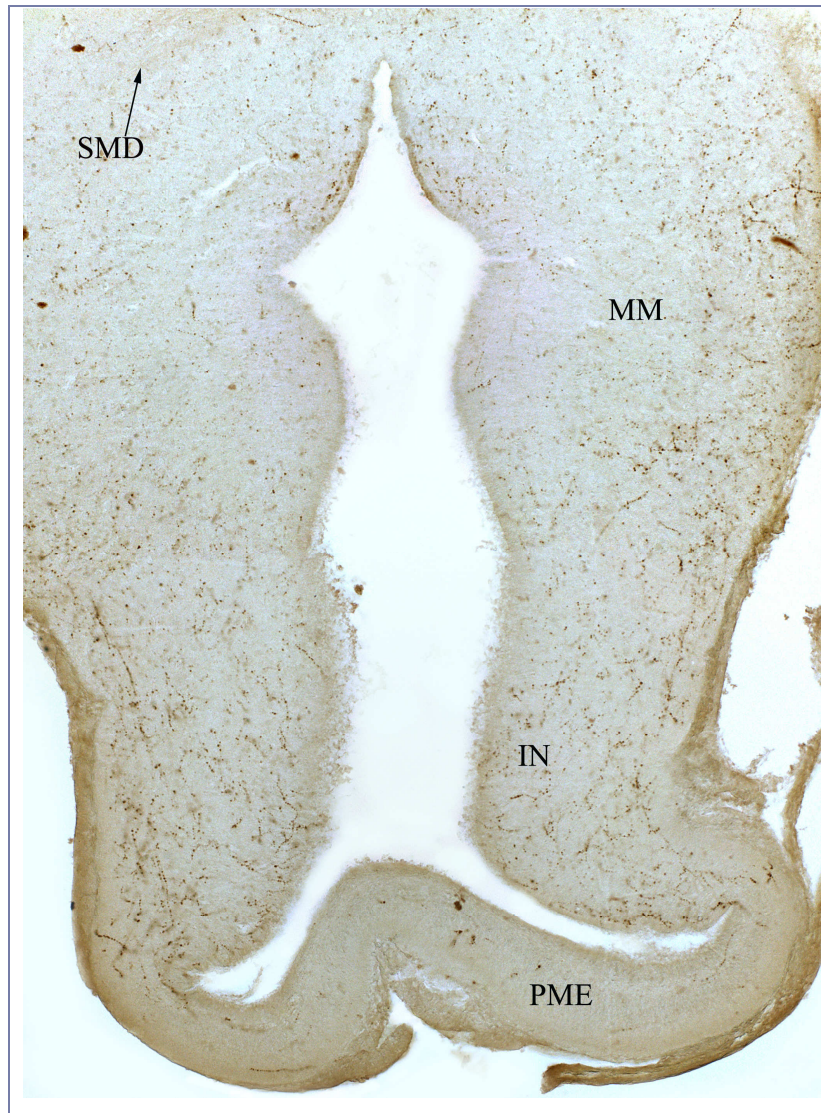


Fig. 15 Microphotograph reconstruction showing orexin-ir fibers in the tuberal hypothalamic region. Immunoreactive fibers were seen under the supramammillary decussation (SMD), in the area occupied by the nucleus mamillaris medialis (MM) and nucleus infundibuli (IN) and in posterior median eminence (PME). PAP method. 250X. See abbreviations on page 34.

