Universidade do Minho
Escola de Ciências

Diana Filipa Pereira Rodrigues

Study of the retinoic acid regulated genes in the rodent hypothalamus controlling growth and appetite and the potential for the epigenetic control

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Mestrado em Bioquímica, especialização em Biomedicina

Trabalho efetuado sob a orientação do
Professor Doutor Peter McCaffery
e do
Professor Doutor Pedro M. Santos

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DECLARAÇÃO

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Study of the retinoic acid regulated genes in the rodent hypothalamus controlling growth and appetite and the potential for the epigenetic control.

Estudo de genes regulados pelo ácido retinóico na região do hipotálamo de roedores que controla o crescimento e apetite, e potencial regulação epigenética.

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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"The brain is a tissue. It is a complicated, intricately woven tissue, like nothing else we know of in the universe, but it is composed of cells, as any tissue is. They are, to be sure, highly specialized cells, but they function according to the laws that govern any other cells. Their electrical and chemical signals can be detected, recorded and interpreted and their chemicals can be identified; the connections that constitute the brainís woven feltwork can be mapped. In short, the brain can be studied, just as the kidney can."

David Hubel, "The Brain"

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ABSTRACT

Study of the retinoic acid regulated genes in the rodent hypothalamus controlling growth and appetite and the potential for the epigenetic control.

In obese people the physiological system that should maintain normal energy balance no longer works correctly. Seasonal animals, such as hamsters and F344 rats, provide excellent models for investigating growth- and weight-related changes in the hypothalamus, the major region of the brain regulating feeding behaviour, metabolism and body weight. Evidence suggests that epigenetic mechanisms regulate the expression of hypothalamic genes affecting energy balance. At same time, it is also known that inflammatory responses are one key to changes in the hypothalamus. This study explored a potential role of HDAC4 and HDAC6, enzymes that set up epigenetic change, in modulating the inflammatory signalling pathways in the hypothalamus, as well the possible role of these HDACs in controlling RA and TH signalling pathways which are known to be key for hypothalamic regulation of energy balance.

This study showed that *HDAC4* and *HDAC6* were photoperiodically regulated in the hypothalamus of F344 rats with higher expression in rats under long-day photoperiod (16h light:8h dark), relative to short-day (8h light:16h dark). The NF-κB pathway, a central mediator of inflammatory responses, was considered a possible target of HDAC4 and HDAC6 action. To investigate this HDAC4/5 inhibitor LMK235 and HDAC6 inhibitor tubastatin A were used to evaluate the effect of HDAC inhibition on gene expression of NF-κB target genes. Inhibition of these HDACs were found to potentially influence the control, by TNFα and LPS, of expression of genes required for AP1, protein kinase C and PTP1B pathways. The effect of HDAC6 inhibition on the activation of MAPK pathways was also studied. Only p38 kinase activation by TNFα was shown to be affected by HDACs inhibition. However p38 inhibition did not change the capacity of HDAC inhibitors to alter TNFα gene regulatory activity. Immunocytochemistry experiments indicated that both HDAC4 and HDAC6 were mainly localized in the nucleus of GT1-7 cells and SH-SY5Y cells, two neuronal cell lines. Further, the histone deacetylation activity of HDAC4 and HDAC6 was evaluated by studying the influence of HDACs inhibition in regulating the expression of the RAR-regulated genes, *Cyp26A1* and *RARβ,* and the THR-regulated gene *Nrgn*. The results pointed to possible regulation of TH- and RA-regulated genes by HDAC4 and 6. Finally, results suggested that RA and T3 metabolism was under control of inflammatory regulators.

Taken together, the results suggest a possible function of HDAC4 and HDAC6 in modulating the activity of inflammatory pathways in the hypothalamus.

RESUMO

Estudo de genes regulados pelo ácido retinóico na região do hipotálamo de roedores que controla o crescimento e apetite, e potencial regulação epigenética.

Em pessoas obesas, o sistema fisiológico existente responsável pela manutenção do balanço energético não se encontra a funcionar corretamente. Animais sazonais tais como hamsters e ratos F344 são excelentes modelos para estudar alterações relacionadas com o crescimento e o apetite no hipotálamo, a principal região do cérebro que regula o comportamento alimentar, metabolismo e peso corporal. Estudos prévios sugerem que, no hipotálamo, a expressão de certos genes que afetam o equilíbrio energético estão sob o controle epigenético. Para além do referido, é também sabido que os processos inflamatórios desempenham um papel proeminente nas alterações hipotalâmicas. No presente trabalho investigou-se a potencial função da HDAC4 e HDAC6, duas enzimas responsáveis pela remoção de grupos acetil das histonas, na modulação dos processos inflamatórios no hipotálamo, bem como o possível papel destas HDACs no controlo das vias de sinalização do ácido retinóico (RA) e da hormona da tiroide (TH), duas vias conhecidas por desempenhar um papel crucial na regulação hipotalâmica do balanço energético.

Este estudo demonstrou que a *HDAC4* e a *HDAC6* são fotoperiodicamente reguladas no hipotálamo de ratos F344 com maior expressão em ratos sob condições que mimetizam dias de verão (16h de luz: 8h escuro) comparativamente a ratos sob condições que mimetizam dias de inverno (8h de luz: 16h escuro). O papel destas duas enzimas na regulação da expressão de genes regulados pelo NF-κB, um mediador central da resposta inflamatória, foi avaliada recorrendo ao uso de um inibidor para a HDAC4/5, o LMK235, e de um inibidor para a HDAC6, a tubastatin A. A inibição destas HDACs revelou uma potencial função da HDAC4 e HDAC6 em regular, através do TNFα e do LPS, a expressão de genes requeridos para as vias de sinalização do AP-1, proteína cinase C e PTP1B. O efeito da inibição da HDAC6 na ativação das vias de MAPK foi também estudado, tendo-se verificado que apenas a ativação do p38 pelo TNFα foi afetada pela inibição desta HDAC. No entanto a inibição do p38 não afetou a capacidade dos inibidores de HDACs em alterar a atividade reguladora do TNFα. Estudos de imunocitoquímica realizados indicaram que tanto a HDAC4 como a HDAC6 se encontram localizadas principalmente no núcleo das células GT1-7 e SH-SY5Y, duas linhas de células neuronais. Além disso, a capacidade da HDAC4 e da HDAC6 em remover os grupos acetil das histonas foi também avaliada através do estudo da expressão dos genes regulados pelo RAR, *Cyp26A1* e *RARβ*, e do gene regulado pelo THR, o *Nrgn*, na presença dos inibidores LMK235 e tubastatin A. Os resultados apontam para uma possível regulação da via de sinalização do RA e da TH por estas enzimas. Por fim, foi ainda verificado que o metabolismo do RA e da TH poderá estar sob o controle de reguladores inflamatórios.

Em suma, este trabalho sugere uma possível função da HDAC4 e da HDAC6 em modular a atividade das vias inflamatórias no hipotálamo.

LIST OF PUBLICATIONS

Rodrigues, D., Stoney, P., Helfer, G., Hay, E., Starr, R., Johnson, L., Bownman, E., Morgan, P., McCaffer, P. "The function of Histone Deacetylase HDAC4 in the Hypothalamus", Poster Communication – Rowett - IMS Winter Symposium, Aberdeen, 2 December 2014

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Stoney, P., Rodrigues, D., Morgan, P., McCaffery, P. A Seasonal switch in histone deacetylase gene expression in the rat with the potential to modulate both Tumour Necrosis Factor alpha and nuclear receptor activity". To be submitted to *Neuroscience*.

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SB202190 (9 μM) or vehicle (DMSO) for 16 hours. They were then treated with or without TNFα (1 ng/mL) and LMK235 (1 μM) for 4 hours (TNFα was added 1 hour after LMK235). The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test. 48

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List of Abbreviations

ADH	Alcohol dehydrogenase
AgRP	Agouti-related peptide
ANOVA	Analysis of variances
ARAT	Acyl-CoA:retinol acyltransferase
ARC	Arcuate nucleus
ATCC	American type culture collection
ATRA	All-*trans*-retinoic acid
bp	Base pairs
BMM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CaMKIV	Calcium/calmodulin-dependent kinase IV
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine-regulated transcript
cDNA	Complementary DNA
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
CoAS	Co-activators
CYP26	Cytochrome P450 26 subfamily
DBD	DNA-binding domain
DIO2	Type II iodothyronine deiodinase
DIO3	Type II iodothyronine deiodinase
DMEM	Dulbecco's modified Eagle's medium
DMN	Dorsomedial
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferases
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FSH	Follicle-stimulating hormone
FXR	Farnesoid X receptor
GnRH	Gonadotropin-releasing hormone
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	heat shock protein
IKK	IκB kinase
IL	Interleukin
IRAK	Interleukin receptor-associated kinase

JNK	c-Jun N-terminal kinase
LBD	Ligand-binding domain
LD	Long-day
LH	Lateral hypothalamic
LPS	Lipopolysaccharide
LRAT	Lecithin:retinol acyltransferase
LXRs	Liver X receptors
MKP-1	MAPK phosphatase 1
MAPK	Mitogen-activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MBD	Methyl-CpG binding domain
ME	Median eminence
MEF2	Myocyte enhancer factor-2
MyD88	Myeloid differentiation factor 88
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NCoR	Nuclear co-repressor
NF-κB	Nuclear factor- κB
NIK	NF-kappa-B-inducing kinase
NPY	Neuropeptide Y
OCT	Optimal cutting temperature compound
PBS	Phosphate buffered saline
PET	Positron emission tomography
PFA	Paraformaldehyd
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PPARs	Peroxisome proliferator activated-receptors
PTP1B	Protein-tyrosine phosphatase 1B
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RALDH	Retinaldhyde dehydrogenase
RAR	Retinoic acid receptor
RAREs	Retinoic acid response elements
RBP	Retinol-binding protein 4
RDH	Retinol dehydrogenase
RIP	Receptor-interacting protein
RXR	Retinoid X receptors
RXRE	Retinoid X response element
rT_3	3,3',5' – triiodothyronine (Reverse T_3)
SCN	Suprachiasmatic nucleus

SD	Short-day
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
T_2	3',3'-diiodothyronine
T_3	3,5,3' – triiodothyronine
T_4	Tetraiodothyronine
TBS	Tris-buffered saline
TH	Thyroid hormone
THR	Thyroid hormone receptor
TKR	Tyrosine kinase receptors
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFα	Tumour necrosis factor α
TNFR	Tumour necrosis factor receptor
TH	Thyroid hormone receptor
TRADD	TNFR-Associated Death Domain
TRAF	TNF-receptor-associated factor
TREs	Thyroid response elements
TRIF	TIR-domain-containing adaptor inducing IFN-β
VDR	1,25- dihydroxy-vitamin D3 receptor
VMH	Ventromedial region of the hypothalamus
Zn	Zinc
WHO	World Health Organization
α-MSH	α-melanocyte-stimulating hormone

CHAPTER I

INTRODUCTION

Obesity is one of the greatest health problems of the 21st century in developed countries. Between 1980 and 2008 the number of obese worldwide doubled, and there is no reason to expect this alarming rate of increase to change over the next years. According to the World Health Organization (WHO), in 2008, 24% of the Portuguese adult population (≥20 years) were obese and 59.1% were overweight. In the United Kingdom and North Ireland, the percentage of adult population obese is 26.9% and the percentage of the adult population overweight is 64.2% (WHO 2013). The terms obesity and overweight are defined as an abnormal or excessive accumulation of fat in the body and results from an imbalance between food intake and energy expenditure. Thus, it is important to understand why the physiological systems that control energy balance allow an increase of food intake beyond the amount required for the body and why energy expenditure is not increased in these cases to restore the initial equilibrium. Seasonal animals, such as the F344 rat, which allow the hypothalamic control of energy balance to be manipulated simply by changing the length of day, provide an excellent model to study the energy balance regulation (Heideman & Sylvester 1997).

1.1. Seasonal Changes

The biological rhythm of many mammals and birds are profoundly affected by the geographic cycles such as daily, seasonal, lunar and tidal. To cope with these environmental variations, animals living in temperate and arctic regions usually show a host of physiological and behavioural changes that include, hibernation, reproduction, migration, feeding pattern, energy metabolism, body weight, changes in pelage colour and thickness, immune function and much else (Barrett *et al.* 2007; Klingenspor *et al.* 2000). Throughout the seasons, one of the environmental aspects that changes most dramatically is the availability of food. Consequently, during the winter, when food availability is minimal and energy demand is maximum, animals display a set of behaviours that allow them to survive. Also important is the timing of reproduction to ensure that offspring born just in the most favourable time of year, when the food viability is maximum and temperatures are warm. The switch in such physiological and behavioural responses is controlled by the hypothalamus, the major portion of brain that links the nervous system to the endocrine system via pituitary gland, being responsible for maintain the internal balance of the organism - homeostasis. Many important features, such as circadian rhythms, reproductive status, hormone release,

feeding, and energy balance, are regulated by hypothalamus by sending out signals as hormones and neuropeptides.

The seasonal variations can be predicted by animals through the changes in day length (photoperiod). Photoperiodism can be defined as *a process whereby organisms are able to use both absolute measures of day length and the direction of day length change as a basis for regulating seasonal changes in physiology and behaviour* (Goldman 2001). The molecular mechanism of the seasonal timing system is not fully understood in any organism studied so far. However, over the last years many progresses have been made in the comprehension of the mechanisms by which photoperiod drive the physic and behaviour changes. The effects of photoperiod can be studied in laboratory by changing the animals from long-day (LD) "summer-like" conditions (16:8 hrs light:dark cycle) to a short-day (SD) "winter-like" conditions (8:16 hrs light:dark cycle).

Studies on many species of seasonal mammals and birds showed that photoperiod is transduced by a photoneuroendocrin system where melatonin, a hormone secreted by the pineal gland with receptors located on several regions of hypothalamus (Morgan *et al.* 1994), acts as a chemical mediator of day length changes. In mammals, melatonin has many biological effects, being involved in the regulation of physiological functions such as sleeping time, seasonal reproduction, immune function, circadian entrainment and many others.

Alterations in the secretion of melatonin provoked by seasonal change is responsible for reproductive seasonality by regulation of testosterone production, testicular size, and sperm production in males, and ovulatory cycles in females (Gerlach & Aurich 2000). The biosynthesis and secretion of melatonin is tightly regulated by sunlight that is transmitted to pineal gland through the retina, suppressing its synthesis. The synthesis of melatonin is directly related to the length of darkness. Thus, winter-like days are associated with a greatest quantity of melatonin secreted whereas summer-like days are associated with a short secretion of this hormone. The amount of melatonin is inversely proportional to gonadotropin-releasing hormone (GnRH) secretion such that an increase in melatonin's secretion is associated with a decrease GnRH release. In turn, the GnRH released regulates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH; Grosse *et al.* 1993). In this way, melatonin controls seasonal reproduction by indirectly modulating the secretion of gonadotropins.

Another hormone known to be involved in seasonal physiology in mammals and birds is trioiodothyronine (T_3), a hormone that plays an important role in photoperiodic control of energy balance and reproduction (Dawson *et al.* 2001; Shinomiya *et al.* 2014). Within the brain, the availability of T_3 hormone is controlled by the activity of type II iodothyronine deiodinase (DIO2) and type III iodothyronine deiodinase (DIO3). DIO2 catalyses the conversion of the prohormone thyroxine (T_4) to the more active form T_3 whilst DIO3 converts T_4 to inactive T_3 (reverse T_3; rT_3) and T_3 to inactive 3',3'-diiodothyronine (T_2; Bernal 2014; **Figure 1**). The expression of both *DIO2* and *DIO3* is photoperiodically regulated in tanycytes, specialized hypothalamic glia that line the floor and the basolateral walls of the third ventricle, with higher expression of *DIO2* in rats under long-day photoperiod, relative to short-day (Yoshimura *et al.* 2003) resulting in higher thyroid hormone activity. Conversely, *DIO3* is weakly expressed in rats under LD conditions and strongly expressed in rats under SD conditions (Yasuo *et al.* 2005) leading to decreased thyroid hormone activity. Interestingly, it has been shown that the regulation of *DIO2* and *DIO3* expression in Siberian hamster (*Phodopus Sungorus*) and Syrian hamster (*Mesocricetus auratus*), two of the most widely used models for photoperiodic studies, are melatonin dependent, and that injection of these hamster species with melatonin under long-day conditions for 7 days inhibits *DIO2* expression to that of short day levels (Revel *et al.* 2006; Watanabe *et al.* 2004).

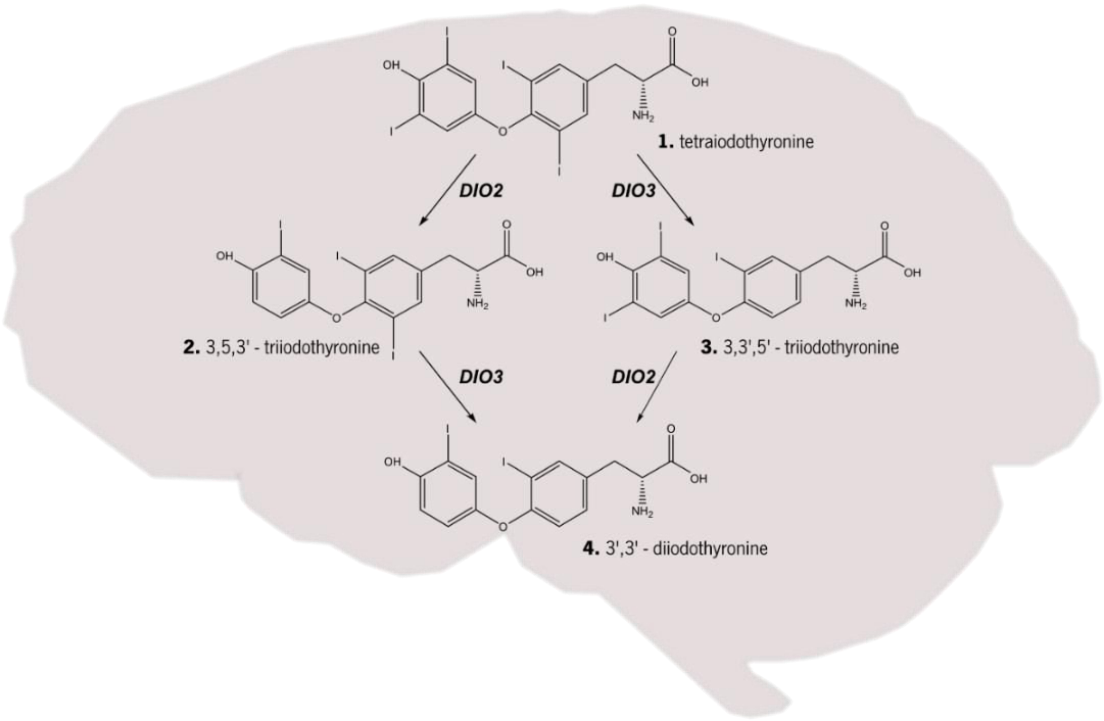

Figure 1 | Thyroid hormone metabolism. Within the brain the tetraiodothyronine (T4; structure 1) can be converted into the more active form 3,5,3 - triiodothyronine (T3; structure 2) by the action of type II iodothyronine deiodinase (DIO2) or the inactive form 3,3,5'- triiodothyronine (rT3; structure 3), an isomer of T3, by type III iodothyronine deiodinase (DIO3). T3 and rT3 can be converted into 3',3'-diiodothyronine (T2; structure 4) by action of DIO3 and DIO2, respectively. Rodrigues (2015)

The importance of photoperiod in seasonal reproduction has been extensively studied over the last years. However, its influence on metabolism and energy balance is poorly understood. It is well known that animals living in temperate regions have a weight decrease during the winter and an increase in summer. Siberian hamsters, for example, lose between 30 and 40% of their body weight in response to photoperiod (Morgan *et al.* 2003). These models can be used to study the hypothalamic signalling systems required to regulate energy balance. Several hypothalamic nuclei are known to be involved in control of metabolism and energy balance regulation; the main hypothalamic nuclei responsible are the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic (LH) area, dorsomedial (DMN), and ventromedial region of the hypothalamus (VMH).

