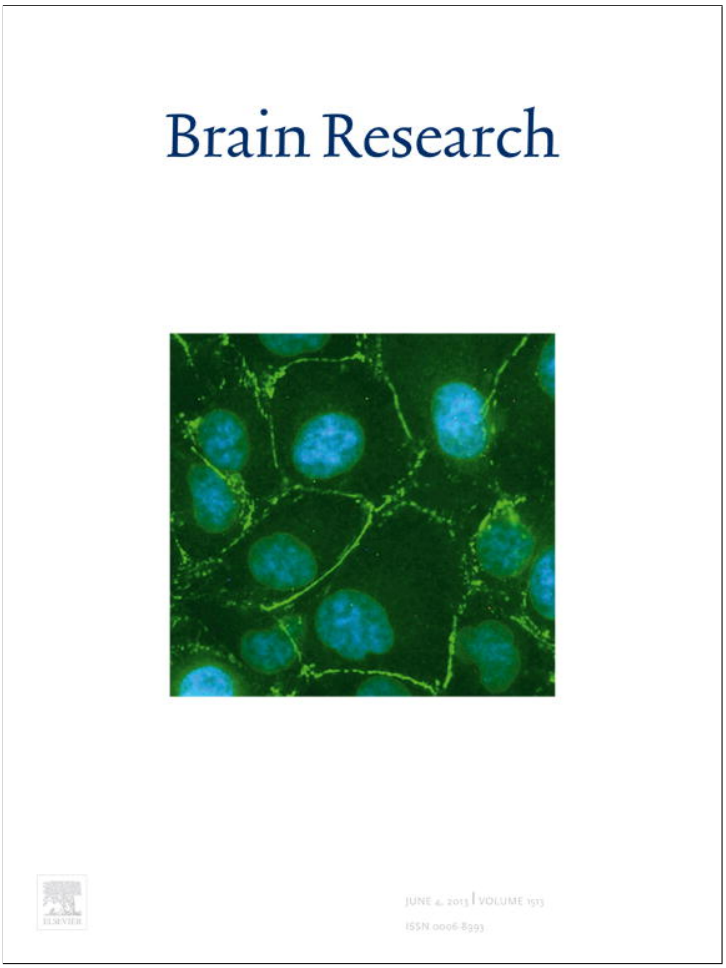

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Research Report

Orexin in the chicken hypothalamus: immunocytochemical localisation and comparison of mRNA concentrations during the day and night, and after chronic food restriction

Bernadette Miranda[a,*], *Vincenzo Esposito*[a,*], *Paolo de Girolamo*[a], *Peter J. Sharp*[b], *Peter W. Wilson*[b], *Ian C. Dunn*[b]

[a]*Department of Structures, Functions and Biological Technologies, University of Naples FedericoII, via Delpino1, I-80137 Naples, Italy*
[b]*The Roslin Institute, The University of Edinburgh, Easter Bush, Midlothian EH25 9RG, Scotland, UK*

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ABSTRACT

In mammals Orexin-A and -B are neuropeptides involved in the hypothalamic regulation of diverse physiological functions including food intake and the sleep-wake cycle. This generalisation was investigated in meat-(broiler) and layer-type juvenile domestic chickens by immunocytochemical localisation of orexin A/B in the hypothalamus, and by measurements of hypothalamic hypocretin mRNA which encodes for orexin A/B after chronic food restriction, and during the sleep-wake cycle. Orexin immunoreactive fibres were observed throughout the hypothalamus with cell bodies in and around the paraventricular nucleus. No differences were observed in the pattern of immunoreactivity using anti- human orexin-A, or -B antisera. The amount of hypothalamic hypocretin mRNA in food -restricted broilers was higher than in broilers fed *ad libitum*, but the same as in layer- type hens fed *ad libitum*. Hypothalamic hypocretin mRNA was increased ($P<0.01$) in 12-week-old broilers fed 25% of their *ad libitum* intake between 6–12 weeks of age. No difference in hypothalamic hypocretin mRNA was seen in 12-week-old layer- type hens when they were awake (1–2 h after lights on) or sleeping (1–2 h after lights off). It is concluded that in the chicken, we could not find evidence that hypothalamic orexin plays a role in the sleep-wake cycle and it may be involved in aspects of energy balance.