4. DISCUSSION

The antibodies against orexin-A and orexin-B used in the present study revealed patterns of immunoreactivity that were constant from animal to animal. No differences were observed in the pattern of immunoreactivity when using anti-orexin-A or anti-orexin-B antisera and, therefore, I will refer to orexin-ir structures. The antisera used in this study (both of Santa Cruz Biotechnology) were raised against a peptide mapping at the C-terminus of orexin A or B of human origin. Both neuropeptides are very preserved in all vertebrates, especially in his half C-terminus (Sakurai et al., 1998; Shibahara et al., 1999). This would explain why there were no differences in the pattern of staining that they yielded (Singletary et al., 2005, 2006; Lopez et al., 2009 a,b; present study) and thus, these antibodies are good tools for detecting these neuropeptides in non-mammalian vertebrates. Moreover, there were no substantial differences in the pattern of orexin-ir obtained with the PAP method compared to those obtained with the ABC method. My immunocytochemical observations shown orexin-ir neurons in a hypothalamic paraventricular population that expands to the stratum cellulare externum. They are in agreement with previous findings of the orexin-ir cell brain distribution in birds (Ohkubo et al., 2002; Phillips-Singh et al., 2003; Singletary et al., 2006) and confirm the opinion of an high level of conservation of this orexin-ir cell population among the birds. This pattern of distribution appear to be intermediate between the medial periventricular distribution described for amphibians (Shibahara et al., 1999) and the dorsolateral localization reported for laboratory rodents (Sakurai et al., 1999). Orexin-ir distribution varies slightly across vertebrates, suggesting the orexin system may support different functions based on diverging physiological needs. Given the location of cells in birds, studies were done to determine if orexin was involved in the regulation of feeding as in mammals. However, while food deprivation up-regulates prepro-orexin in rats [Cai et al. 1999; Karteris et al. 2005], fasting does not enhance

orexin expression in Japanese quail (Phillips-Singh et al. 2003). Furthermore, while injections of orexin A increase feeding in rats (Dube et al. 1999), and infusions of orexin A increase feeding in goldfish (Volkoff et al. 1999), no orexigenic effect has been seen in chicken or pigeons (Furuse et al. 1999; da Silva et al. 2008). It is also interesting to note that unlike mammals, a receptor that preferentially binds orexin A in chickens has not yet been identified (Okhubo et al. 2003). Though only one receptor has been found in birds, it is the most similar to orexin receptor 2 which binds orexin A or orexin B with the same affinity. Several studies in mammals suggest a role for orexin A as orexigenic and orexin B as a modulator of the sleep/wake cycle (Sakurai et al. 1998; Archer et al., 2002; Fujiki et al, 2003; Zieba et al., 2011), but these roles could be dependent on the receptors that have evolved in different vertebrates (Chen and Randeve, 2004). In addition, orexin A injections in pigeons increase arousal and orexin antagonist injections increase sleep behavior in sparrows (da Silva et al. 2008) similar to mammals. It is possible that as the orexin system evolved in birds, the regulation of the sleep/wake cycle was conserved but feeding modulation was lost. However, I had not be able in this study to demonstrate the influence of day-night cycle on the expression of hypothalamic orexin mRNA in chick. In addition, in the present study, no breed differences were found in hypothalamic orexin mRNA levels with real-time RT-PCR. This result is in agreement with data reported for other neuropeptides (Yuan et al., 2009). The principal finding of the present study is the demonstration of increased hypothalamic orexin gene expression after food restriction. In our study we used a quantitative PCR, a technique more sensitive than semi-quantitative PCR used previously (Okhubo et al. 2002). The model adopted was the chronic restriction routinely employed for broiler breeders as opposed to the 24 hr fast imposed (Okhubo et al. 2002). This represent approximately a 25% quantitative restriction of what would be consumed by a bird with ad libitum access to food. In