The arcuate nucleus has two types of neurons that control fuel intake and metabolism: orexigenic neurons (appetite-stimulating) and anorexigenic neurons (appetite-suppressing). Orexigenic neurons stimulate eating by producing and releasing neuropeptide Y (NPY), whereas anorexigenic neurons exerts the opposite effect physiologic producing α-melanocyte-stimulating hormone (α-MSH; Nelson & Cox 2013). The release of NPY and α-MSH are regulated by various factors including the serum levels of leptin, a hormone synthesized in adipose tissue and whose receptors are highly expressed in ARC (Sahu 2003). When there is an increase in the size of fat tissue, leptin is released into the bloodstream and binds to the hypothalamic leptin receptors, influencing the expression of NPY and α-MSH (Klok *et al.* 2007). An increase in leptin levels in blood serum stimulates α-MSH production and inhibits NPY production. Therefore, leptin exerts its function stimulating hypothalamic anorexigenic pathways and inhibiting orexigenic ones. On the other hand, food restriction decreases leptin levels and increase the ghrelin levels, and consequently increases the expression of some orexigenic genes such as NPY and Agouti-related peptide (AgRP) and suppresses the expression of some anorexigenic genes such as pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) in the ARC (Kalra *et al.* 1999; **Figure 2**). Studies developed in Siberian hamsters showed that animals under LD conditions subject to food restrictions have the same changes in gene expression that normally result from a decrease in leptin levels. However the gene expression pattern of hamsters under SD conditions are completely different suggesting that photoperiod exerts its effect on the energy balance through a different regulatory system to that regulated by leptin.

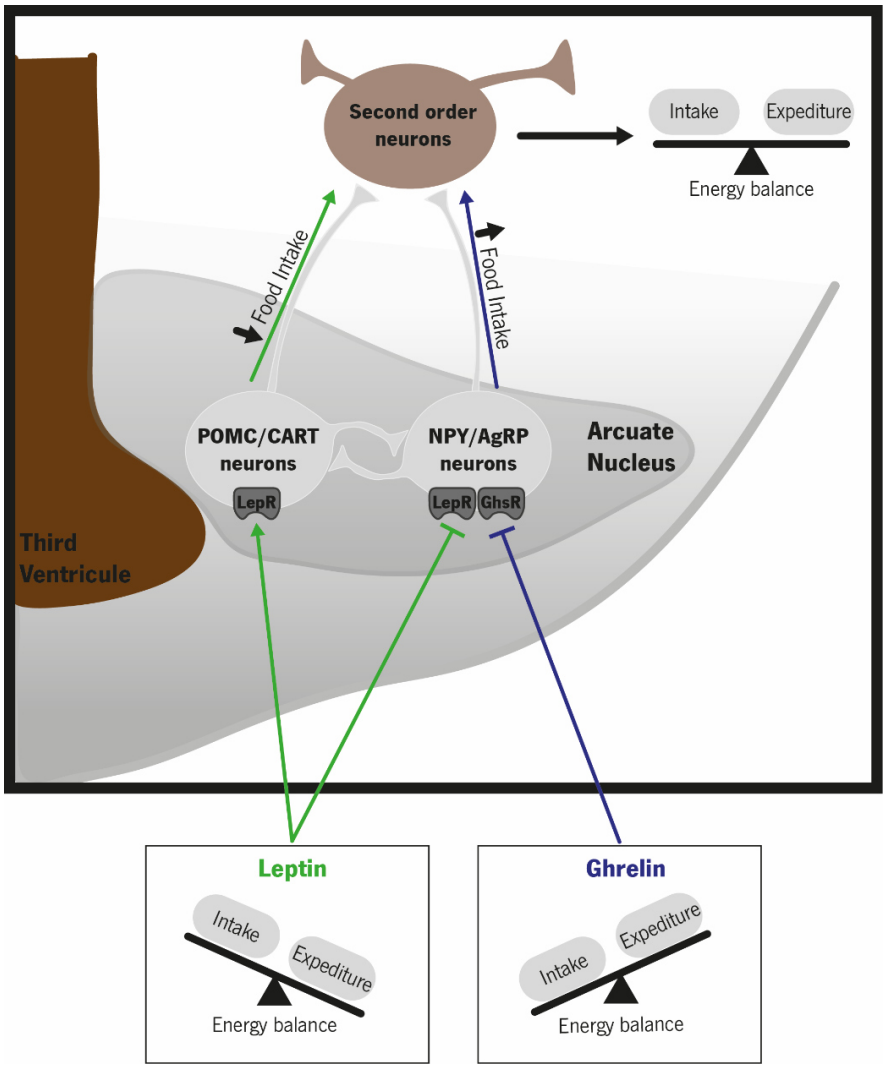

Figure 2 | Regulation of energy balance by leptin and ghrelin. In the arcuate nucleus of the hypothalamus, orexigenic and anorexigenic neurons receive hormonal inputs and relay neuronal signals. Stimulation of these neurons leads to production of orexigenic peptides (promoting appetite), such as Neuropeptide Y (NPY) and Agouti-related peptide (AgRP), or anorexigenic peptides (decreasing appetite), as α-melanocyte-stimulating hormone (α-MSH), one of several cleavage products of pro-opiomelanocortin (POMC), and cocaine amphetamine - related transcript (CART). Leptin is a hormone released from adipose tissue when there is an increase in the size of body-fat mass. Release of leptin leads to a decrease in appetite and an increase in fuel metabolism by stimulation of melanocortin-producing neurons, inhibiting neurons that produce the molecules NPY and AgRP. On the other hand, when of body-fat mass decreases ghrelin is released by the stomach, which leads to an activation of NPY/AgRP-expressing neurons, and consequently stimulates appetite. In this way, energy balance is restored, ensuring the homeostasis of the organism. Rodrigues (2015)

In Siberian hamsters melatonin receptors are localised to the DMN, one of the hypothalamic nuclei involved in the regulation of metabolism and energy balance, as well as the suprachiasmatic nucleus (SCN; Morgan *et al.* 1994). Siberian hamsters infused for 10 hours with melatonin, to mimic the nocturnal durations of this hormone in serum, showed a decrease in body and fat pad weights, however these effects were blocked by lesion of the SCN (Bartness *et al.* 1991). These results suggest a role of the SCN in the photoperiodic regulation of body weight. Although the melatonin receptors are expressed at a low level and in just a few neurons of the DMN, it was

shown that the increase in the duration of melatonin secretion leads to an increase of these receptors in the DMN which, in turn, increases lipolysis in white adipose tissue and decreases adiposity (Song & Bartness 2001). These findings support the idea that energy balance may be regulated by the melatonin receptors in the DMN. Although the action of photoperiod and melatonin in energy balance regulation is far from completely understood, it is known that the several hypothalamic nuclei involved in these pathways have interrelated connections, which can explain the interface between the neuronal pathways involved in energy balance regulation and circadian rhythms (**Figure 3**). However, melatonin is presumed to work via an intermediate since in isolated adipocytes from Syrian hamsters, Sprague-Dawley rats and rabbits melatonin does not directly affect lipolysis and lipogenesis (Ng & Wong 1986). Although this intermediate is still unidentified, several signalling pathways including thyroid hormone (TH) and retinoic acid (RA) signalling pathways, are known to be regulated by melatonin and to control energy balance and metabolism.

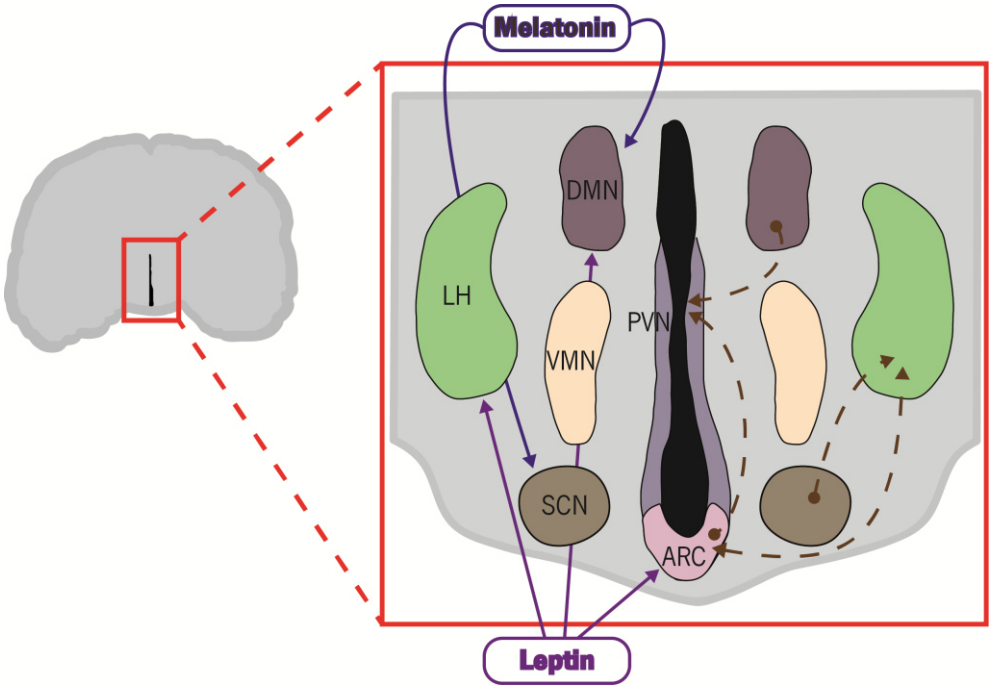

Figure 3 | Link between photoperiod and energy balance regulation. Schematic representation showing the main hypothalamic nuclei involved in energy balance and their interrelated connections (right side) as well the action of the hormones melatonin and leptin in these nuclei (left side). Melatonin, a hormone photoperiodically regulated, has its receptors expressed in dorsomedial (DMN) and suprachiasmatic nucleus (SCN). In turn, these nuclei have connections with others nuclei, namely the paraventricular nucleus (PVN) and lateral hypothalamic (LH), respectively, both connected to the arcuate nucleus (ARC), the most important nucleus in the regulation of energy balance. The ARC regulates feeding and energy expenditure though two different neuronal types, anorexigenic and orexigenic neurons, that project from the ARC to the PVN. Rodrigues (2015)

The importance of thyroid hormone in the regulation of appetite and energy expenditure is indicated from studies of patients with thyroid dysfunction; either hypothyroidism, the underproduction of TH, or hyperthyroidism, the overproduction of TH. Patients with hypothyroidism have a body weight gain while hyperthyroid patients decrease their body weight. In addition, in hyperthyroid rats, TH has a similar effect to the 'hunger hormone" ghrelin, increasing the expression of *Agrp* and *NPY,* and decreasing the expression of *Pomc*, in ARC (Varela *et al.* 2012). Concordant with that, fasting results in an increase in the conversion of T_4 into T_3 in the hypothalamus (Diano *et al.* 1998).

A study developed by Ross *et al.* in 2004 showed that in Siberian hamster, the expression of some genes involved in the RA signalling pathway are photoperiodically regulated, with expression higher in the hypothalamus of animals under long day "summer-like" conditions. Furthermore, the alterations in gene expression appeared to be in dependent on melatonin. These results suggest an important role of the RA signalling pathway in the photoperiodic regulation of body weight. However, the link between RA and food intake has remained relatively unexplored. Studies by Ziouzenkova *et al.* (2007) showed that vitamin A metabolism and responses to a high-fat diet are linked. *Raldh1*, which is necessary for RA synthesis, when deficient in mice, had lower leptin and RBP4 plasma levels, and gained less weight than wild type mice when on a high-fat feeding, suggesting that the RA can play an important role as a mediator of energy balance.

1.2. Retinoic Acid Metabolism and its Signalling Pathway

Vitamins are compounds that, just in small amounts, are required for growth and are essential to health of humans and other vertebrates. Vitamin A, one of the earliest vitamins to be discovered is an essential dietary micronutrient belonging to the group of fat-soluble vitamins. This vitamin found in animal tissue most commonly in the form of retinol or retinyl ester, or as provitamin A carotenoids in plant tissues, among which β-carotene is the most important.

Retinoids, a generic term introduced in 1976 by Sporn *et al.*, are a family of signalling molecules related to vitamin A with respect to their chemical structures (Tang & Gudas 2011; Sodhi & Singh 2014). They are low molecular weight molecules derived from terpenoids with a cyclic end group, a 6 carbon β-ionone ring, a polyene side chain, and a polar end group.

Diversification of side chains and end groups creates the various classes of retinoids (Tang & Gudas 2011; Ransom *et al.* 2013).

Unlike plants and microorganisms such as some bacteria, algae, and fungi, mammals lack the biosynthetic machinery to synthesize vitamin A *de novo*. The principal source of vitamin A for mammals is dietary, namely provitamin A carotenoids (mainly β carotene, α carotene and, β cryptoxanthin) found in plant-derived products and, preformed vitamin A in the form of retinyl esters, which is found in foods of animal origin (Orfali *et al.* 2014; Paik *et al.* 2014). The most stable storage form of vitamin A in animal tissues is the fat-soluble retinyl esters (Tanumihardjo 2011; Ransom *et al.* 2013). In humans, vitamin A can be found in three forms, retinol, retinal and retinoic acid, the latter being the main and most abundant intracellular metabolite in mammals.

Retinol homeostasis, as well as the distribution of RA in different tissues, is tightly regulated by several proteins and enzymes involved in retinoid signalling (Rhinn & Dollé 2012). The main sources of vitamin A in the diet are fish-liver oils, eggs, milk, butter, and plants and are present in the form of retinyl esters and provitamin A carotenoids (Marill *et al.* 2003). In the intestinal lumen the majority of food-derived retinoids are converted to retinol prior to uptake by the enterocytes by a series of reactions. Retinyl esters are hydrolysed to unesterified retinol by enzymes such as pancreatic triglyceride lipase and intestinal phospholipase B. Carotenoids are oxidatively cleaved by β-carotene 15,15-oxygenase into two molecules of all-*trans*-retinaldehyde followed by their reversible reduction to retinol, a reaction catalysed by the retinol dehydrogenase (RDH; During & Harrison 2007). The retinol resulting from this reaction, the form absorbed into the intestinal mucosal cell, is then re-esterified in enterocytes with long chain fatty acids, particularly palmitic acid, before secretion into the intestinal lymph (Lane & Bailey 2005; Blomhoff & Blomhoff 2005). This esterification reaction is catalysed by the enzyme lecithin:retinol acyltransferase (LRAT), an enzyme that uses the acyl group at the *sn*-1 position, using phosphatidylcholine from the membrane bilayer as a source of fatty acids. Esterification of retinol can also be carried out by acyl-CoA:retinol acyltransferase (ARAT) which catalyses the fatty acyl-CoA-dependent esterification of retinol (Eroglu & Harrison 2013). LRAT and ARAT are distinguished by the capacity of the former to esterify retinol when bound to cellular retinol binding protein (CRBP), as opposed to the latter which does not require substrate bound to CRBP (O'Byrne & Blaner 2013).

Retinol binds with high affinity to cellular retinol binding protein II (CRBP-II), playing this protein important roles in the regulation of retinol absorption by enterocytes and its intracellular

metabolism, directing the retinol substrate more specifically toward LRAT, thus preventing its free access to cytosol reductases (Eroglu & Harrison 2013; Ransom *et al.* 2013). The absorbed vitamin A can also be directly secreted into the portal circulation as unesterified retinol (Lane & Bailey 2005; Blomhoff & Blomhoff 2005).

After re-esterification of retinol, the retinyl esters are incorporated with other dietary lipids into chylomicrons for transport to the liver, where they are hydrolysed in hepatocytes. The unesterified retinol formed is then associated with retinol-binding protein 4 (RBP4), a protein found in high concentrations in hepatocyte endoplasmic reticulum. Binding of retinol to RBP4 apparently results in the translocation of retinol-RBP4 from endoplasmic reticulum to the Golgi complex, and therefore the secretion of retinol-RBP4 into the blood plasma for transportation to peripheral tissues (Ransom *et al.* 2013; Orfali *et al.* 2014; Blomhoff & Blomhoff 2005). Retinol is transported in the bloodstream to its target tissue bound in a 1:1 ratio to RBP4/transthyretin (Lane & Bailey 2005). However, some of unesterified retinol can be transferred as retinol to another liver cell type, the perisinusoidal stellate cell, for storage (Blomhoff & Blomhoff 2005). The liver is the major site of retinoid metabolism and storage in the body, storing the retinol secreted in response to the body's needs, thus relieving animals from the daily acquirement of vitamin A from food (Paik *et al.* 2014; Lane & Bailey 2005).

Uptake of retinol by cells that require this retinoid is mediated by a multi-transmembrane domain protein that is the product of the RA-inducible gene *STRA6* (Kawaguchi *et al.* 2007). This protein acts like a RBP4 receptor, removing retinol from the RBP4 complex and transporting it across the plasma membrane, where it can be metabolized. However, studies developed in *Stra6*-deficient mice suggest the presence of additional non-receptor-mediated pathways for retinol uptake into cells. Nevertheless, the uptake of retinol via the RBP4 receptor Stra6 is the most important one (O'Byrne & Blaner 2013; Eroglu & Harrison 2013), and mutations in STRA6 leads to a wide variety of diseases in humans, including anophthalmia, heart defects, lung hypoplasia, and mental retardation (Mongan & Gudas 2007).

Once inside the cell, retinol binds to CRBP and can be esterified by LRAT for storage or metabolized to its biologically active metabolite, all-*trans*-retinoic acid (ATRA), by two sequential oxidation steps that convert first retinol to retinaldehyde by RDH or alcohol dehydrogenase (ADH) and then to retinoic acid by retinaldhyde dehydrogenase (RALDH). When necessary, the cells can mobilize the retinyl esters from storage by retinyl esterase to yield retinol, which can then be

converted in ATRA (Khillan 2014; Napoli 1996; Lane & Bailey 2005; Orfali *et al.* 2014). ATRA is the most important metabolite of vitamin A and is involved in a wide variety of biological processes, being responsible for regulating the expression of many genes by binding to retinoic acid receptors (RAR) when bound to cellular retinoic acid binding protein 2 (CRABP-2), and which will be discussed in more detail later (Lane & Bailey 2005). In addition, RA can bind to CRABP-1 and be destroyed by the enzymes of the cytochrome P450 26 subfamily (CYP26A1, CYP26B1 and CYP26C1). CYP26 enzymes catalyse the oxidation reactions that convert RA into 4-hydroxy-RA and then 4-oxo-RA (**Figure 4**). This is an important mechanism that allows tight control of RA levels in cells and tissues, and thereby regulate the retinoid signalling (Rhinn & Dollé 2012).