1. Introduction

Orexin-A and -B, also known as hypocretin-1 and -2, are hypothalamic peptides derived from the precursor protein, prepro-orexin, and are generally thought to be involved in the sleep–wake cycle, food intake, energy balance, gastric secretion, cardiovascular function, insulin secretion, reproduction, the control of autonomic function and release of adenohypophyseal hormones (reviews, Sakurai and Mieda (2011); Wong et al. (2011)). The gene which encodes for both the peptides is also known in the HUGO and Chicken Gene Nomenclature Consortium (http://www.agnc.msstate.edu/)

*Corresponding authors. Fax: +39 081 2536097.
E-mail address: espovin@unina.it (V. Esposito).

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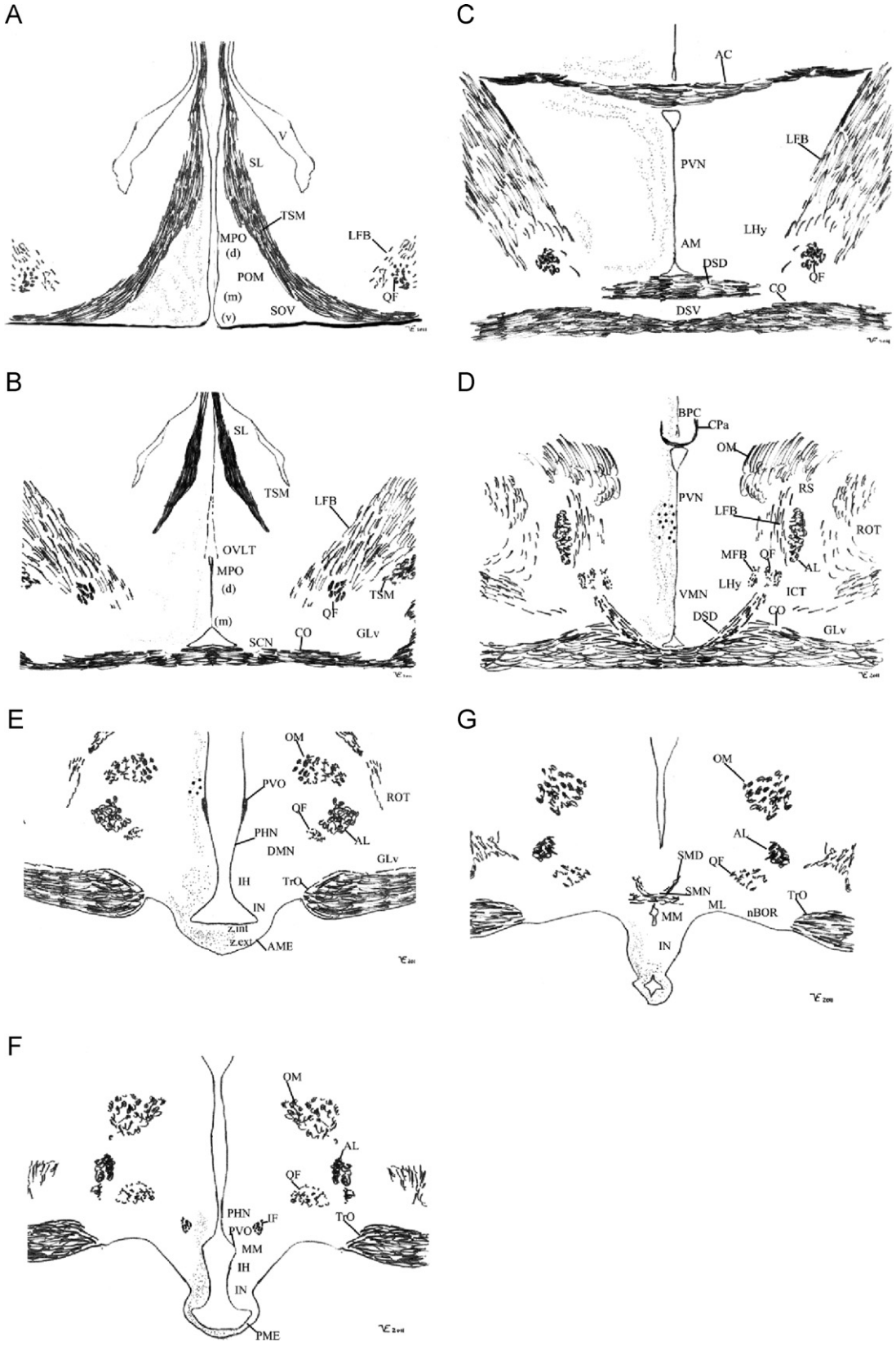