this physiological state I highlighted that in broiler occurs an increase of hypothalamic orexin mRNA. Similar findings have been previously reported in zebrafish (Novak et al., 2005), goldfish (Nakamachi et al., 2006) and Atlantic cod (Xu and Volkoff, 2007). This reflect probably the involvement of orexins in mechanisms of adaptation to fasting and to fluctuations in food availability to which the animals are submitted in their natural environment. When faced with a negative energy balance due to reduced food availability, mammals respond behaviorally with phases of increased wakefulness and locomotor activity that support food seeking and orexins might have important roles in the molecular and physiological basis of this phenomenon (reviewed by Sakurai, 2005, 2007). Hypothalamic orexin neurons monitor indicators of energy balance and mediate adaptive augmentation of arousal in response to fasting. Activity of isolated orexin neurons is inhibited by glucose and leptin and stimulated by ghrelin. Transgenic mice, in which orexin neurons are ablated, fail to respond to fasting with increased wakefulness and activity. These findings indicate that orexin neurons provide a crucial link between energy balance and arousal (Yamanaka et al., 2003a). These mechanisms may be important in the regulation of energy homeostasis that helps to ensure survival in nature (Sakurai, 2005, 2007; Kelley et al., 2005). Further studies are needed to confirm if also in birds the orexins may play an important role in these regulatory mechanisms. In this regard, previous findings in pigeon suggesting the orexin system is involved in modulating arousal states and species-typical behaviors in birds (da Silva et al., 2008). Finally, the present study is the first to describe the distribution of orexin-ir fibers and projections in the hypothalamus of a galliformes specie. The distribution of immunoreactive fibers in this study was consistent with previous description in the house finch (Singletary et al., 2006), the only other study reported a fibers distribution in birds. However, in the chick diversely to that reported in house finch, orexin-ir fibers were detected also in the posterior hypothalamic region (Kuenzel

and Van Tienhoven, 1982) and in the internal and external layer of median eminence (ME). Orexin nerve fibers were detected in the ME, which consists of the internal layer continuous with the neurohypophysis and the external layer connected with the portal vasculature (Mikami and Yamada, 1984). In particular, the presence of orexin-ir fibers in proximity of vessels of the hypothalamo-hypophysial portal system suggests that orexins may be released in the portal circulation and may have influence on biosynthesis or the secretion of anterior lobe hormones as well as in mammals (reviewed by Voisin et al., 2003). Previously, orexin-ir fibers have been observed in the ME of reptiles (Dominguez et al., 2010) and mammals (Peyron et al., 1998; Date et al., 2000). What orexin cells project to the neurosecretory zone of the ME and release orexins in the portal system will require further investigation. In any case, some data could indicate a role for orexins at this level. For example orexin A directly administrated into the ME has an inhibitory effect on the luteinizing hormone release in female rats (Small et al., 2003). In addition, orexins modulate proopiomelanocortin (POMC) axons in the ME (Acuna-Goycolea and van den Pool, 2009). On the other hand, intraventricular injections of orexins stimulate the liberation of gonadotropins (Pu et al., 1998) and ACTH (Kuru et al., 2000) and inhibit the secretion of LH, prolactin (Kohsaka et al., 2001) and GH (Blanco et al., 2001; Seoane et al., 2004; Molik et al., 2008). The findings of this study have furthered our understanding of orexin circuit in the neuroendocrine regulation in the hypothalamo- hypophysial tract. Together these anatomical and physiological results support the idea that orexins play a role in the control of several endocrine systems (Lopez et al., 2010). Special consideration deserves the presence of orexin-ir observed in the mSCN of chick (Cantwell and Cassone, 2006 a,b) in the present study. In the SCN of birds orexin-ir is conspicuous (Singletary et al., 2006, present results) in line with results in amphibians (Lopez et al., 2009), reptiles (Dominguez et al., 2010) and mammals (Date

et al., 1999; Mc Granaghan and Piggins, 2001; Mintz et al., 2001). Therefore, in non-mammalian vertebrate species orexins can also alter suprachiasmatic neuronal activity, as in mammals, and may influence the transmission of information from this nucleus to other brain regions (Brown et al., 2008). Though much work needs to be completed to determine the receptors in birds and in other non-mammalian vertebrates, we can begin to narrow down function by comparing distributions of cells and fibers, orexin sequences, and the few physiological studies available. Given the information today the orexin system seems to regulate homeostatic function across vertebrates, with specialization of function depending on the species. Furthermore, they is likely to function as one of the essential modulators for orchestrating the circuits that control autonomic functions and behavior (Kuwaki, 2011).

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