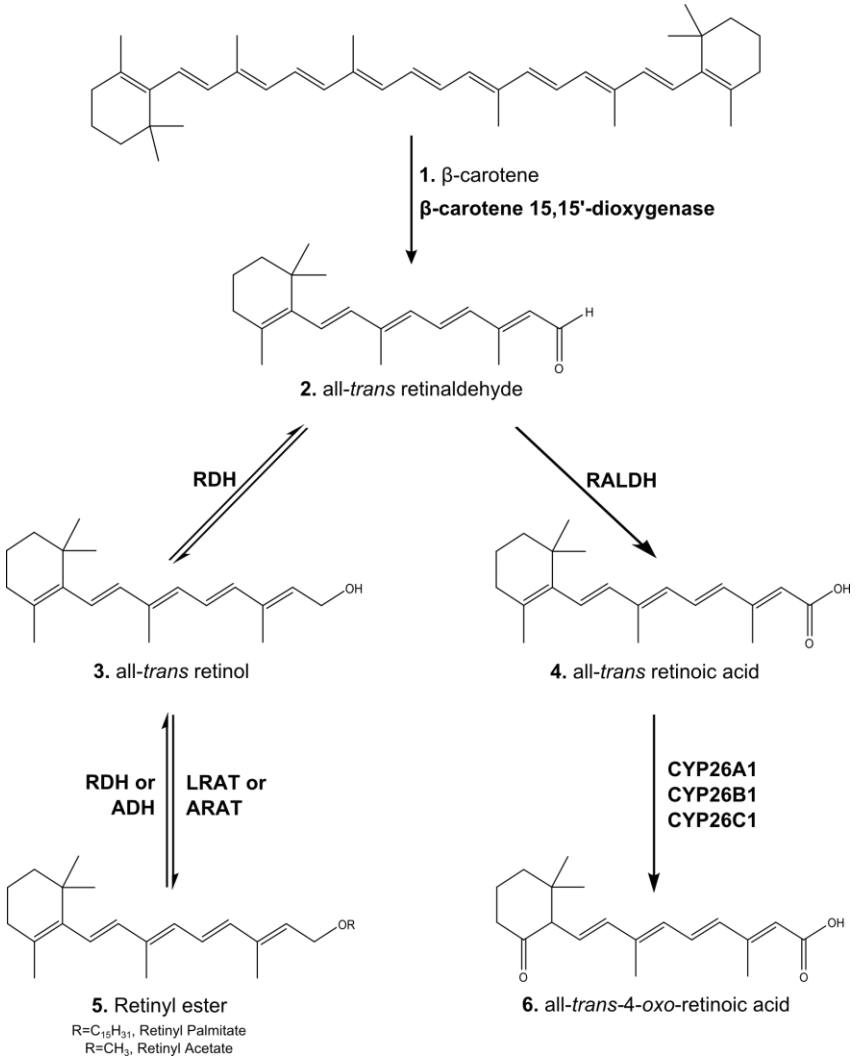

Figure 4 | The major metabolic pathway of retinol in nonvisual mammalian cells. Retinol is obtained from dietary in the form of retinyl esters (structure 5) and/or β-carotene (structure 1). β-carotene 15-15' – dioxygenase catalyse the oxidative cleavage of the β-carotene in all-*trans* retinaldehyde (structure 2), which in turn can be reducted to all-*trans* retinol (structure 3) by retinol dehydrogenases (RDHs) and alcohol dehydrogenase (ADH), or converted in all-*trans* retinoic acid (structure 4) by retinaldehyde dehydrogenase (RALDH) family members. The retinol can be stored in form of retinyl esters, being the esterification reaction of retinol carried out by lecithin:retinol acyltransferase (LRAT) or acyl-CoA:retinol acyltransferase (ARAT). The degradation of retinoic acid is carried out by the enzymes of the cytochrome P450 26 subfamily (CPY26A1, CPY26B1 and CPY26C1), originating all-*trans*-4-*oxo*- retinoic acid (structure6) and other polar retinoic acid metabolites. Rodrigues (2015)

Retinoic acid, a major biologically active metabolite of vitamin A, has critical biological functions, including regulation of proliferation, differentiation, and apoptosis (Lerner *et al.* 2012), exerting their pleiotropic effects through nuclear receptors termed retinoic acid receptors (RARα, β, and γ), which are activated by all-*trans* RA and its stereoisomers 9-*cis*-RA and 13-*cis*-RA, and retinoid X receptors (RXRα, β, and γ), whose ligand is 9-*cis* RA (Krezel *et al.* 1999) . Each of the three RAR and RXR subtypes (α, β, and γ) are encoded by a unique gene (*Rara*, *Rarb* and *Rarg* genes that encode RAR receptors and *RXRa*, *RXRb* and *RXRg* genes that encode RXR receptors (Maden 2007)), wherein their transcription product can undergo differential splicing, resulting for each subtype at least two different receptor isoforms (for example, RARβ1, RARβ2, RARβ3 and RARβ4, Sodhi & Singh 2014; Orfali *et al.* 2014), differing only in their N-terminal region (Germain *et al.* 2006). This complexity gives rise to numerous receptor combinations between RAR and RXR, mediating these heterodimers the physiological actions of RA. *In vitro* studies demonstrated that RAR-RXR heterodimers acting as ligand-inducible transcriptional factors of several genes by binding to specific *cis*-acting DNA sequences named retinoic acid response elements (RAREs) located in the promoter and/or enhancer region of retinoid target genes (Germain *et al.* 2006; Orfali *et al.* 2014; Blomhoff & Blomhoff 2005; Lohnes *et al.* 1994). The RAREs sequences are composed of repeated hexameric half-sites with consensus sequences (5'-PuG(G/T)TCA-3'), separated most commonly by five nucleotides (DR-5) or, less commonly, by two nucleotides DR-2 (Marill *et al.* 2003; Blomhoff & Blomhoff 2005; Rhinn & Dollé 2012). The activation of RAR-RXR heterodimers, and consequently the modulation of the transcription of target gene, is dependent of RAR agonists even in the absence of RXR agonists. However the opposite is not the case, the presence of RAR agonist being necessary for RXR agonists to activate RAR-RXR heterodimers (Sodhi & Singh 2014).

In absence of an agonist, or in response to antagonist activation, RAR-RXR heterodimers binds to the RARE and recruit histone deacetylase (HDAC) complexes via co-repressor proteins such as nuclear co-repressor (NCoR) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT). The end result of these interactions is the silencing of target gene promoter regions, as a consequence of the deacetylation of histone proteins by HDACs and the compaction of chromatin. The binding of an agonist to a RXR-RAR heterodimer induces an allosteric change in the ligand binding domain resulting in the dissociation of co-repressors and recruitment of co-activators (CoAs) such as CBP. These CoAs are responsible for the recruitment of histone acetyltransferases (HATs), an enzyme that catalyse the histone acetylation, resulting in the

decondensation of chromatin, thus facilitating target gene expression (Nagy *et al.* 1997; Altucci & Gronemeyer 2001; Mongan & Gudas 2007; Tang & Gudas 2011; **Figure 5**).

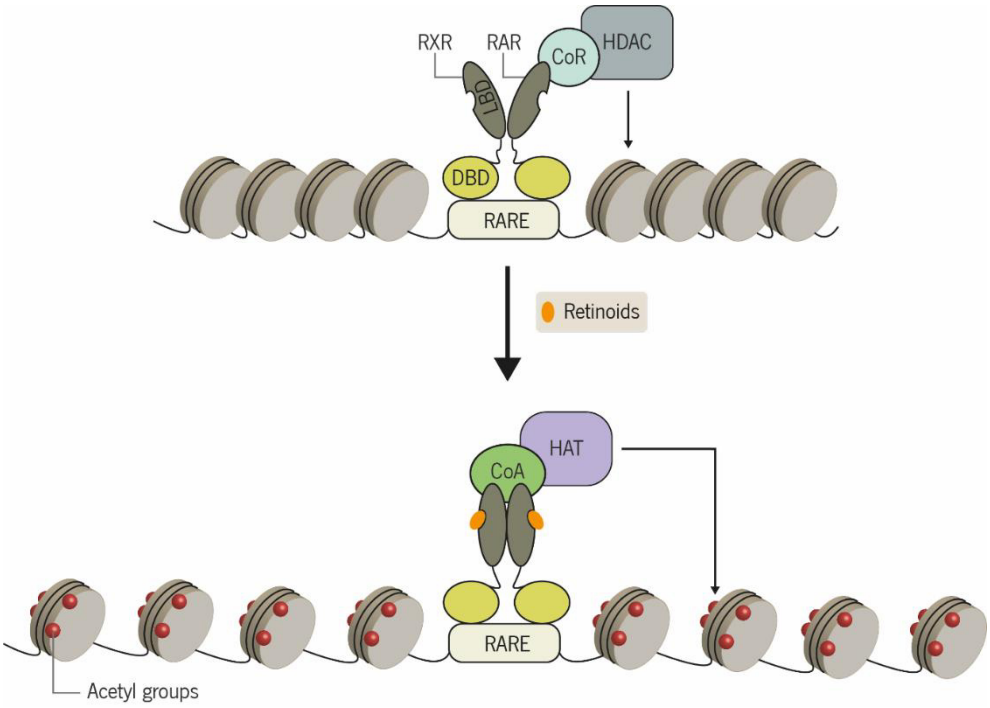

Figure 5 | Mechanism of retinoid action. In the absence of retinoids, the corepressor complex consisting of corepressor proteins (CoR) such as NCoR and SMRT, and histone deacetylases (HDACs) is bound to the RAR-RXR heterodimer. As result, the HDACs removes the acetyl groups from histones proteins, promoting condensation of chromatin and consequently the repression of gene transcription. When retinoids are present in the nucleus, they bind to the RAR-RXR heterodimer inducing an allosteric conformational change in the ligand-binding domain (LBD). This structural change leads to the recruitment of co-activators (CoAs) which in turn recruit histone acetyltransferases (HATs). HAT action results in acetylation of histone and therefore chromatin decondensation and gene expression activation. DBD, DNA-binding domain; RAR, retinoic-acid receptor; RARE, retinoic-acid response element; RXR, rexinoid receptor. Rodrigues (2015)

Despite the action of retinoids in transcriptional regulation being mediated mainly by RAR-RXR heterodimers, the RXRs are able to form homodimers (Lohnes *et al.* 1994), activating the transcription of gene targets by binding to a retinoid X response element (RXRE). The RXRE consists of direct repeats of AGGTCA separated by one nucleotide (DR-1; Soprano *et al.* 2004; Marill *et al.* 2003). RXR can also form multimeric complexes with diverse nuclear receptor partners, such as liver X receptors (LXRs), peroxisome proliferator activated-receptors (PPARs), farnesoid X receptors (FXRs; Rhinn & Dollé 2012), thyroid hormone receptor (THR) and the 1,25-dihydroxy-vitamin D3 receptor (VDR; Ross *et al.* 2000), affecting numerous receptor signalling pathways. The crucial role played by retinoid receptors in development processes is shown in gene knockout studies of single or multiple RAR and/or RXR receptors resulting in the lack of hindbrain

and cerebellar structures (Lohnes *et al.* 1994), as well behaviour abnormalities (Krezel *et al.* 1998).

In the last decade it has been demonstrated that RAR and RXR are also involved in activation of several kinase cascades, including the mitogen-activated protein kinase (MAPK) signalling pathway, thus having an additional extra-nuclear and non-transcriptional effect (Rochette-Egly 2014). Hence the RAR–RXR heterodimers play two distinct functions: first, they modulate the transcription of target genes; and second they have the potential to affect the signalling of many other pathways by mechanisms that remain elusive (Altucci & Gronemeyer 2001).

1.3. Inflammatory Signalling Pathways

Cells are able to recognize and respond to extracellular stimuli triggering specific intracellular mechanisms. Inducible transcription factors regulate cellular responses crucial for normal physiologic functions and allows the organism to adapt to diverse stresses such as environmental, mechanical, chemical, and microbiological stresses (Hayden & Ghosh 2008). Thus, the cascade of evolutionary conserved signal transduction pathways permit cells to convert environmental inputs into cellular responses that protect the organisms against potentially damaging environmental cues. One of the responses that can be triggered by a damaging environmental input via a set of specific signalling pathways is inflammation. Inflammatory pathways are well understood in the body's response to injury or infection. Over the past 15 years some of the same inflammatory pathways have been described to occur in tissues such as adipose as part of the abnormal physiology of obesity (Wellen & Hotamisligil 2003). More recently the tissues in which inflammatory processes occur as a result of obesity have expanded to include the brain (Gregor & Hotamisligil 2011), and lead to further changes in feeding behavior, metabolism and body weight, the three of which are commonly designated as energy balance (Wang & Ye 2014). The nuclear factor- κB (NF-κB) and the MAPK pathways are two of the most studied signal transductions pathways involved in the inflammatory response, promoting the expression of pro-inflammatory cytokines and chemokines (Wang & Liu 2007).

1.3.1. NF-κB Pathway

The NF-κB is a signalling pathway expressed in almost all animal cells types. This evolutionarily conserved signalling pathway plays pivotal roles in inflammatory and immune responses, as well as other biological processes including cell proliferation, differentiation, and survival (Chen & Greene 2004; Oeckinghaus & Ghosh 2009).

Mammalian NF-κB is a family of inducible dimeric transcription factors comprised of five structurally-related proteins – p50 (and its precursor p105), p52 (and its precursor p100), p65 (RelA), c-Rel, and RelB – that are found mostly as inactive homo or heterodimers through association with IκB family proteins (Pahl 1999; Karin & Ben-neriah 2000; Hayden & Ghosh 2004). When associated with these inhibitory proteins, NF-κB is normally sequestered and consequently unable to translocate to the nucleus and promote gene transcription. In the canonical pathway, and in response to a range of stimuli, IκB family proteins are phosphorylated on two N-terminal serine residues by the IκB kinase (IKK) complex. Phosphorylation of IκB proteins leads to recognition of these proteins by the ubiquitin ligase machinery and consequently to their polyubiquitination and subsequent proteasomal degradation, freeing NF-κB (Hayden & Ghosh 2004; Israel & Kroemer 2006). On the other hand, in non-canonical pathway, the activation of IKKα, by activation of kinase NF-kappa-B-inducing kinase (NIK), leads to C-terminal phosphorylation of p100, and consequently to ubiquitination and proteasomal processing to p100 (Oeckinghaus & Ghosh 2009). Once freed, NF-κB is translocated to the nucleus where either alone or in combination with other transcription factor families, binds to specific 10 bp DNA sites within the promoter or enhancer regions, called κB sites, regulating the expression of several target genes, through the recruitment of coactivators or corepressors (Hayden & Ghosh 2008; **Figure 6**).

The transcription factor NF-κB activity is induced by a wide range of stimuli, including cytokines such as tumour necrosis factor α (TNFα), IL-1, IL-6 and IL-8, prostaglandin E2, bacteria, and bacterial products, such as lipopolysaccharide (LPS), viruses and viral products, physical and chemical stresses, and many other stimuli (Ghosh *et al.* 1998). The classical pathway is mainly activated by cytokines such as TNFα and interleukins, stress-inducing agents and lipopolysaccharide (LPS), whereas the non-canonical pathway is activated by certain members of the TNF-cytokines family (Liang *et al.* 2004).

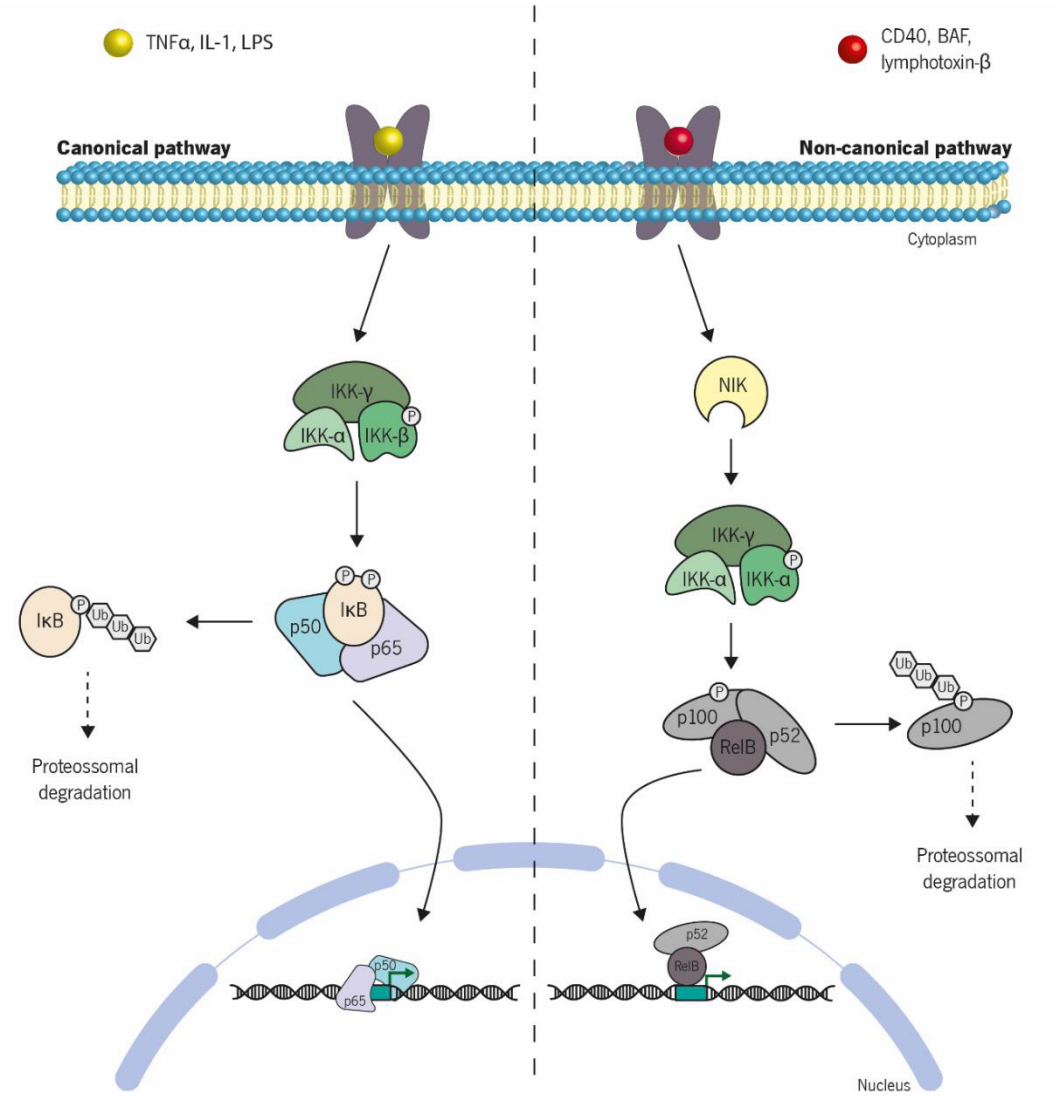

Figure 6 | Schematic representation of the canonical and non-canonical pathways of NF-κB activation. Under resting conditions, the nuclear factor- κB (NF-κB) is bound to the inhibitory IκB proteins, being this way sequestered in the cytoplasm. Stimulation by a range of specific stimuli leads to the activation of two different signalling cascades: canonical (left) and non-canonical (right) pathways. In the canonical pathway activation of IKK complex leads to phosphorylation of IκB and consequently its ubiquitination and proteossomal degradation. In contrast, the non-canonical pathway induces the activation of NF-kappa-B-inducing kinase (NIK), which phosphorylates and activates the IKKα dimers. Once activated, IKKα dimers phosphorylates p100 which is subsequently ubiquitinated and degradated. This results in the release and activation of NF-κB and p52-RelB, respectively, which induce transcription of target genes. Rodrigues (2015)

1.3.2. MAPK Pathway

MAP kinases are a conserved family of protein kinase cascades only found in eukaryotes that have two specific and conserved threonine and serine residues susceptible to phosphorylation. These residues are localized within a conserved TXY motif in the loop between the kinase subdomains VII and VIII (Chang & Karin 2001). MAPKs are involved in regulating a gamut of cellular activities, such as proliferation, metabolism, apoptosis, stress responses, differentiation

and immune response (Roux & Blenis 2004; Whitmarsh 2007). MAPK signalling pathway has been associated with a range of processes in the adult brain, such as memory formation and long-term potentiation (Impey *et al.* 1999; Schafe *et al.* 2001; English & Sweatt 1997; Atkins *et al.* 1998), neuroprotection (Wang *et al.* 2005) and also play a crucial role in the setup of synapses (Shatz 1990; Constantine-Paton *et al.* 1990).