Fig. 1 – Map of the distribution of orexin fibres and cells bodies in the broiler (meat type) chicken hypothalamus. Observations were made on two week-old-females ($n=2$) and males ($n=2$). No sex differences were observed. Fibres are shown as small light dots and cell bodies as larger dots. The schematic drawings and nomenclature of chick hypothalamus were based on the schemes of Kuenzel and van Tienhoven (Kuenzel and Vantienhoven, 1982).

nomenclature as hypocretin (HCRT). The molecular structure of orexin peptides is evolutionarily conserved making it possible to use antibodies against mammalian orexins to localise immunoreactive cells and fibres in the brains of other classes of vertebrates (review, Wong et al., (2011)). The two orexins are colocalized in cells in the hypothalamus (Zhang et al., 2002) and give rise to networks of fibres throughout the brain (Cutler et al., 1999; Dominguez et al., 2010; Ferguson and Samson, 2003; Kirouac et al., 2005; Peyron et al., 1998; Singletary et al., 2006; Zhang et al., 2004).

In the avian hypothalamus, orexin cells are grouped in a continuous population, centred on the periventricular hypothalamic nucleus of chicken (Ohkubo et al., 2002) and Japanese quail (Phillips-Singh et al., 2003) or the paraventricular hypothalamic nucleus of the house finch (Singletary et al., 2006). The highest density of orexin fibres occur in the pre-optic area, hypothalamus and thalamus (Singletary et al., 2006).

The chicken orexin gene encodes a 148 amino acid peptide that is proteolytically cleaved to produce orexin A (33 amino acids) and orexin B (28 amino acids) which are 85% and 65% similar to their mammalian homologues, respectively (Ohkubo et al., 2002). Semi-quantitative PCR measurements indicated that there are no differences in the amounts of hypothalamic hypocretin mRNA between chickens fed *ad libitum*, or deprived of food for 24 h (Ohkubo et al., 2002) and similar observations have been reported in the Japanese quail using *in situ* hybridisation and autoradiography (Phillips-Singh et al., 2003). Despite marked differences in appetite, there is no difference in the reported expression of hypothalamic hypocretin mRNA in layer- and broiler-type chickens (Yuan et al., 2009). Central nervous injections of mammalian orexins in birds did not induce feeding behaviour (da Silva et al., 2008; Furuse et al., 1999; Katayama et al., 2010) which is contrary to findings in mammals and fish (reviews, (Sakurai and Mieda, 2011; Wong et al., 2011). However orexin-A may be involved in the sleep-wake cycle in birds since intracerebroventricular injection of orexin-A induces arousal in neonatal chicks (Katayama et al., 2010) and reduced sleep bouts in pigeons (da Silva et al., 2008). In order to further increase knowledge of the functions of orexins in birds, the distribution of immunoreactive orexin cells and fibres were mapped in the chicken hypothalamus and hypothalamic hypocretin mRNA concentrations were compared during the day-night cycle, and in fully fed and chronically food-restricted birds and between strains with different growth and food intake.

2. Results

2.1. *Immunocytochemical distribution of orexin cells and fibres in the hypothalamus*

No differences were observed in the pattern of immunoreactivity using anti-orexin-A or anti-orexin-B antisera, and either ABC, or PAP immunocytochemistry. Furthermore, no differences were observed in the pattern of immunoreactivity between the layer and broiler chickens examined.