The MAPKs remain in the cytoplasm of unstimulated cells or in quiescent cells in an inactivated form being activated only when both the critical tyrosine and threonine residues are phosphorylated. Binding of ligands to their specific tyrosine kinase receptors (TKR) promotes the autophosphorylation of the receptor on tyrosine residues. The autophosphorylated receptors recruit other proteins that associate with small GTPases activating them (Meloche & Pouysségur 2007). Once activated GTPases recruit the MAPK kinase kinase (MAPKKK) to the membrane for subsequent activation by phosphorylation (Roux & Blenis 2004). Consequently, activated MAPKKK phosphorylates the MAPK kinases (MAPKKs), a group of proteins responsible for phosphorylation and therefore activation of MAPKs. Then MAPKs translocate to the nucleus where they activate some transcription factors, such as AP-1 (Whitmarsh & Davis 1996), c-Myc, SAP-1 and Elk-1 (Zhang & Liu 2002). In this way, the MAPK pathway works as a cascade of activator and effectors proteins.

In mammals, there are four well-defined groups of MAPKs, extracellular signal-regulated kinase (ERK) 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) 1, 2 and 3, p38 isoforms α, β, γ, and δ, and extracellular signal-regulated kinase 5 (ERK 5). ERK 1/2 (also called p48 and p42) is activated by MAPKK-1/2, respectively, p38 by MAPKK-3/6, JNKs by MAPKK-4/7, and ERK-5 by MAPKK-5. However, each MAPKKK can activate different MAPKKs in response to specific stimuli (Hunter 1995; Wang & Liu 2007; Whitmarsh 2007; **Figure 7**).

The different MAPK pathways respond to a plethora of extracellular stimuli: the ERK pathway is mainly activated by growth factors, hormones and mitogens whereas p38, ERK-5 and JNK pathways are activated by stress stimuli and pro-inflammatory cytokines (Roux & Blenis 2004; Zhang & Dong 2005). Of these MAPKs, p38 and ERK1/2 are the most studied families.

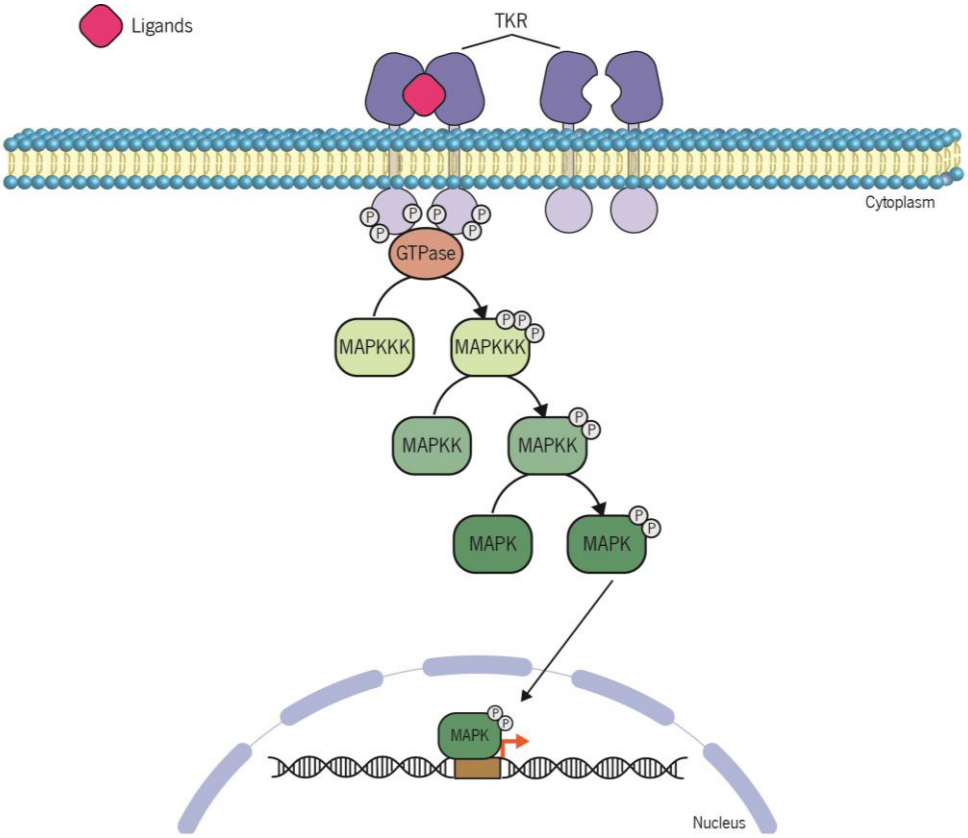

Figure 7 | Schematic representation of the MAPK pathway activation. The extracellular ligand binding to the tyrosine kinase receptor (TKR) and its dimerization cause a conformational change that allows the autophosphorylation of the cytosolic portion of each receptor monomer. The activation of receptors by autophosphorylation triggers a sequential phosphorylation of MAPKKK, MAPKK and MAPK. Once phosphorylated, MAPK translocates to the nucleus where it acts as a transcription factor promoting gene transcription. MAPKKK, MAP kinase kinase kinase; MAPKK, MAP kinase kinase; MAPK, MAP kinase. Rodrigues (2015)

1.3.3. Activation of NF-κB and MAPK signalling pathways by LPS and TNFα

Endotoxin LPS is a glycolipid that constitutes the major constituent of the outer membrane of Gram-negative bacteria and one of the prime inducers of both pro-inflammatory and a type I interferon response, via downstream NF-κB and MAPK signalling (Barton & Kagan 2009; Pålsson-McDermott & O'Neill 2004). The molecule is composed of three parts: lipid A, a core oligosaccharide and an O side chain. The lipid A domain is a conserved component of LPS among the Gram negative bacteria and seems to be responsible for much of the biological toxicity of this glycolipid (Raetz 1990). This endotoxin activates NF-κB and MAPK pathways through Toll-like receptor-4 (TLR4), a receptor that belongs to the TLR family. This family of receptors recognize a range of microbial ligands, including viral nucleic acids and several bacterial and fungal products (Medzhitov 2007), and is necessary for pathogen recognition and innate immune system activation. Little is known about the TLR signalling pathways; however the activation of this pathway

seems to require some adaptors, which are required to determine which signalling pathway will be activated. Once LPS is bound to TLR4, a complex signal-transduction cascade is initiated, which can activate two distinct signalling pathways: the MyD88-dependent pathway, if it depends on the adaptor molecules myeloid differentiation factor 88, and MyD88-independent pathway if it depends on TIR-domain-containing adaptor inducing IFN-β (TRIF, Sharif *et al.* 2007; Lu *et al.* 2008). Both signalling pathways lead to NF-κB and MAPK activation, by recruiting other proteins to the complex – the protein kinases interleukin receptor-associated kinase (IRAK) and TNF-receptor-associated factor (TRAF; Barton & Kagan 2009; Miller *et al.* 2005; Lu *et al.* 2008), and consequently triggers the transcription of some mediators of inflammation such as TNFα, IL-6 and IL1β (Miller *et al.* 2005; Raetz & Whitfield 2008; **Figure 8**).

The pro-inflammatory cytokine TNFα is one of the most powerful physiological activators of the NF-κB pathway in a wide variety of cell types, which is consistent with the role of this pathway in the defensive response. This cytokine belongs to the tumour necrosis factor superfamily and is produced primarily by macrophages, playing an important role in the inflammatory response, activating many genes involved in this physiologic process (Schiitze *et al.* 1992; Cheshire & Baldwin 1997). The biological action of TNFα is mediated by members of a superfamily of receptors, called tumour necrosis factor receptors (TNFRs; **Figure 8**). Several different isoforms of these cell surface receptors exist, being TNFR-1 (or CD120 a) and TNFR-2 (or CD120 b) two of the most important receptors belonging to this family of receptors. The binding of TNFα to TNFR-1 or TNFR-2 results in the recruitment of signal transducers such as TRADD (TNFR-Associated Death Domain), TRAF and RIP (Receptor-Interacting Protein) that activate distinct effectors (Park *et al.* 2000; Bouwmeester *et al.* 2004; Gregory *et al.* 2012). As result, and through complex signalling cascades and networks, different signalling pathways can be activated including the apoptotic programmed cell death pathway through the involvement of caspases (Miscia *et al.* 2002). Both the activation of NF-κB and MAPK results from cooperation of the TNFR with TRADD, which recruits TRAF2 and RIP, and activates the IKK kinase complex and MAPKK, respectively (**Figure 8**).

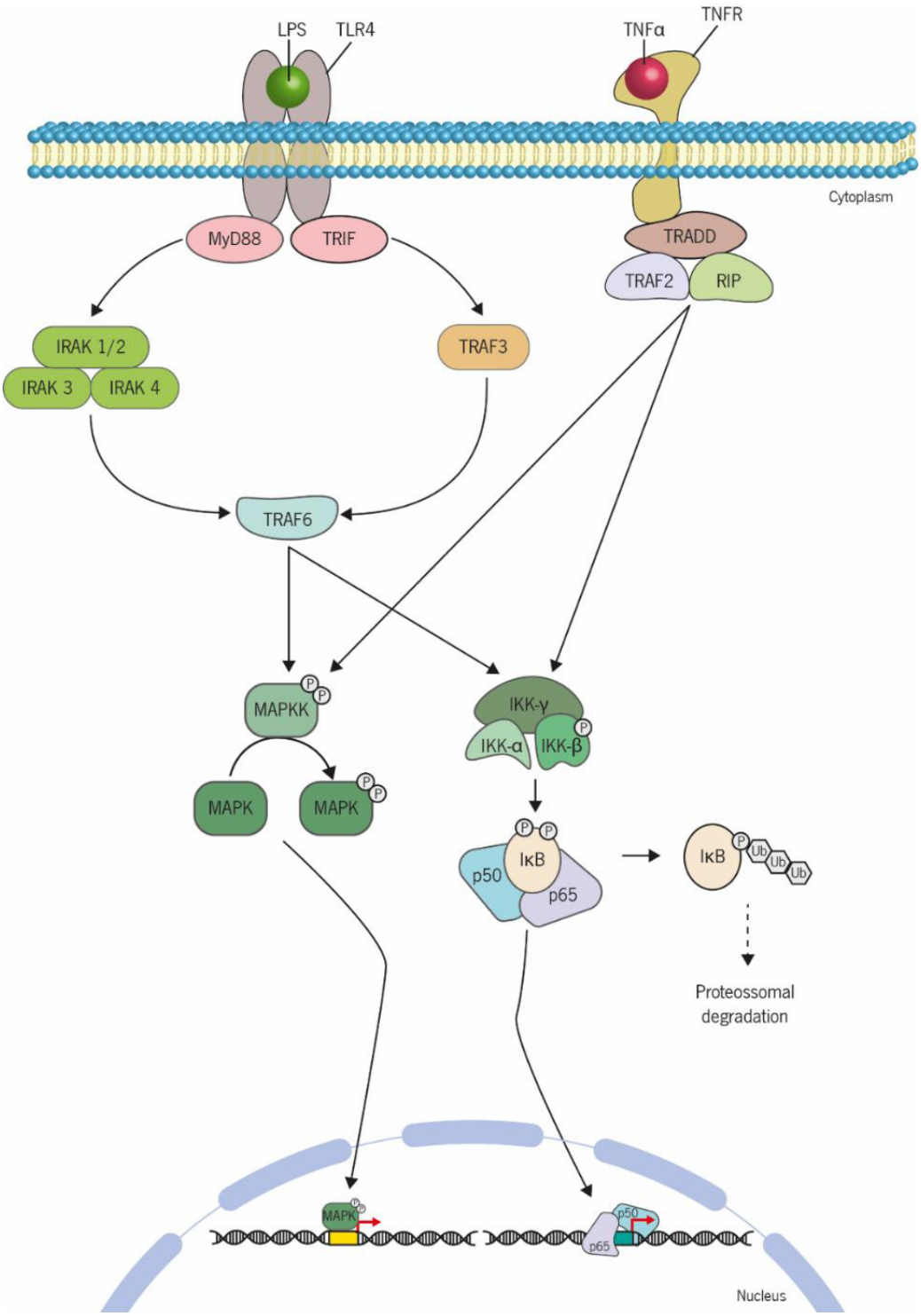

Figure 8 | Schematic overview of activation of NF-κB and MAPK pathways by LPS and TNFα. The binding of tumor necrosis factor α (TNFα) to the TNF receptor type 1 (TNFR1), one isoform present in almost every cell type, or TNFR2, expressed mostly in immune cells, unleashes different signalling pathways responsible for regulating important biological processes such as cell proliferation, differentiation and apoptosis. The NF-κB and MAPK pathways are two of the cascades activated by TNFα by recruitment of the proximal receptor-associated complex consisting of TRADD, TRAF2 and RIP1, what leads to activation of the IKK complex and MAPKK proteins, respectively. The IKK complex and MAPKK proteins may also be activated by the binding of lipopolysaccharides (LPS) to the Toll-like receptor-4 (TLR4). Once bound, two different signalling pathways can be activated: myeloid differentiation factor 88 factor (MyD)88-dependent and MyD88-independent signalling via different adaptor proteins. In the first case, MyD88 is associated with the intracellular domain of cell surface receptors, activating an intracellular complex constituted by Interleukin-1 receptor-associated kinase (IRAK)4, IRAK3 and IRAK1 or IRAK2. The MyD88-independent pathway is mediated by the TIR domain-containing adaptor inducing interferon-β (TRIF), which activates TNF-receptor-associated factor (TRAF)3. Both the IRAK complex and TRAF3 leads to the activation of TRAF6 which in turn phosphorylates the IKK complex and MAPKK proteins, allowing translocation of NF-κB and phosphorylated-MAPK from the cytosol into the nucleus. Rodrigues (2015)

1.4. Epigenetics

Several studies on photoperiod in mammals and birds have indicated that the response to changes in day length can be modulated by epigenetic mechanisms.

The term "epigenetics" was first coined in middle of 20th century by Conrad Waddington (1939) as "*the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being*". Nowadays, epigenetics is defined as *"the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence"* (Wu & Morris 2000).

Over the last 15 years, epigenetic regulation has been intensely studied and it is becoming increasingly clear that epigenetics modification plays a critical role in regulation and remodelling of chromatin structure, interfering with DNA-based processes, such as transcription, DNA repair, and replication (Dawson & Kouzarides 2012). Chromatin is a dynamic macromolecular complex of DNA and histone proteins that forms chromosomes within the nucleus of eukaryotic cells. The fundamental unit of chromatin is designed by nucleosome and consists of 146bp of DNA wrapped around an octamer of histone proteins, with two of each histone H2A, H2B, H3, and H4. The second level of packaging of DNA is called solenoid and consists of numerous closely coiled nucleosomes, where histone H1 supports the chromatin structure (Klug *et al.* 2009). The histone N-terminal tails are able to undergo reversible posttranslational modification such as methylation, acetylation, ubiquitination, phosphorylation, SUMOylation, ADP-ribosylation, and glycosylation since they are not bound to the nucleosome core (Cheung *et al.* 2000; Suto *et al.* 2000).

In addition to histone modification, epigenetic mechanisms also include modifications in DNA (such as DNA methylation), and noncoding RNA-mediated posttranslational regulation (Klug *et al.* 2009). These epigenetic mechanisms seem to be involved in regulation of specific gene expression in the nervous system. However, DNA methylation together with histone modifications, are the most extensively studied epigenetic changes. So far, at least four different DNA modifications and sixteen classes of histone modifications are known (Dawson & Kouzarides 2012).

1.4.1. DNA Methylation

DNA methylation consists of an enzyme-mediated addition of methyl groups, most often to the 5′ position of the pyrimidine ring of cytosine residues, being the primary sites for DNA methylation

the CpG dinucleotides. In mammals, this reaction is catalysed by a family of DNA methyltransferases (Dnmt), including Dnmt3a, Dnmt3b, and Dnmt1 (Feng *et al.* 2007). Dnmt1 is the main enzyme responsible for the maintenance of methylation over multiple cell divisions. These enzymes recognize the hemi-methylated DNA and methylate the non-methylated DNA strand, maintaining the methylation of DNA through mitoses. Dnmt3a and Dnmt3b are responsible for catalysing *de novo* DNA methylation at former sites of un-methylated DNA (Handel *et al.* 2010).

Generally, DNA methylation inhibits the expression of downstream genes by interfering with the binding of transcription factors to DNA (Watt & Molloy 1988) and recruiting methyl-CpG binding domain (MBD) proteins which are capable of binding specifically to methylated DNA and recruit histone deacetylase, thus causing the deacetylation of the DNA and consequent compaction of chromatin and repression of transcription (Rottach *et al.* 2009; Fan & Hutnick 2005). However, in some cases, methylation can lead to an increase in gene transcription by methylation, thereby blocking a silence element (Suzuki & Bird 2008).

Recently it has been shown that photoperiod induces changes in methylation of the *DIO3* proximal promoter, regulating in this way hypothalamic *DIO3* expression, and consequently seasonal reproduction. Animals exposed to SD photoperiod have reduced expression of DNA methyltransferase, the enzyme responsible for DNA methylation of the *DIO3* proximal promoter, and consequently leads to reduced *DIO3* proximal promoter methylation. Thus *DIO3* in animals under short-day winter-like conditions have up-regulated *DIO3* which induces regression of the gonads in hamster. This methylation pattern is reversed when the animals is exposed to LD photoperiod (Stevenson & Prendergast 2013).

1.4.2. Histone Modifications

While DNA methylation often leads to inhibition of gene transcription histone modification can inhibit or activate the same. These epigenetic mechanisms influence chromatin structure, determining the accessibility of the underlying DNA sequences (Yao & Jin 2014). Despite the existence of different classes of histone modifications, lysine acetylation is one of the most studied.

Dynamic histone acetylation and deacetylation of lysine residues is catalysed by HATs and HDACs, respectively, a mechanism which controls several cell signalling events. As mentioned above, the acetylation of N-terminal histone tails leads to a decrease in the stabilization of the

nucleosome, by decreasing the electrostatic interactions between positively charged histone tails and the negatively charged phosphate backbone of DNA. On the other hand, histone deacetylation results in a compression of chromatin preventing the access of transcriptional activators and, consequently, the inhibition of transcription (Hsieh & Gage 2005). To date, eighteen HDACs have been identified in humans and clustered into four distinct groups based on localization and homology to three *Saccharomyces cerevisiae* orthologous - Rpd3, Hda1 and Sir2. Class I HDACs, consisting of HDAC1, -2, -3 and -8, are widely expressed in most tissues and cell lines and have homology to Rpd3. Class II HDACs are homologous to yHDA1 and divided into two subclasses: HDAC IIa, that includes HDAC4, -5, -7, -9, and HDAC IIb, consisting of HDAC6 and HDAC10. Class III HDACs, are homologous to the yeast SIR2 protein. Unlike class I and II that require Zn for catalytic activity, class III HDACs are nicotinamide adenine dinucleotide (NAD)-dependent enzymes. Finally, the class IV HDACs have only a single member, HDAC11, an enzyme that displays similarity with class I and - II but has distinct physiological roles (Thiagalingam *et al.* 2003; Grozinger & Schreiber 2002; Verdin *et al.* 2003; Broide *et al.* 2007). Members of the class II HDACs display several unique features. In addition to class II HDACs being expressed in a restricted number of tissues and cell types, these enzymes shuttle between the cytoplasm and nucleus where they promote the repression of transcription through interaction with tissue-specific transcription factors and co-repressors, via N-terminal domain. The localization of these enzymes is determined by the phosphorylation of two or three conserved serine residues presents in the regulatory N-terminal domain. In this way, when phosphorylated, class II HDACs are sequestered in cytoplasm through association with other proteins (Verdin *et al.* 2003; Mielcarek, Seredenina, *et al.* 2013). This class of HDAC is highly expressed in the brain (Volmar & Wahlestedt 2015), and have been associated with a wide-range of brain disorders such as Huntington's and Alzheimer's disease (Mielcarek *et al.* 2015).