Orexin immunoreactivity was observed in punctate structures in fibres throughout the hypothalamus (Fig. 1A–G; Fig. 2B,C), and in fusiform cell bodies with one or two primary dendrites (Fig. 2A) in the caudal parvocellular component of the paraventricular nucleus (PVN) (Fig. 1D) and in an area

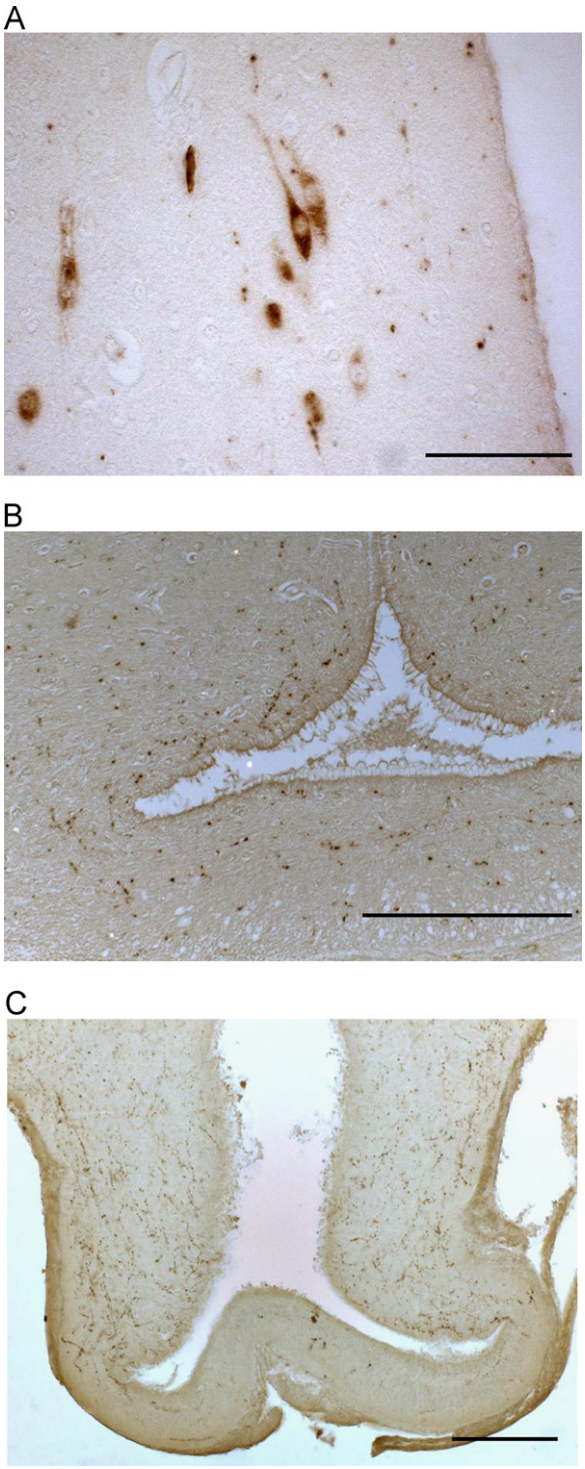

Fig. 2 – Orexin cell bodies in the medial basal hypothalamus (A), and orexin fibres in the anterior median eminence (B), basal hypothalamus and posterior median eminence (C) of a 2-week old female broiler chicken. The scales bar is 100 μm on photo (A) and 200 μm on (B) and (C).

dorsal to the paraventricular organ (Fig. 1E). In the preoptic hypothalamus, orexin fibres were in a region bounded by the tractus septomesencephalus (TSM) that included the nucleus pre-opticus paraventricularis magnocellularis (PPM), nucleus preopticus medialis (POM), and nucleus supraopticus ventralis (SOV) (Fig. 1A), while more caudally orexin fibres were found in the suprachiasmatic nucleus (Fig. 1B). In the anterior, mid- and posterior hypothalamus orexin fibres were seen adjacent to the walls of the third ventricle (Fig. 1B–G), extending laterally around the anterior commissure (Fig. 1C), and also dorsal to the nucleus commissura palli (CPa) (Fig. 1D), and in the lateral hypothalamic area (LHy) (Fig. 1C.) In the posterior (infundibular) hypothalamus sparse orexin fibres were observed in the zona interna and zona externa of the anterior median eminence (Fig. 2B) and a few fibres were also present at these sites in the posterior median eminence (Fig. 2C).