HDAC4 is a key member of the class IIa histone deacetylases, and like its counterparts, can shuttle between the cytoplasm and nucleus. This shift in its location is regulated by calcium/calmodulin-dependent kinase IV (CaMKIV), an enzyme that catalyses the phosphorylation of HDAC4. When phosphorylated, HDAC4 binds to the 14-3-3 family of protein chaperones which allows it to be exported from the nucleus. HDAC4 can subsequently shuttle to the nucleus after dephosphorylation, and consequent dissociation from 14-3-3 (Mielcarek, Seredenina, *et al.* 2013). In the nucleus, HDAC4 binds to chromatin and promotes the deacetylation of nucleosomal histones. HDAC4 is also able to bind to myocyte enhancer factor-2 (MEF2), through the MEF2 -

binding site present in its regulatory domain N-terminal. This prosurvival transcription factor is highly expressed in neurons and is involved in the transmission of extracellular signals to the genome, regulating several calcium signalling pathways involved in cellular processes such as cell division, differentiation and death (McKinsey *et al.* 2002; Potthoff & Olson 2007). The binding of HDAC4 to MEF2 represses its activity and thereby represses the transcription of MEF2-dependent genes (Mielcarek *et al.* 2013). In the cytoplasm, HDAC4 seems to be involved in the regulation of other proteins, such as HIF-1α, MEKK2 and STAT1, by deacetylating them through zinc-containing catalytic domains present in its C-terminal (Wang *et al.* 2014). It has been reported that HDAC4 is abundantly and heterogeneously distributed in the brain (Darcy *et al.* 2010; Broide *et al.* 2007), where its mRNA is highly expressed (Wang *et al.* 1999) as compared to the other class IIa enzymes. Taken together, these studies suggest an important biological role of HDAC4 in brain. However its precise function and regulation is still unknown; HDAC4 seems to be associated with obesity and energy balance once its expression levels increases in the medial hypothalamus of mice in response to fasting (Funato *et al.* 2011). In addition, this histone deacetylase has also been linked with eating disorders (Cui *et al.* 2013), as well obesity, being downregulated in obese subjects and induced by physical exercise (Abu-Farha *et al.* 2013).

1.5. Aim

It is well-known that the control of hypothalamic energy balance, including feeding and body weight, have an important genetic component, but surprisingly few single genes have been directly linked to regulation of these processes. This has led to growing interest in the epigenetic regulation of hypothalamic function wherein some evidence suggests that the expression of some of the genes affecting energy balance are regulated by epigenetic mechanisms. At same time, it is also known that inflammatory responses are one key to changes in the hypothalamus. Finally, the HDACs are key in the activity of several nuclear receptors involved in these pathways. A future challenge consist in understand and identify the mechanisms that regulate neuroinflammatory responses involved in energy balance.

Data obtained in our laboratory showed that *HDAC4* expression is photoperiodically regulated in the hypothalamus of F344 rats, with higher expression in rats under long-day photoperiod (16h light:8h dark), relative to short-day (8h light:16h dark). This result suggested that HDAC4 may be involved in the regulation of energy balance by photoperiod. Immunohistochemical analysis also

showed that HDAC4 was predominantly expressed by neurons and tanycytes, two of the hypothalamic cell types emerging as components of the networks that control body weight and energy balance (Bolborea & Dale 2013), and was largely absent from astrocytes or microglia. In addition, expression of the NF-κB target gene *NFKB1* was increased in the hypothalamus of F344 rats under short-day conditions, when HDAC4 expression is lower, relative to rats in long-day conditions. Given the known function of HDAC4 in regulating cytoplasmic proteins, we therefore hypothesised that HDAC4 may regulate energy balance through NF-κB signalling in hypothalamic neurons.

Thus, in order to clarify the role of epigenetic mechanisms and inflammatory pathways in the regulation of energy balance by photoperiod, the present study aims to explore the association of HDAC4 with hypothalamic function by investigating seasonal changes in gene expression, as well as the possible role of this HDAC in modulating the inflammatory signalling pathways in the hypothalamus. In addition RAR and THR are studied as possible factors modulating energy balance and acted upon by HDACs.

To achieve this goal, the following specific objectives were defined:

- Identify NF-κB - regulated genes in GT1-7 hypothalamic neurons and primary cultures of hypothalamic tanycytes;
- Use HDAC inhibitors to understand the link between HDAC4 and hypothalamic inflammatory signalling pathways;
- Use HDAC inhibitors to understand the possible role of HDAC4 in regulating the activity of RAR and THR in hypothalamic cells.

CHAPTER II

MATERIALS AND METHODS

2.1. Chemicals

For activation of NF-κB, p38 and p44/p42 signalling pathways, cells were treated with a final concentration of 1 µL/mL LPS (L3012, Sigma) or 1 ng/mL TNFα (510-RT-010, R&D Systems). For studies of HDAC inhibition, cells were treated with LMK235 (4830, Tocris) or Tubastatin A (SML0044, Sigma). Both the inhibitors were dissolved in 100% DMSO (D2650, Sigma) to a final concentration of 1 µM and 5 µM, respectively (the final concentration of DMSO was 0.1%). 5-Aza-2'-deoxycytidine (A3656, Sigma) was used for studies of DNA methylation inhibition. This inhibitor was dissolved at 50 mg/ml in 50% acetic acid. The p38 inhibitor SB202190 (57067, Sigma) was used at a concentration of 9 µM and was dissolved in 100% DMSO. Cells were also treated with a final concentration of 1 µM all-*trans*–retinoic acid (R2625, Sigma), which was dissolved in 100% DMSO and 50 mM triiodothyronine (T2877; Sigma), dissolved in 1 M NaOH. When DMSO, NaOH or acetic acid were used to dissolve the reagents, a negative control with the same final concentration of these solvents was employed.

2.2. Cell cultures

GT1-7 cells, a mouse hypothalamic neuronal cell line (Mellon *et al.* 1990), were kindly provided by Prof. Alexander Ross (Rowett Institute of Nutrition and Health, University of Aberdeen, Scotland), and SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured in 75 cm^2 tissue culture flasks containing 12 mL Dulbecco's modified Eagle's medium (DMEM; 21885-025, Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS; S020, Invitrogen) and 1% penicillin/streptomycin (INV220, Invitrogen), at 37°C in an incubator containing 5% CO2. The medium was replaced twice a week.

Primary tanycyte cell cultures were prepared from the median eminence (ME) of 10-day-old rat pups as described previously (Prevot *et al.* 2003). Cultures from this age mainly consist of tanycytes, having few astrocytes (Chauvet *et al.* 1996). Briefly, the brains were carefully removed and washed in tanycyte culture medium (DMEM/F-12; 31331-028, Invitrogen) supplemented with 10% of FCS and 1% penicillin/streptomycin). Hypothalamus was dissected submerged in the same medium, and the median eminences were collected. The ME were washed with phosphatase buffered saline (PBS) 1x, incubated for 20 min, at 37°C, with trypsin, and the tissue was subsequently triturated using a P1000 pipet. The dissociated cells were collected and cultured in

tanycyte culture medium in 40 cm^2 tissue culture flasks for five days. After that, the medium was replaced twice a week, and the cultures maintained a maximum of three weeks. For gene expression studies and western blotting analysis, the tanycytes were isolated from contaminant cells by overnight shaking at 220 r.p.m. and plated in twelve-well plates.

2.3. Organotypic slice culture

The hypothalamic organotypic slice cultures were prepared from 10-day old Sprague-Dawley rats as described by Stoney *et al.* (2015). In detail, brains were carefully removed under sterile conditions and the hypothalami were dissected submerged in slice medium composed of 50% Minimum Essential Medium Eagle (M5775, Sigma), 25% Horse Serum Bovine (H1138, Sigma), 2.5% GlutaMax (10388-582, Invitrogen), 1% HEPES Buffer (H0887, Sigma), 18% HBSS (11590-466, Invitrogen) and 1% penicillin/streptomycin, and additional 5 mg/mL of D-Glucose. A McIlwain tissue chopper (Mickle Laboratory Engineering Ltd) was used to cut each hypothalamus in 400-μm-thick transverse slices. Slices were transferred and gently separated in ice-cold slice medium. The hypothalamic slices including the arcuate nucleus and the paraventricular nucleus were selected, trimmed laterally and dorsally by the ARC and PVN, respectively and cut along the midline. This way two equivalent set of slices per animal were obtained, which were individually placed onto a millicell semiporous membrane (10412511, Millipore). The membranes were transferred to a six-well culture plate with 950 μL slice medium. After 24 hours the slice medium was replaced with Neurobasal medium 1x (10770-954, Invitrogen) containing B-27 (11500-446, Invitrogen) supplemented with 1% GlutaMax, 1% penicillin/streptomycin, and an additional 5 mg/mL of D-Glucose. Cultures were maintained in a humidified 5% CO2 atmosphere at 35 °C and medium was refreshed every two days.

2.4. RNA extraction and gene analysis by qPCR

The GT1-7 cells, primary tanycytes and organotypic slices underwent different treatments depending on the goal of the experiment. Total RNA was extracted using Isolate II RNA Mini Kit (BIO-52043, Bioline) according to the manufacturer's instructions and quantification of RNA was measured using a NanoDrop spectrophotometer (Thermo Scientific). RNA from macrophages

stimulated with LPS (1 µg/mL) for 2 hours were kindly provided by Dr. Heather Wilson (Institute of Medical Sciences, University of Aberdeen, Scotland)

From each sample, the cDNA was synthesized from 250 ng of total RNA by reverse transcription using a High capacity RNA-to-cDNA kit (Applied Biosystems) in a reaction performed at 37°C for 1h, and in a final volume of 20 µL.

Quantitative real-time PCR expression analysis was carried out using the LightCycler 480 thermal cycler (Roche). For each qPCR reaction, 3 µL cDNA (diluted 1:4 in ultra-pure distilled water) was used in a final reaction volume of 15 µL that also contained 7.5 µL SensiMix SYBR Green Master Mix (QT650-05, Bioline) and 0.25 µM of each primers. The qPCR reaction cycles and temperatures were as follows: pre-incubation (10 min at 95°C), 45 cycles of amplification and quantification program (95°C for 15s for denaturation, 15s at 60°C for primer annealing and 15s at 72°C for extension with fluorescence acquisition), followed by a melting curve program (5s at 95°C; 1min at 58°C with a single fluorescence measurement). Standard curves and blank controls were run for all genes. The expression values of the studied genes were normalized by the average of the expression of the reference gene *mActb* (actin). All biological samples were tested in triplicate, and standard deviation values of the means were calculated using standard statistical methods. Quantitative PCR primer sequences are listed in **Table 1**.

Table 1 | Sequences of the primers used in the qPCR.

Symbol	Sequence (5' → 3')		Amplicon lenght
	Forward primer	Reverse primer	
mPrkcd	GGGACCCTGACAAGAGGCTGGG	GGTCAAAGTTGCTGTAGTCTGAAGGGG	150
mPrkca	CTGGCCCTGCTGGACAAGCC	AGGTCCCCGCCGTTGACGTA	104
mFos	CTGGGGCTTACGCCAGAGCG	GGAGATAGCTGCTCTACTTTGCCCCT	94
mJun	TGTCCTGCCCAGTGTTTGTA	GAGGTTGGGGGCTACTTTTC	155
mPTP1B	GGAACTGGGCGGCTATTTACC	CAAAAGGGCTGACATCTCGGT	117
mCyp26A1	GCCTGTACCGGGGCGTGAAG	GTGACGTCGCAGCACTGGCT	240
mRALHD2	CAAGGAGGCTGGCTTTCCACCC	GGGCTCTTCCCTCCGAGTTCCA	198
mCYP26B1	CGTGCGTGTGCTGCTAGGC	AGCTTGGATGCCCCTCCGGT	139
mRARb	CGCGAGCCCTTCCTCCTGC	AAAAGCCCTTGCACCCCTCGC	254
mDio2	TGCCTACAAACAGGTTAAACTGGGTGA	TGGCATTCGGCCCCATCAGC	120
rHDAC6	ACAGGCTCTGTCCTCAGGCTGG	CTGCGCTGAGCATGGTGTCCA	98
rHDAC4	GCTCGAGGGGGAGCAGCATC	CGAAGTCCCACTGGCAGCGT	112
rDNMT1	CGCCGGTGTGGTGTCTGTGA	CCGTCCGGTGCCACCAAACTT	93

Symbol	Forward primer	Reverse primer	Amplicon lenght
rRALDH1	AGGGTCACGCTGGAGCTTGGG	GAAAATCCGGGATGCCGCGACG	138
rRALDH2	TGGTGGAACGGGACAGGGCA	CGGGTGGAAAGCCAGCCTCCT	336
rDio2	GCTGCTTCTCAGCCGCTCCA	GCACCACACTGGAATTGGGAGCA	149
rNFKB1	AGCAATTGCCCCGGCATCCA	CCGCGTAGTCGAAGAGGGCG	125
rNos2	ACCTTGGTGAGGGGACTGGACTTT	CCAACTCTGCTGTTCTCCGTGGG	105
rFcgr1	ACCAACCTGAGTCACAGCGGC	CACTCTCAGCACTGGTGTGGCA	117
rIL1b	AGCAGCTATGGCAACTGTCCCTG	CAGCCCAAGTCAAGGGCTTGGAA	132
rIL6	AGACAAAGCCAGAGTCATTCAGAGCA	GGAGAGCATTGGAAGTTGGGGTAGG	113
rTnfa	TCTGGGTCCAACTCCGGGCTC	TGAGCAGCCCGCAATCCAGG	94
rCyp26A1	CCCCCGGGTACCATGGGCTT	GCCCATCACTCGCACCGTGG	147
rCyp26B1	TCCATTGGCGACATCCACCGC	GGCTGCTCCAGGCTCGAAGTG	127
mActb	CCACACCCGCCACCAGTTCG	TACAGCCCGGGGAGCATCGT	112
hCyp26A1	CACCGTACGGGTGATGGGCG	GCTGGCCAGTGGACCGACAC	81
hCyp26B1	ACCGGCCACTGGCTGCTG	ACGTTGATGGCCTCGGGGTG	134
hRALDH1	CAAGGCAGTGAAGGCCGCAAGA	ATTGACTCCATTGTCGCCAGCAGC	144
hRALDH2	CACTGAGCAGGGTCCCCAGATTGA	AACCCCTTTCGGCCCAGTCCT	123
hActb	CCGCCGCCAGCTCACCATG	CGATGGAGGGGAAGACGGCC	119

2.5. Western blotting analysis

Western blotting was used to evaluate the activation of NF-κB, p38 and p44/42 pathways. For that purpose, GT1-7 cells, primary tanycyte cultures and organotypic slices were treated with TNFα and LPS at different times of incubation according to the setup of the experiment. After treatment, the proteins were extracted using lysis buffer (150 mM NaCl, 50 mM HEPES, 1% Triton, 0.1% SDS) supplemented with 1x protease inhibitor cocktail (04693159001, Roche Life Science) and 1x phosphatase inhibitor cocktail (P5726, Sigma). For organotypic slices, the protein quantification was performed using Micro BCA™ Protein Assay Kit (Thermo Scientific) with bovine serum albumin (BSA) as protein standard.

For western blotting analysis, equal amounts of sample (10 μL for GT1-7 and tanycytes, and 12.5 μg for slices) were electrophoresed in 12% SDS-PAGE gel at 150 V for 1hr 20 min, and electroblotted to nitrocellulose membranes (GE Healthcare), using a Mini Trans-Blot Cell (Bio-Rad) for 1hr 30min at 100V. Western blotting was performed following conventional protocols; in summary blots were washed 3 x 5 min with 1x TBS-Tween and blocked with 5% Milk in TBS-Tween

for 1h at RT. Primary antibodies were incubated overnight at 4°C in 2% BSA in TBST-Tween. Anti-NF-κB (1:2000), anti-phospho- NF-κB (1:000), anti-p38 (1:1000), anti-phospho-p38 (1:1000), anti-p44/42 (1:2000) and anti-phospho-p44/42 (1:4000) were the primary antibodies used. Afterwards, the blots were washed 3 x 5 min with 1x TBS-Tween, and incubated with anti-rabbit horseradish peroxidise-conjugated secondary antibodies (1:5000) during 1h at RT, followed by 3 x 5 min washes with TBS-Tween. The immunoreactive bands were visualized using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica), acquired using a ECL imager (Thermo Scientific), and the data was analyzed using Image J.

2.6. Immunostaining

For immunocytochemistry, GT1-7 cells, SH-SY5Y cells and primary tanycytes were grown on poly-L-lysine (PLL)-coated coverslips for 24 hours. After that, cells were washed 3x 5 min in PBS and fixed in 4% PFA at room temperature for 15 min and then washed again 3x 5 min in PBS. Fixed cells were blocked in 1x PBS containing 5% v/v of horse serum (H1138, Sigma) and 0.01% Triton X-100 (28313, ThermoScientific) for 1 hour at room temperature. The blocking buffer was removed and blocking buffer containing primary antibody was added. Cells were incubated in HDAC4, HDAC6, vimentin and α-β-tubulin primary antibodies (**Table 2**) overnight at 4 °C. After washing in blocking buffer, cells were incubated with the corresponding secondary antibodies (**Table 2**) for 1 hour at room temperature. The cells attached to coverslips were then washed 3x 5 min in blocking buffer and 1x 5 min in PBS, and mounted on slides in fluorescence mounting medium (4% paraformaldehyde in 0.1 M phosphate buffer containing 50% glycerol, 1 mg/mL bisbenzimide and 2.5% DABCO), before imaging by fluorescence microscopy.

As a positive control, anti-vimentin immunocytochemistry was performed on tanycytes and anti- α-β-tubulin immunocytochemistry was performed on GT1-7 and SH-SY5Y cells. The negative control was performed with the same protocol except that the primary antibodies were not added to the blocking buffer.

For immunohistochemistry, the rat brains were perfused in 4% paraformaldehyde dissected out, kept in 4% paraformaldehyde overnight and sunk in 30% sucrose in PBS at 4°C before embedding in optimal cutting temperature compound (OCT). Serial coronal sections were sectioned at 40 μM using a cryostat (Leica, CM1850) and placed in a 12 well plate containing

phosphate-buffered saline (PBS), placing each sequential section into a new well and cycling around the 12 wells until completed. This way, each well had approximately 15-18 representative sections from all region of the brain. Sections were stored at -20°C in PBS buffer just before use. Antigen retrieval was carried out by incubation in 0.01 M sodium citrate buffer (pH 6.0) and heating for 20 min at 85 °C, using a thermoblock. The sections were left at RT for 20 min to allow the slides to cool down and then washed 4x 10 min in PBS and blocked in blocking buffer (1x PBS, 10% donkey serum (AS-228/100, Labtech international), 0.1% Triton X-100) for 3 hours. The blocking buffer was removed and primary antibodies in blocking buffer was added. The sections were incubated in HDAC6 and GFAP primary antibodies (**Table 3**) overnight at 4 °C. After washing in PBS buffer, sections were incubated with the corresponding secondary antibodies (**Table 3**) for 2 hour at room temperature and then washed 4x 10 min in PBS buffer. The sections were mounted on glass slides, allowed to air dry and coverslipped with mounting medium, before imaging by fluorescence microscopy.

2.7. Statistical analysis

Statistical analysis was performed on at least three independent experiments using GraphPad Prism® software (Prism 5 for Windows). All statistics for the variables in the studies were expressed as mean ± standard error (SEM). The two-tailed unpaired Student's t-test was used to determine significance of differences in means between two independents populations. For multiple groups, one-way ANOVA was used and *post hoc* comparisons were performed using Tukey's test.

Differences were considered at the significance level of *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Table 2 | Antibodies used for fluorescent immunocytochemistry.