2.2. *Effect of chronic food restriction on body weights and hypothalamic hypocretin mRNA*

The body weights of broilers fed *ad libitum*, food-restricted broilers and layers, were 2373±121 g, 1245±41 g and 1028±40 g, respectively, and were significantly different from each other ($P<0.01$). The amounts of hypothalamic hypocretin mRNA in broilers fed *ad libitum*, food-restricted broilers and layers fed *ad libitum* were significantly different (ANOVA; $P=0.019$) (Fig. 3). Further analysis showed that hypothalamic hypocretin mRNA in the food-restricted broilers was significantly higher ($P<0.01$) than in broilers fed *ad libitum,* but was not higher than in layers fed *ad libitum*. There was no difference between the amounts of hypothalamic hypocretin mRNA in food-restricted broilers and layers fed *ad libitum*, but they were lower ($P<0.05$) in broilers fed *ad libitum* than in layers fed *ad libitum* (Fig. 3).

2.3. *Effect of the day-night cycle on hypothalamic hypocretin mRNA*

The amount of hypothalamic hypocretin mRNA 1–2 h after the lights were switched on (i.e. day) was not significantly different from the amount 1–2 h after the lights were switched off (i.e. night) (28.9±5.1 *vs.* 37.1±6.5; $P=0.621$).

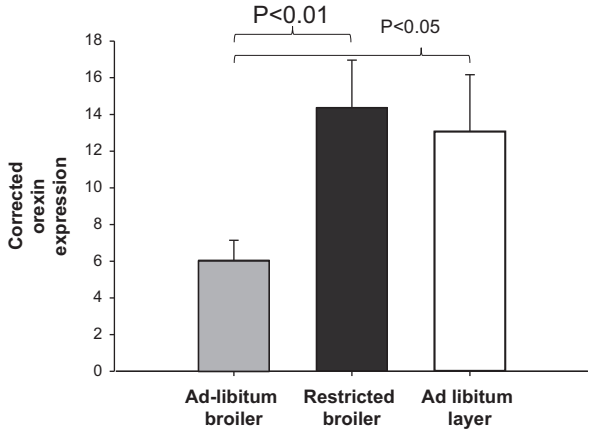

Fig. 3 – Comparison of concentrations of basal hypothalamic hypocretin mRNA in 11-week-old female broiler chickens, fed *ad libitum*, or fed 25% of *ad libitum*, and 11-week-old White Leghorn layer-type chickens fed *ad libitum*. The data are expressed as ± SEM ($n=10$).

3. Discussion

In this study we describe the distribution of orexin neurones and fibres in the chicken, and the differences in the amount of hypocretin mRNA in the basal hypothalamus during day *vs.* night, and in fed and food-restricted juvenile broiler chicken and juvenile White leghorn chickens. The location of orexin cells bodies centred on the caudal paraventricular nucleus agrees with observations in the house finch (Singletary et al., 2006), but not with earlier studies in the chicken (Ohkubo et al., 2002) and quail (Phillips-Singh et al., 2003) that located these cells more ventrally in the periventricular hypothalamic nucleus. In contrast with these earlier studies, orexin cells were not seen to spread laterally, and this may be a characteristic feature of broilers. This pattern of distribution is similar to the medial periventricular distribution described for amphibians (Shibahara et al., 1999) rather than the dorsolateral localisation reported for laboratory rodents (Broberger et al., 1998).