Primary antibody	Isotype	Host	Supplier (code)	Dilution	Secondary antibody used (used at 1:300 dil.)	Supplier code
anti-HDAC4	IgG	Rabbit	Abcam (ab79521)	1:500	Donkey anti-rabbit IgG, Alexa 594-conjugated	Stratech (711-585-152-JIR)
anti-HDAC6	IgG	Rabbit	Abcam (ab1440)	1:100	Donkey anti-rabbit IgG, Alexa 488- or Alexa 594-conjugated	Stratech (711-545-152-JIR/ 711-585-152-JIR)
anti-α-β-tubulin	IgG	Mouse	———	1:500	Donkey anti-mouse IgG, Alexa 488-conjugated	Stratech (715-545-151-JIR)
Anti-Vimentin	IgG	Rabbit	Sigma (V6389)	1:300	Donkey anti-rabbit IgG, Alexa 488	Stratech (711-545-152-JIR)

Table 3 | Antibodies used for fluorescent immunohistochemistry.

Primary antibody	Isotype	Host	Supplier (code)	Dilution	Secondary antibody used (used at 1:300 dil.)	Supplier code
anti-HDAC6	IgG	Rabbit	Abcam (ab1440)	1:300	Donkey anti-rabbit IgG, Alexa 594-conjugated	Stratech (711-585-152-JIR)
Anti-GFAP	IgG	Mouse	Sigma (G3893)	1:300	Donkey anti-rabbit IgG, Alexa 594	Stratech (711-585-152-JIR)

CHAPTER III

RESULTS

3.1. NF-κB pathway is activated by TNFα in GT1-7 cells and by LPS in tanycytes

In preliminary experiments to determine the optimal way to activate the NF-κB pathway in GT1-7 cells and tanycytes, these cells were incubated with TNFα (1 ng/mL) and LPS (1 μg/mL), two well-known inducers of the NF-κB pathway (Oeckinghaus & Ghosh 2009; Karin *et al.* 2004) for 30 minutes, 1 hour and 3 hours. Western blotting was used to detect the total NF-κB and phosphorylated-NF-κB proteins. The immunoblots showed that exposure of GT1-7 cells to both TNFα and LPS resulted in NF-κB activation (**Figure 9**). However, the NF-κB-inducing capacity of LPS seems to be less pronounced when compared with TNFα, and moreover, it could be observed that activation of the NF-κB pathway by LPS was independent of incubation time, contrary to activation with TNFα that presents a peak after 1hr of incubation.

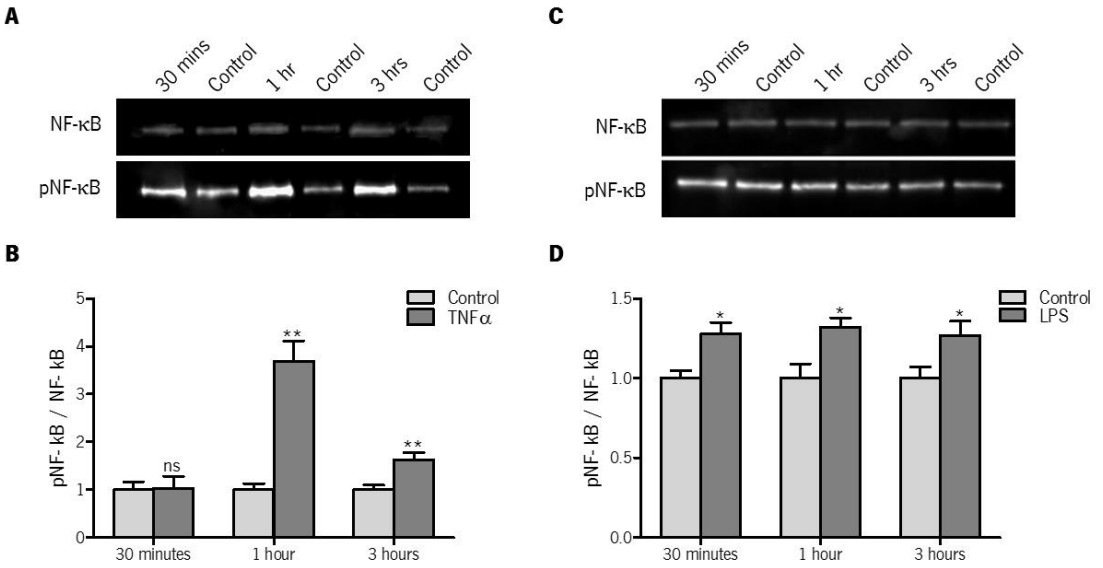

Figure 9 | Western blot analyses of NF-κB pathway activation by TNFα and LPS, in GT1-7 cells. The effect of TNFα **(A,B)** and LPS **(C,D)** in the activation of NF-κB pathway was measured by western blot, after 30 minutes, 1 hour and 3 hours of incubation with TNFα (1 ng/mL) and LPS (1 μg/mL). **(A, C)** Ten μL of total protein obtained from GT1-7 cells samples were subjected to SDS-PAGE 12% gel. The gel with proteins was transblotted onto a nitrocellulose membrane and probed with 1: 2000 dilution of anti-NF-κB and 1:1000 dilution of anti-phospho- NF-κB antibodies. The bands were quantified as described in material and methods. Shown blots are representative of 3 independent experiments with similar results. **(B, D)** The graph shows the ratio between phosphorylated NF-κB and total NF-κB, normalized relatively to controls. Values are mean ± S.E.M. of 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference.

The same experiments were performed in tanycytes which showed a quite different response to LPS and TNFα, with LPS resulting in a much stronger induction of the NF-κB pathway than TNFα (**Figure 10**), which supports what was previously reported by Vries *et al.* (2014).

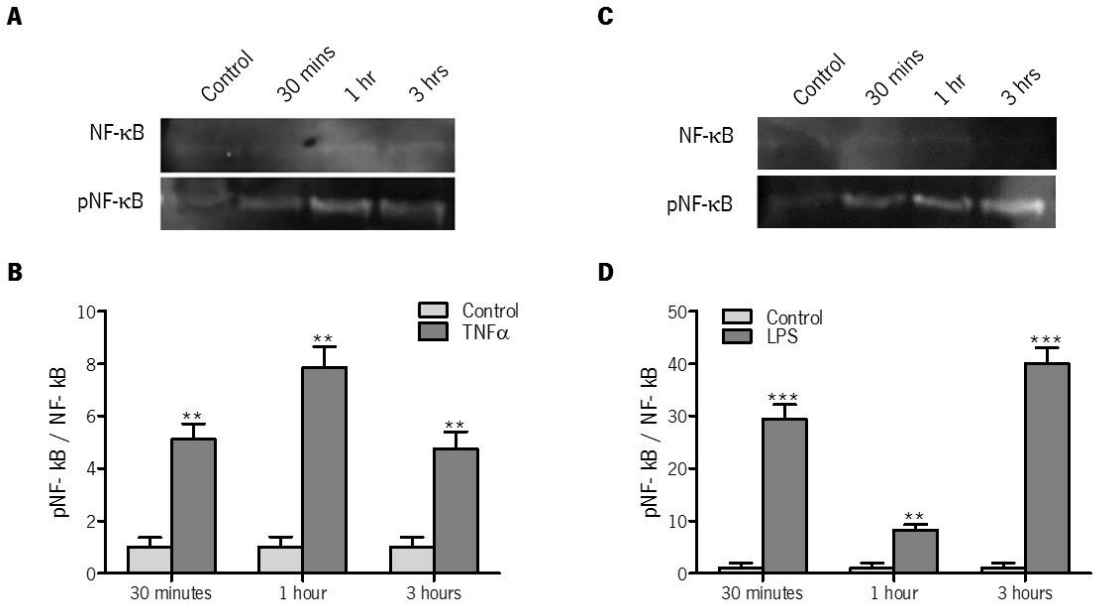

Figure 10 | Western blot analyses of NF-κB pathway activation by TNFα and LPS, in tanycytes. The effect of TNFα **(A and B)** and LPS **(C and D)** on the activation of the NF-κB pathway was measured by western blot, after 30 minutes, 1 hour and 3 hours of incubation with TNFα (1 ng/mL) and LPS (1 µg/mL). **(A, C)** Ten µL of total protein obtained from tanycytes samples was subjected to SDS-PAGE 12% gel. The gel with proteins was transblotted onto a nitrocellulose membrane and probed with 1: 2000 dilution of anti-NF-κB and 1:1000 dilution of anti-phospho- NF-κB antibodies. The bands were quantified as described in material and methods. Shown blots are representative of 3 independent experiments with similar results. **(B, D)** The graph shows the ratio between phosphorylated NF-κB and total NF-κB, normalized relatively to controls. Values are mean ± S.E.M. of 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference.

Thus, for the subsequent experiments performed to study the effect of HDAC4 on NF-κB pathway regulation, TNFα was selected to be used in GT1-7 cells, whereas LPS was selected to be used in tanycyte experiments.

3.2. Class IIa HDACs can decrease TNFα and LPS activity in hypothalamic cells

Although HDAC corepressor proteins are required to regulate several inflammatory transcription factors such as NF-κB (Barneda-Zahonero & Parra 2012), little information is known about their specific function and mechanism of action in the brain. To clarify the possible role of HDAC4 on inflammatory signalling in hypothalamic cells, the influence of HDAC inhibitors on gene expression was evaluated. For that purpose, GT1-7 cells were treated simultaneously with HDACs

inhibitors and TNFα for 3 hours, and the transcript levels of some genes regulated by NF-κB were quantified by qPCR. Since there is no specific inhibitor for HDAC4, two different inhibitors were used: the LMK235 inhibitor which is a selective HDAC4 and HDAC5 inhibitor and also inhibit weakly HDAC6 (Marek *et al.* 2013), and Tubastatin A, a selective inhibitor for HDAC6 (Butler *et al.* 2010).

Jun, *Fos*, *Prkca*, *Prkcd* and and *PTP1B* encode c-Jun, c-Fos, protein kinase C alpha, protein kinase C delta and protein-tyrosine phosphatase 1B (PTP1B), respectively. All of these genes are described in the literature to be regulated by NF-κB pathway (Fujioka *et al.* 2004; Yu *et al.* 2013; García-San Frutos *et al.* 2012; Zhang *et al.* 2013) and can therefore be used as markers to study the activation of this signalling pathway.

C-Jun and c-Fos are two proteins which heterodimerize to form the AP-1 transcription factor capable of binding to specific DNA sequences and regulate the transcription of genes involved in biological processes including proliferation, differentiation and apoptosis (MArk Glover & Harrison 1995). As depicted in **Figure 11A and B**, *Fos* expression was not significantly induced by TNFα. However, HDAC4 and 5 inhibitor, by itself, weakly induced *Fos* expression and enhanced the TNFα induction of this gene (**Figure 11A**). Similar results were observed with HDAC6 inhibition (**Figure 11B**). In contrast, *Jun* expression was induced by TNFα and HDAC inhibition did not enhance its expression (**Figure 11C and D**). Prkca and Prkcd are two isozymes of Protein kinase C (PKC), a family of protein kinases that appear to be involved in obesity (Huang *et al.* 2012; Itani *et al.* 2000; Gao *et al.* 2007). *Prkca* was not induced by TNFα with or without HDAC4 and 5 inhibition (**Figure 11E**) while its expression was slightly and significantly increased by HDAC6 inhibition combined with TNFα (**Figure 11F**). On the other hand, *Prkcd* was not induced by TNFα, whereas HDAC4 and 5, and also HDAC6 inhibition led to an induction of *Prkcd* expression even in absence of TNFα (**Figure 11G and H**). Lastly, PTP1B is a protein which belongs to the protein tyrosine phosphatase family, a family of proteins responsible for the dephosphorylation of tyrosine residues of other proteins. This protein has been implicated in the regulation of the insulin signalling pathway, being a potential therapeutic target in treatment of type 2 diabetes (Johnson *et al.* 2002). Similar to *Fos* and *Prkcd*, TNFα is unable to induce the expression of *PTP1B* (**Figure 11I and J**). However, HDAC4 and 5 inhibition, as well as HDAC6 inhibition, resulted in an increase in the capacity of TNFα to induce *PTP1B*.

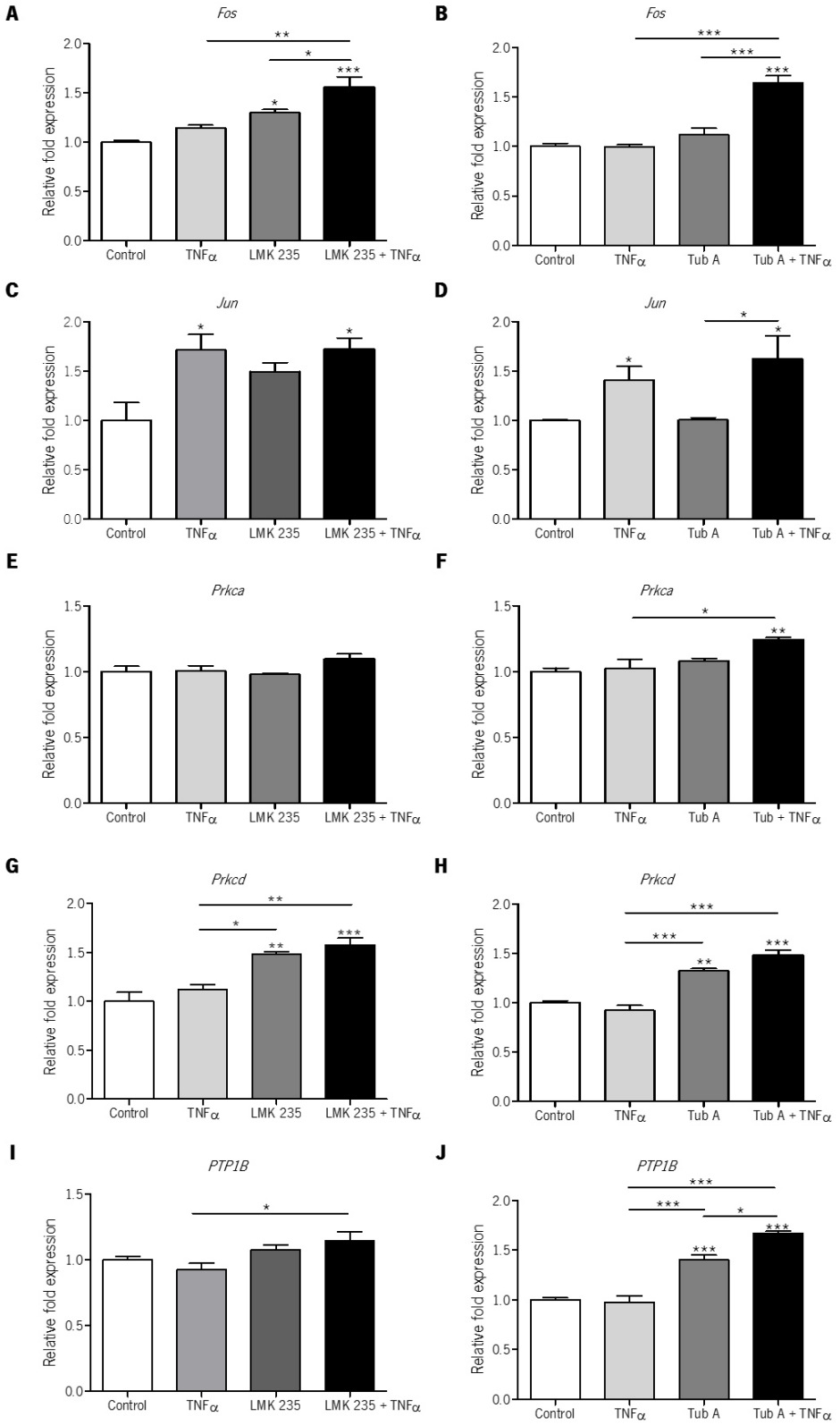

Figure 11 | Effect of deacetylase inhibitors on NF-κB target gene activation by TNFα, in GT1-7 cells. The effect of HDAC4 and 5 inhibition (*left panel*) and HDAC6 inhibition (*right panel*) on expression of the *Fos* **(A,B)**, *Jun* **(C,D)**, *Prkca* **(E,F)**, *Prkcd* **(G,H)** and *PTP1B* **(I,J)** in GT1-7 cells. The GT1-7 cells were treated with LMK235 (1 μM), a HDAC4 and 5 inhibitor, or Tubastatin A (5 μM), a specific HDAC6 inhibitor, for 1 hour. After that TNFα (1 ng/mL) was added and the cells were incubated for more 3 hours. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test. Tub A, Tubastatin A

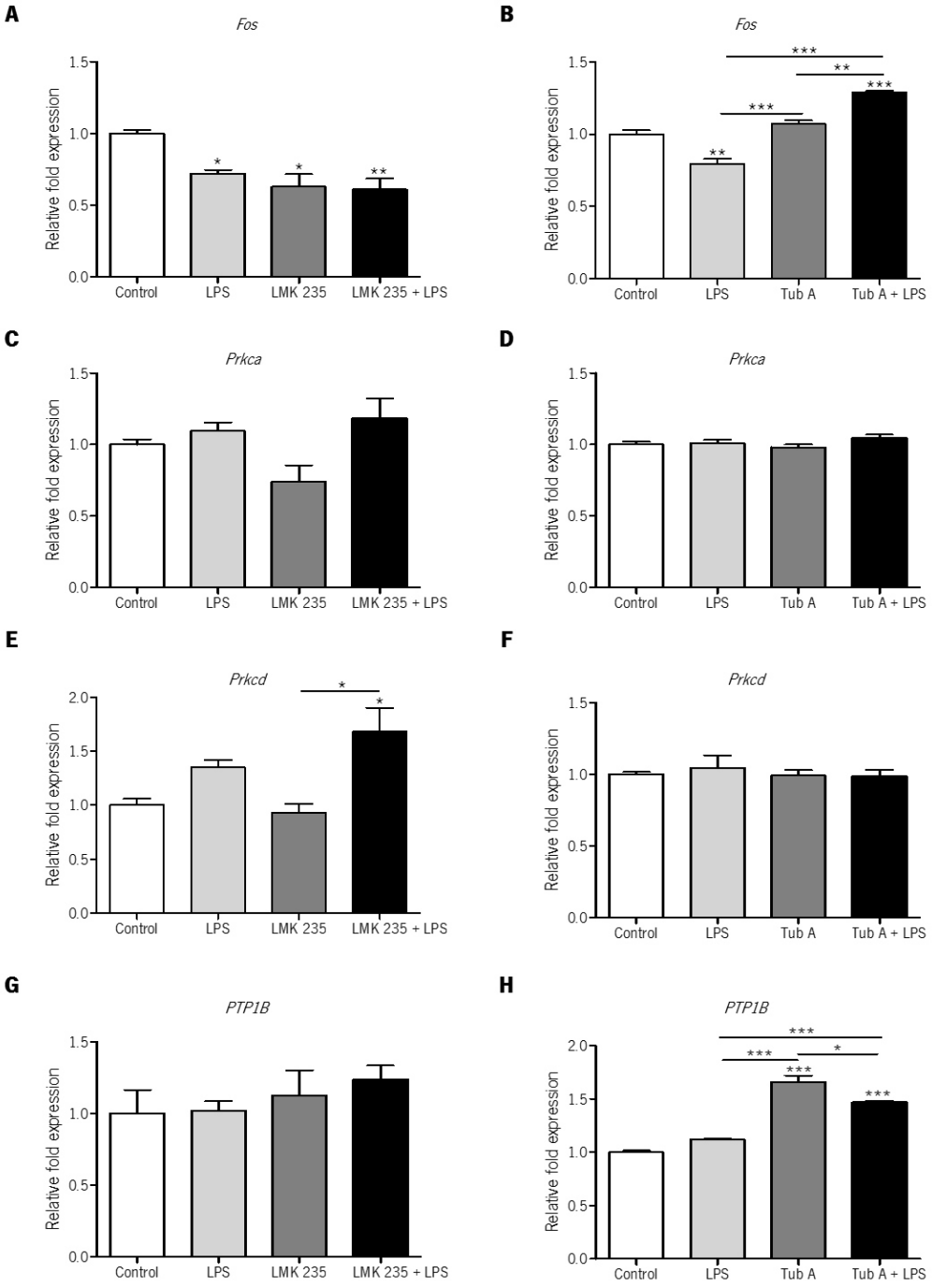

Figure 12 | Effect of deacetylase inhibitors on NF-κB target gene activation by LPS, in tanycytes. The effect of HDAC4 and 5 inhibition (*left panel*) and HDAC6 inhibition (*right panel*) on expression of the *Fos* **(A,B)**, *Jun* **(C,D)**, *Prkca* **(E,F)**, *Prkcd* **(G,H)** and *PTP1B* **(I,J)** in tanycytes. The tanycytes were treated with LMK235 (1 µM), a HDAC4 and 5 inhibitor, or Tubastatin A (5 µM), a specific HDAC6 inhibitor, for 1 hour. After that LPS (1 µg/mL) was added and the cells were incubated for 3 hours. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test. Tub A, Tubastatin A

In parallel, the same experiments were performed in primary tanycyte cultures. As discussed above, in tanycytes, LPS is an NF-κB activator stronger than TNFα. For that reason, LPS was used to activate NF-κB signalling pathway. In this cell type, *Fos* expression was repressed by LPS (**Figure 12A and B**), but HDAC6 inhibition resulted in a significant induction of this gene by LPS (**Figure 12B**) whereas HDAC4 and 5 inhibition decreased *Fos* expression (**Figure 12A**). *Prkca* was not induced by LPS with or without HDACs inhibitions (**Figure 12C and D**). LPS did not increase *Prkcd* expression (**Figure 12E and F**) but HDAC4 and 5 inhibition resulted in an induction of this gene by LPS (**Figure 12E**). *PTP1B* was not induced by LPS (**Figure 12G and H**) but its expression was induced by HDAC6 inhibition (**Figure 12H**).

Some studies show that HDAC activity may promote activation of the NF-κB pathway by acting upstream or downstream of NF-κB phosphorylation. HDAC3, for example, promotes deacetylation of STAT3, and that when acetylated dimerizes and promotes the cleavage of p100 to p52, activating NF-κB, and RelA, promoting the binding of IκB to NF-κB and its nuclear export (Barneda-Zahonero & Parra 2012). Thus, the direct effect of HDAC inhibition on NF-κB phosphorylation was examined by western blotting. For this propose, the GT1-7 cells were treated with LMK235 (1 μM) and Tubastatin A (5 μM), for 3 hours. As can be seen in **Figure 13**, HDACs inhibition didn't change the ability of TNFα to activate NF-κB pathway and HDACs inhibitors by themselves didn't interfere with the pathway activation. These results suggest that the increased expression of some of the genes studied induced by HDAC4 and 5, and HDAC6 inhibitors is independent of the molecular NF-κB pathway regulation, possibly acting at a nuclear level by remodelling the chromatin structure.

3.3. HDAC6 expression is photoperiodically regulated in the hypothalamus of F344 rats

The prominent effects of HDAC6 inhibition in enhancing the capacity of TNFα and LPS to regulate several genes that act in different inflammatory pathways stimulated the study to understand whether HDAC6 is photoperiodically regulated in the rat brain. As already discussed, inflammatory pathways are one mechanism of change in the hypothalamus to regulate energy balance. Thus, the expression of HDAC6 in the hypothalamus of F344 rats under long-day photoperiod (16h light:8h dark; LD) and short-day (8h light:16h dark; SD) was assessed by qPCR.

Results showed a significant increase of the expression of *HDAC6* in LD conditions relatively to SD (**Figure 14**) and the results confirmed a similar change in *HDAC4* expression (**Figure 14**). The expression of *Dnmt1*, a gene that codify the DNA methyltransferase 1, was also measured in the same conditions as well the genes *NOS2*, *Fcgr1*, *IL6*, *IL-1β* and *TNFα*. These genes encode the proteins nitric oxide synthase 2, high-affinity Fc-gamma receptor, interleukin 6, interleukin 1β and tumour necrosis factor α, respectively, all playing an important role in the immune response. However, as shown in **Figure 15**, none of these genes showed significant changes between the two different conditions of daylight length.

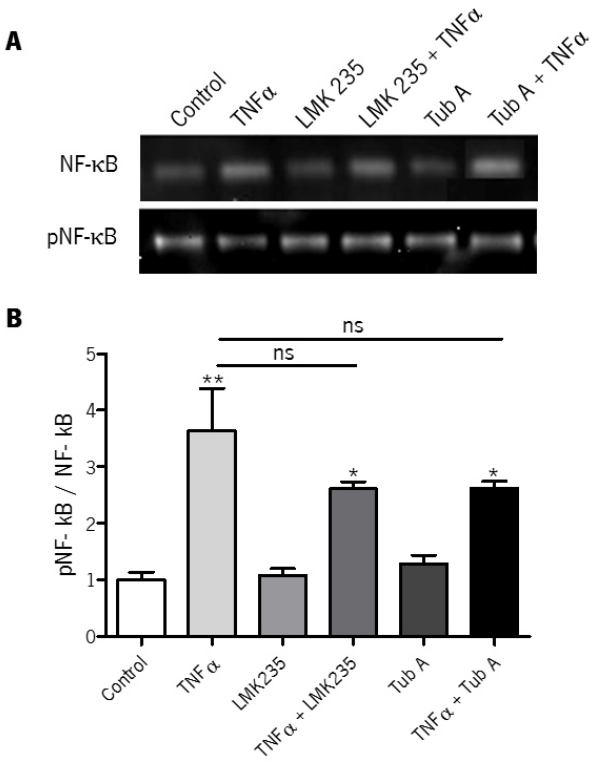

Figure 13 | Western blot analyses of the effect of HDAC4/5 and HDAC6 inhibition in NF-κB pathway activation, in GT1-7 cells. The effect of HDAC4/5 and HDAC6 inhibition in the activation of NF-κB pathway was measured by western blotting, after cell treatment with LMK235 (1 μM) and Tubastatin A (5 μM), respectively, for 3 hours. **(A)** Ten μL of total protein obtained from GT1-7 cells was subjected to SDS-PAGE 12% gel. The gel with proteins was transblotted onto a nitrocellulose membrane and probed with 1: 2000 dilution of anti-NF-κB and 1:1000 dilution of anti-phospho- NF-κB antibodies. The bands were quantified as described in material and methods. The blots shown are representative of 3 independent experiments with similar results. **(B)** The bar graph represents the ratio between phosphorylated NF-κB and total NF-κB, normalized relative to control. Error bars denote SEM, n=3. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test. ns, no statistically significant difference; Tub A, Tubastatin A.

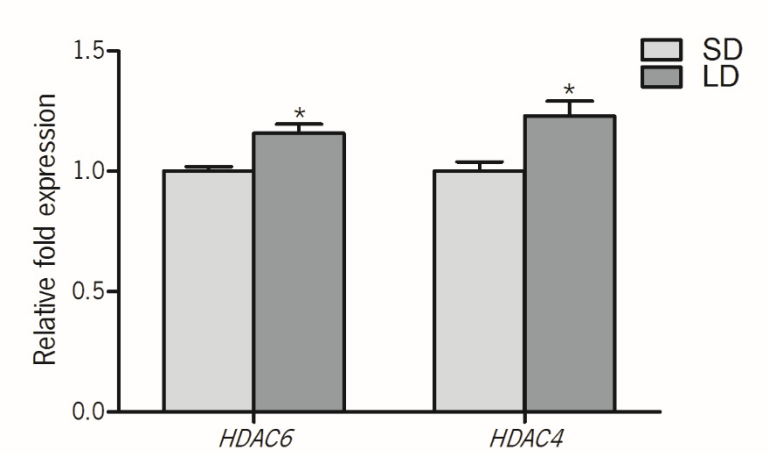

Figure 14 | Effect of photoperiod on regulation of histones deacetylases in the hypothalamus of F344 rats. Gene expression analysis by qPCR of responsiveness of *HDAC6* and *HDAC4* to photoperiod. Transfer of rats from SD to LD conditions increased hypothalamic *HDAC6* and *HDAC4* mRNA expression. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. LD, long-day conditions; SD, short-day conditions.

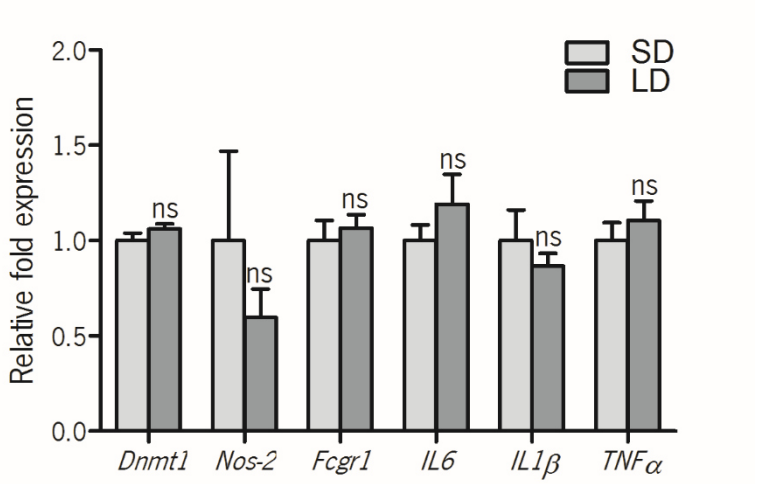

Figure 15 | Effect of photoperiod on regulation of inflammation related genes in the hypothalamus of F344 rats. The regulation of *Dnmt1*, *Nos-2*, *Fcgr1*, *IL6*, *IL1β* and *TNFα* gene expression by photoperiod in the hypothalamus of rats under LD conditions and short day conditions was measured by qPCR. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference; LD, long-day conditions; SD, short-day conditions.

3.4. HDACs inhibition reduced the ability of TNFα to activate p38 kinase, but p38 inhibition did not change the capacity of HDAC inhibitors to alter TNFα gene regulatory activity

To explore a possible signalling pathway acted upon by TNFα and modulated by the HDACs the p38 and ERK1/2 signalling pathways were investigated in GT1-7 cells. Western blotting analysis showed that as expected TNFα induces p38 and ERK1/2 phosphorylation and LMK235

inhibited de activation of p38 signalling pathway (**Figure 16A and B**) but not the ERK1/2 signalling pathway (**Figure 16C and D**).

The specific p38 inhibitor SB202190, one of most widely inhibitor used, was used to study a possible mechanism of HDAC4 and 5 in regulating p38 signalling pathway. Thus, GT1-7 cells were incubated with SB202190 (9 μM) for 16 hours and then added LMK235 (1 μM) and incubated for more 4 hours. qPCR approaches were performed to study the effect of p38 inhibition in the expression of TNFα regulated genes. As depicted in **Figure 17**, and contrary to what was expected, p38 inhibition increased the effect of LMK235 inducing slightly the expression of *Jun* (**Figure 17A**), *Fos* (**Figure 17B**) and *Prkca* (**Figure 17C**). No changes were verified in *Prkcd* (**Figure 17D**) and *PTP1B* (**Figure 17E**) expression.

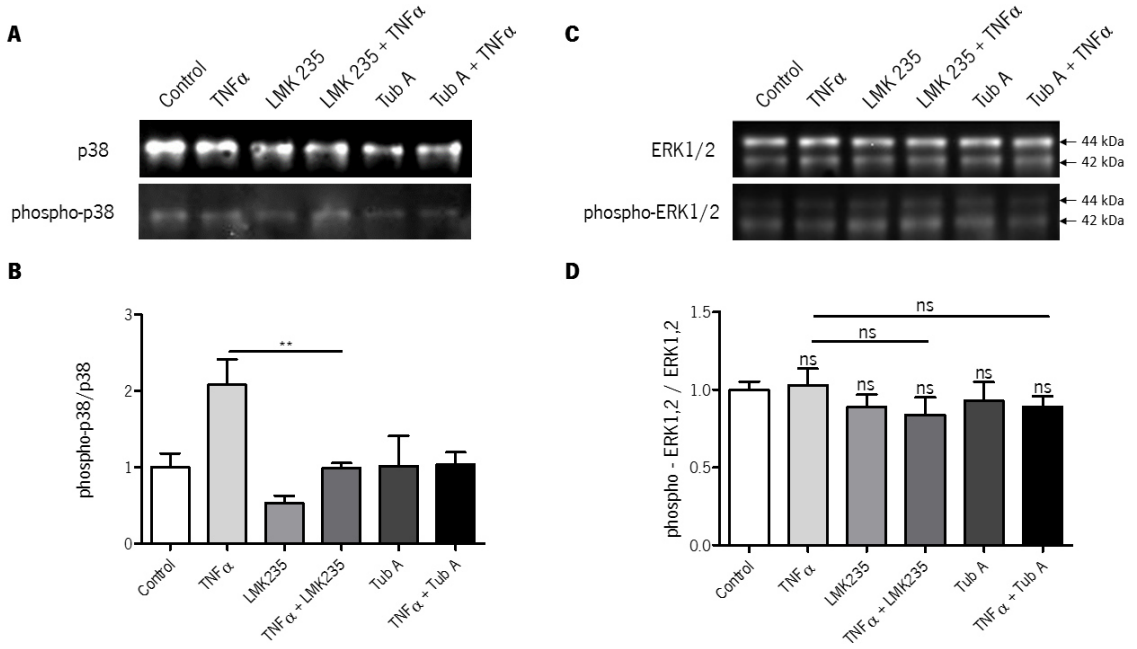

Figure 16 | Western blot analyses of the effect of HDAC4/5 and HDAC6 inhibition in MAPKs pathways activation, in GT1-7. The effect of HDAC4/5 and HDAC6 inhibition on the activation of the p38 pathway (*left panel*) and ERK1/2 pathway (*right panel*) was measured by western blotting, after cell treatment with LMK235 (1 μM) and Tubastatin A (5 μM), respectively, for 3 hours. **(A, B)** Ten μL of total protein obtained from GT1-7 cells was subjected to SDS-PAGE 12% gel. The gel with proteins was transblotted onto a nitrocellulose membrane and probed with 1: 1000 dilution of anti-p38 and 1:1000 dilution of anti-phosphorylated p38 or 1:2000 dilution of anti-ERK1/2 antibodies and 1:4000 dilution of anti-phosphorylated ERK1/2. The bands were quantified as described in material and methods. The blots shown are representative of 3 independent experiments with similar results. **(C, D)** The bar graphs represent the ratio between phosphorylated p38 and total p38, and between phosphorylated ERK1/2 and total ERK1/2, normalized relative to controls. Error bars denote SEM, n=3. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test. ns, no statistically significant difference; Tub A, Tubastatin A.

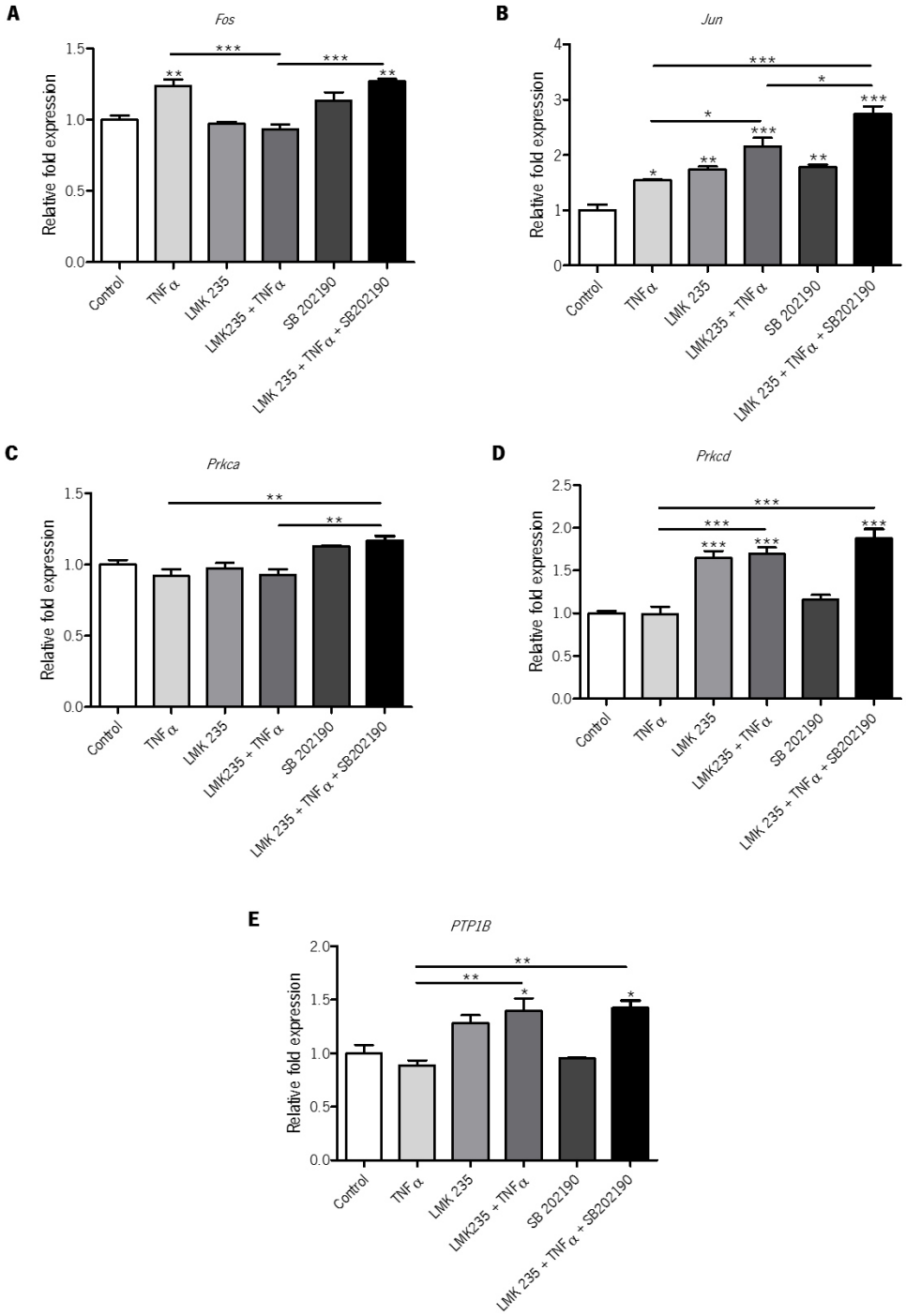

Figure 17 | Regulation of p38 signalling pathway by histone deacetylases, in GT1-7 cells. The TNFα regulated genes *Fos* **(A)**, *Jun* **(B)**, *Prkca* **(C)**, *Prkcd* **(D)** and *PTP1B* **(E)** expression levels were evaluated after SB202190 treatment by qPCR. GT1-7 cells were treated with the p38 inhibitor SB202190 (9 μM) or vehicle (DMSO) for 16 hours. They were then treated with or without TNFα (1 ng/mL) and LMK235 (1 μM) for 4 hours (TNFα was added 1 hour after LMK235). The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test.

3.5. TH and RA regulated genes were controlled by HDACs

Although HDAC4 and HDAC6 are described in the literature as playing an important role in the deacetylation of cytoplasmic proteins, HDACs are also known for their ability to remodel chromatin structure through histone deacetylation. The binding of nuclear receptors to DNA is strongly affected by chromatin compaction and the activity of HDACs are crucial in the regulation of nuclear receptor activity. Thus, the potential function of HDAC4 and HDAC6 in regulating the nuclear receptors RAR and THR was explored. RAR and THR are two important nuclear receptors that mediate signalling pathways controlling energy balance and growth in the hypothalamus.