The presence of orexin fibres in the area of the suprachiasmatic nucleus (SCN) is consistent with observations in the house finch (Singletary et al., 2006) and other vertebrate species (Date et al., 1999; Dominguez et al., 2010; Lopez et al., 2009) and with a role in circadian organisation (Klisch et al., 2009). However, in the chick there is evidence that there are orexin-ir fibres in the tuberal region and in the median eminence which was not reported in the house finch (Singletary et al., 2006). The presence of orexin-ir fibres in the ME in proximity of vessels of the hypothalamo–hypophysial portal system suggests orexin involvement in the regulation of hypophysial functions or in the modulation of other neuropeptides release. Although not all neural sites that affect food intake in birds show the presence of orexin-ir, the presence of orexin ir-cells in the parvocellular component of PVN might be related to regulatory function exerted by this nucleus on food intake in birds (Kuenzel et al., 1999).

The principal finding of the current study on levels of expression is the confirmation that the expression of mRNA for hypocretin does appear to change in response to chronic food restriction. Chronic restriction has been studied in mammals but does not appear to change orexin expression in sheep (Archer et al., 2002) or rats (Johansson et al., 2008) but it is elevated in fish after chronic restriction (Novak et al., 2005). Additionally we have not been able to demonstrate any effect of differences in expression between night and day analogous to that observed in the rat. We also observed a difference between the expression of hypocretin between layers and broiler chickens which have relatively low and high growth rates and food intake respectively. In the experiments reported here, we used quantitative PCR, a technique more sensitive than the semi-quantitative PCR used previously and we confirmed that hypocretin mRNA in the chicken does change after chronic food restriction. The model employed was the chronic restriction routinely employed for broiler breeders compared with birds with *ad libitum* access to food for 6 weeks as opposed to the 24 h fast

previously reported (Ohkubo et al., 2002). At the age studied this represents approximately a 25% quantitative restriction of that consumed by a bird with *ad-libitum* access to food.

These results suggest that contrary to earlier observations orexin neurones may play a part in the control of energy balance in the chicken, as in other vertebrates. The differences in expression between strains which have very different growth characteristics due to intensive selection, whilst potentially supporting a role of orexin in energy balance are not as one might expect, with levels higher in the low growth layer genotype. This is contrary to a study comparing 1 week old layers and broilers measuring orexin using *in situ* hybridisation which showed no difference (Yuan et al., 2009). It would however be consistent with the hypothesis that orexin's role in birds is to regulate energy expenditure, in which case lower levels of orexin would favour growth. This must be balanced against the fact that chronic food restriction in broilers elevated hypocretin expression and it may be that there is a dichotomy between the food intake and energy expenditure effects, but it is premature to speculate further. For example there may be developmental differences, or a secondary genetic effect underlying the line differences. There is also the possibility that chronic restriction may produce a stress response or differences in activity that effects expression, although the effects of restriction on welfare provoke much debate (D'Eath et al., 2009). As in other studies the distribution of cell bodies and fibres supports the possibility of orexin mediating multiple roles in arousal associated with food seeking and other behaviours associated with a positive energy balance. Our failure to demonstrate a night day difference in orexin expression does not rule out a role in arousal in the chicken but may reflect differences in the importance of the system, although functional studies do support a role in birds. The lack of functional evidence for a role in energy balance observed in previous studies that have administered mammalian orexin may be due to differences in the structure from chicken orexin and their inability to interact correctly with the appropriate receptor and may reflect differences in receptor roles for arousal and energy balance.

4. Experimental procedures

4.1. Birds and experimental designs

Meat type birds (Ross 503 broilers) were obtained from a commercial breeder and layer-type birds from Roslin Institute stock (White Leghorn) and were reared on 14 h light per day in groups of 4/5 in floor pens. Birds were sacrificed with an overdose of intra-venous sodium pentobarbital. Immunocytochemical observations were made on two-week-old female ($n=2$) and male ($n=2$) broilers and on female ($n=2$) and male ($n=2$) layers. To study effects of chronic food restriction on hypothalamic hypocretin mRNA, observations were made on 12-week-old female broilers fed a commercially recommended restricted diet from hatch (~25% of *ad-libitum*) ($n=9$), or the same diet to 6 weeks of age, and thereafter fed *ad-libitum* ($n=10$), and in 12 week-old female White Leghorns fed *ad libitum* ($n=10$). To study the effects of the day-night cycle on hypothalamic hypocretin mRNA, observations were made on 6-week-old female White Leghorns fed *ad libitum* from hatch and sampled 1–2 h after lights off ($n=8$) and 1–2 h after light on ($n=8$)

4.2. Immunocytochemistry

After dissection, brains were placed in Bouin's fixative for 48 h and embedded in paraffin wax as described previously (Esposito et al., 1993), and 5–7 μm sagittal or coronal sections were cut through the area of the brain containing the hypothalamus. Immunohistochemistry was performed using the peroxidase anti peroxidase (PAP) and avidin biotin complex (ABC) systems (Vector Laboratories, DBA Italia SRL, Segrate, Italy). Deparaffinized and rehydrated sections were initially washed in 0.01 M phosphate-buffered saline (PBS; pH 7.4), subsequently incubated in 3% H_2O_2 for 20 min at room temperature (RT) to inhibit endogenous peroxidase activity, rinsed for 10 min 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 orexin A or B primary antibodies (orexin-A (C-19): sc8070; orexin-B (C19): sc-8071; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500 and 1:1000. These antibodies were raised against epitopes at the C-terminus of human orexin A or B which are up to 85% homologous to the corresponding chicken sequences (Sakurai et al., 1998). After several rinses in PBS, the sections were incubated for 30 min at RT with secondary antibody anti-goat IgG (Dako Italia, Portaluppi, Italy) diluted 1:50 in PBS, washed in PBS, and treated for 30 min at RT with the peroxidase-antiperoxidase (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 H_2O_2 at 0.03%. Negation controls were performed using the blocking peptide, sc-8070 (Santa Cruz Biotechnology).

For the ABC method, the method above was used except the secondary antibody was diluted 1:200 (anti-goat, code F0250, Dako), and then washed in PBS and treated for 30 min at RT using the Vectastain elite ABC system (PK 6101, Vector Laboratories). The peroxidase reaction was revealed as above.

For both methods selected sections were counterstained with Luxol fast blue/Cresyl violet for identification of anatomical structures. All sections were dehydrated, coverslipped, and observed and photographed using a Leitz Aristoplan microscope.

4.3. Hypothalamic hypocretin mRNA measurements

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 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 a dissected basal hypothalamus was 74±3 mg. Dissected tissues were immediately frozen in liquid nitrogen and stored at -80 °C. RNA was extracted using 1 ml Ultraspec II (AMS Biotechnology, Abingdon, UK) as per

protocol using lysing matrix D tubes (ThermoFisher, UK) and a Fast Prep Instrument (MP Biomedicals Cambridge, UK). The amounts of RNA extracted were quantified using a nanodrop ND 1000 spectrophotometer (Fisher Clinical Services, Horsham UK). RNA (1 μg or 5 μg) was reverse transcribed using a first strand cDNA synthesis kit with notI-d(T) 18 primer (GE Healthcare) and stored at −20 °C. QPCR was carried out on cDNA according to a Platinum Sybr green (Invitrogen, Paisley, UK) protocol with duplicates using a standard curve on an MX3000 QPCR instrument (Agilent Technologies UK Limited, Cheshire, UK). Real time PCR primers orex 1F (CTT GGC CAC CTG AAG ACA C) and orex 3R (GGG TCA CCG TAG GCT GAG T) were used. Controls (no template) were run for all primer pairs. To normalise expression and to account for differences in reverse transcription lamin-B receptor was used with the following primers; LBr-F (GGT GTG GGT TCC ATT TGT CTA CA) and LBr-R (CTG CAA CCG GCC AAG AAA) (McDerment et al., 2012).

4.4. *Statistical analysis*

Statistical analysis of the data was carried out by analysis of variance (ANOVA) on the log transformed corrected expression data (hypocretin/ LBR) using Genstat 12 (VSN International Ltd., Oxford, UK).

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