To evaluate the effect of HDAC inhibition on RAR and THR nuclear receptor control of gene expression, GT1-7 cells were treated with LMK235 (1 μM) or Tubastatin A (5 μM) inhibitors and *Cyp26A1, RARβ* and *Nrgn* transcript levels were assessed by qPCR. *Cyp26A1* and *RARβ* are two genes encoding proteins involved in RA metabolism whose expression is regulated through RA response elements (Loudig *et al.* 2005; Thé *et al.* 1990). *Nrgn* encodes the neurogranin protein and contains in its promotor a thyroid response element (Muñoz *et al.* 1991) and therefore its expression is inducible by thyroid hormone (De Arrieta *et al.* 1999). As expected, RA induced the expression of *RARβ* (**Figure 18A and B**), and this induction was enhanced by HDAC6 inhibition (**Figure 18B**), but not by HDAC4 and 5 inhibition (**Figure 18A**). In contrast, both HDAC4 and 5 (**Figure 18C**) and HDAC6 (**Figure 18D**) inhibition significantly increased the induction of *Nrgn* by T3, and also by RA. Moreover, T3 enhanced the ability of RA to induce *Nrgn* expression (**Figure 18C and D**). The HDAC4 and 5 inhibition (**Figure 18D**), as well HDAC6 inhibition (**Figure 18E**), have an opposite effect to that seen before and repressed the ability of RA to induce *Cyp26A1* expression. Furthermore, T3 inhibit the expression of *Cyp26A1* (**Figure 18D**) and represses the ability of RA to induce *Cyp26A1* expression. Surprisingly, GT1-7 cells spontaneously lost their responsiveness to RA (**Figure 19**). It, was hypothesized that the loss of responsiveness was possibly due to an epigenetic modification of the *Cyp26A1* promoter. To investigate the possible role of methylation in the promotor of *Cyp26A1,* GT1-7 cells were incubated with the DNA methyltransferase inhibitor decitabine (50 mg/ml) for three days to reduce DNA methylation. After that, RA and tubastatin A were added, cells were incubated for 3 hours and *Cyp26A1* expression was measured by qPCR. As shown in **Figure 20**, the ability of the *Cyp26A1* gene to respond to RA was restored by inhibition of DNA methylation.

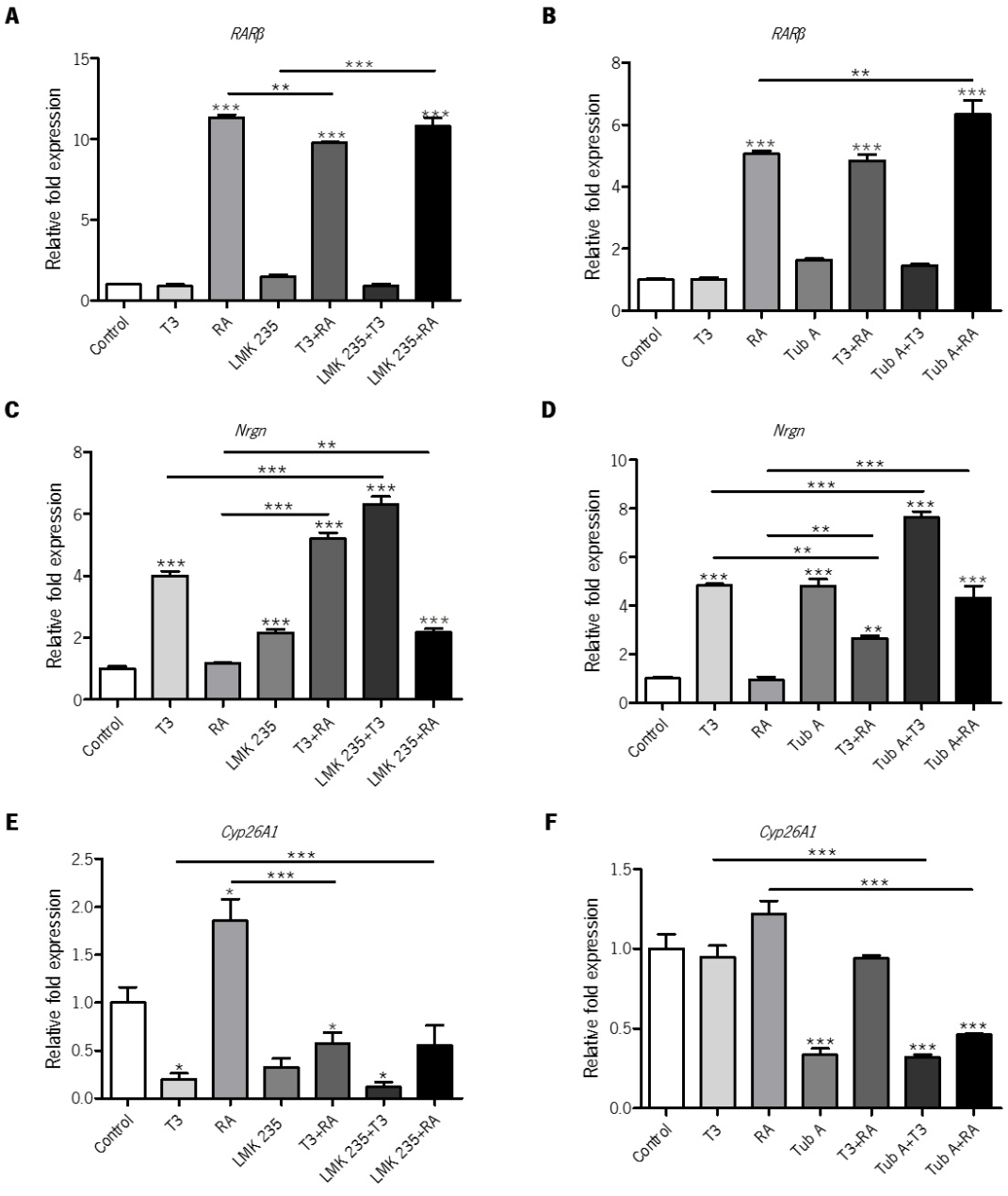

Figure 18 | Influence of HDAC inhibition in the expression of RAR and THR regulated genes. Effects of HDAC4 and 5 inhibition (*left panel*) and HDAC6 inhibition (*right panel*) on expression of the *RARβ* **(A,B)**, *Nrgn* **(C,D)** and *Cyp26A1* **(E,F)** in GT1-7 cells. The GT1-7 cells were incubated with RA (1 μM), T3 (50 mM), LMK235 (1 μM), a HDAC4 and 5 inhibitor, and Tubastatin A (5 μM), a specific HDAC6 inhibitor, for 24 hour. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test. Tub A, Tubastatin A, RA, retinoic acid, T3, thyroid hormone.

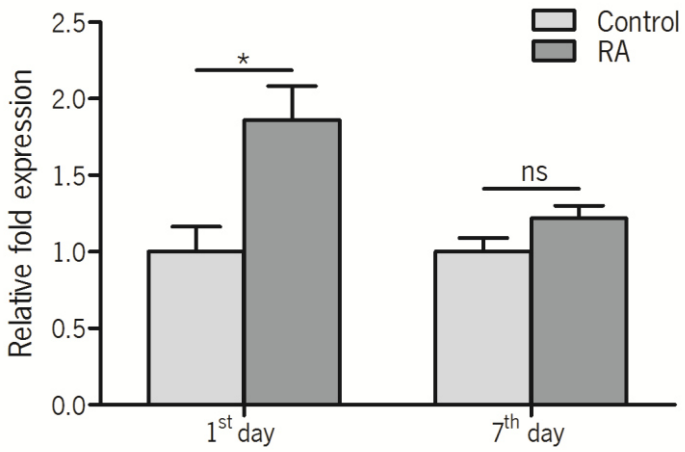

Figure 19 | Loss of *Cyp26A1* responsiveness to RA, in GT1-7 cells. GT1-7 cells were treated with RA (1 μM) for 24 hours and *Cyp26A1* expression was evaluated by qPCR. After 7 days, the same experiment was repeated in the same batch of cells, having verified that GT1-7 cells lost the ability to respond to RA. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference; RA, retinoic acid

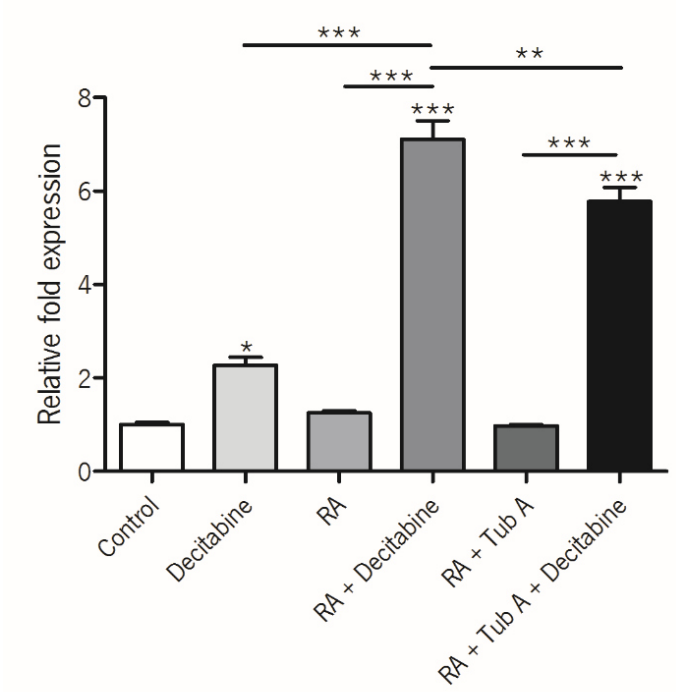

Figure 20 | Epigenetic regulation of Cyp26A1 promoter. Effect of DNA methylation on *Cyp26A1* gene regulation by retinoic acid. GT1-7 cells were treated with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (50 mg/mL) for 72 hours. Every day the medium was refreshed and added the inhibitor. After that, was added RA (1 μM), once *Cyp26A1* expression is induced by retinoic acid, and Tubastatin A (5 μM). The GT1-7 cells recovered their ability to response to retinoic acid after inhibition of DNA methylation. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the one-way ANOVA followed by the Tukey's post-hoc test. RA, retinoic acid; Tub A, Tubastatin A.

3.6. HDAC6 and HDAC4 are localized predominantly in the nucleus in cultured cells

The detailed subcellular localization of HDAC4 and HDAC6 in GT1-7 cells, SH-SY5Y cells and in tanycytes were analysed by immunocytochemistry. SH-SY5Y cells are a human derived cell line used as an *in vitro* model of neuronal function and differentiation. Thus, this cell line were used as a second type of neuronal cell to confirm the subcellular localization of HDAC4 and HDAC6.

In hypothalamic neuronal cells, HDAC6 is manly localized in the nucleus, being also present in the cytoplasm (**Figure 21A**), whereas HDAC4 is exclusively present in the nucleus (**Figure 21B**). Similar distribution patterns were also found in SH-SY5Y human neuroblastoma cells where both HDAC6 and HDAC4 were localised mainly in the nucleus (**Figure 22A and B**). In contrast HDAC6 was not detected in tanycytes (**Figure 23A**).

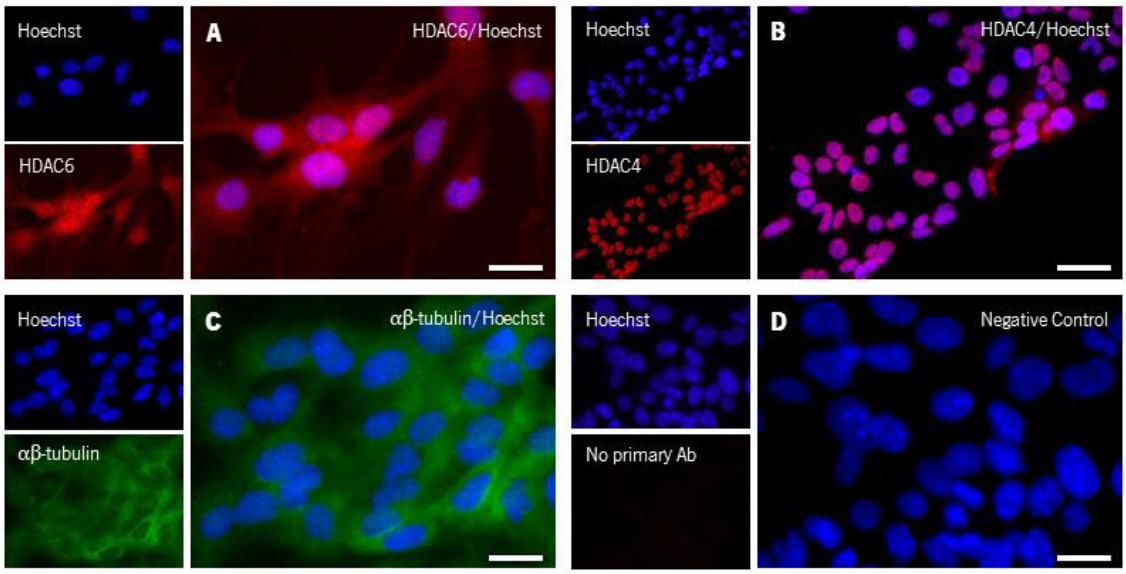

Figure 21 | HDAC6 and HDAC4 localisation, in GT1-7 cells. Immunocytochemistry staining shows that HDAC6 **(A)** was expressed in cytoplasm and nucleus whereas HDAC4 **(B)** was strongly expressed in nucleus. Each image panel shows the separate colour channels as smaller inserts to the left of the main merged image. The αβ-tubulin, a neuronal marker, was used as positive control **(C)**. A negative control sample that was stained with the secondary antibody but not with HDAC4 or HDAC6 antibody was also run alongside the positive samples **(D)**. *Scale bars* 50 µm in a, c, d; 25 µm in b.

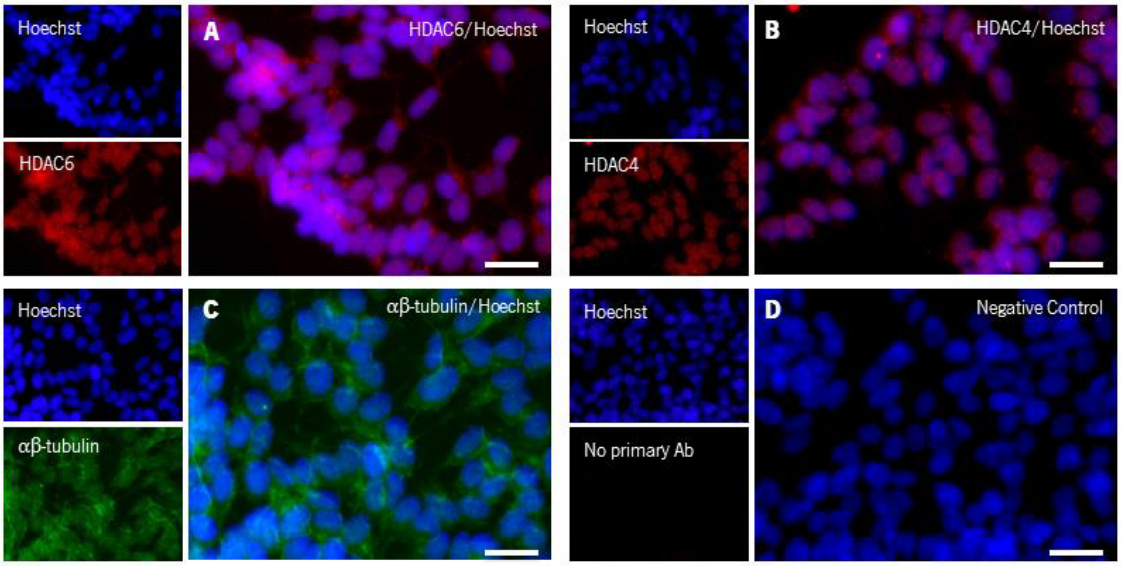

Figure 22 | HDAC6 and HDAC4 localisation, in SH-SY5Y cells. Immunocytochemistry staining shows that HDAC6 **(A)** and HDAC4 **(B)** were mainly expressed in nucleus. The αβ-tubulin, a neuronal marker, was used as positive control **(C)**. A negative control sample that was stained with the secondary antibody but not with HDAC4 or HDAC6 antibody was also run alongside the positive samples **(D)**. Each image panel shows the separate colour channels as smaller inserts to the left of the main merged image. *Scale bars* 25 µm.

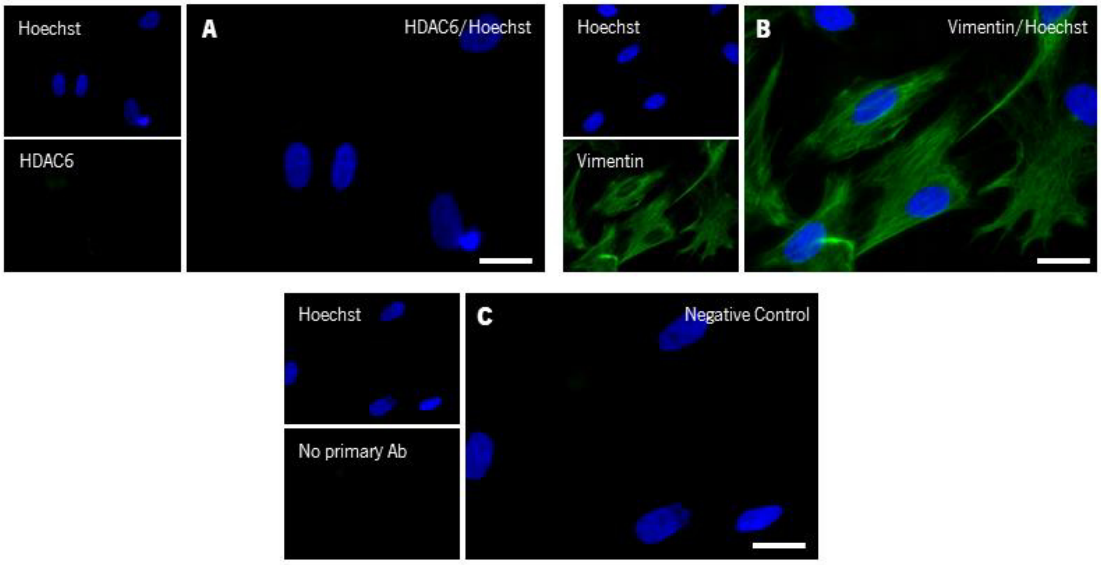

Figure 23 | HDAC6 localisation, in tanycyte primary cells. Immunocytochemistry staining shows that HDAC6 **(A)** was not expressed in tanycytes. Vimentin, a marker for tanycytes, was used as positive control **(B)**. A negative control sample that was stained with the secondary antibody but not with HDAC6 antibody was also run alongside the positive samples **(C)**. Each image panel shows the separate colour channels as smaller inserts to the left of the main merged image. *Scale bars* 50 µm.

3.7. RA metabolism is under inflammation control

Given the known function of retinoic acid in repressing inflammatory responses (Dheen *et al.* 2005; Neerven *et al.* 2010; Matsushita *et al.* 2001; Katsuki *et al.* 2009) and in regulating energy balance and metabolism, the effect of TNFα and LPS in the expression of some genes encoding enzymes involved in RA signalling pathway were evaluated by qPCR in GT1-7 cells and tanycytes. Cyp26A1 and Cyp26B1 are two proteins belonging to the cytochrome P450 superfamily which catalyse the degradation of retinoic acid whereas RALDH1 and RALDH2 catalyse the conversion of retinal into retinoic acid. Thus, Cyp26 and RALDH proteins play an important role in regulating the cellular level of retinoic acid. Dio2 is an enzyme involved in the biosynthesis of T3, the form of thyroid hormone with highest activity. Thyroid hormone exerts its effects by binding to thyroid hormone receptor, regulating the expression of several genes some of which are involved in inflammation (Schanga *et al.* 2014) and metabolism regulation (Song *et al.* 2011). To induce an inflammatory response to assess whether the inflammatory processes interfere with the metabolism of RA and TH, GT1-7 cells were treated with TNFα (1 ng/mL) for 3 hours. As shown in **Figure 24**, TNFα induced the expression of *Cyp26B1* and decreased the expression of *RALDH2*. No changes were found in *Dio2* expression while there was a hint that *Cyp26A1* was repressed by LPS, although this was not significant.

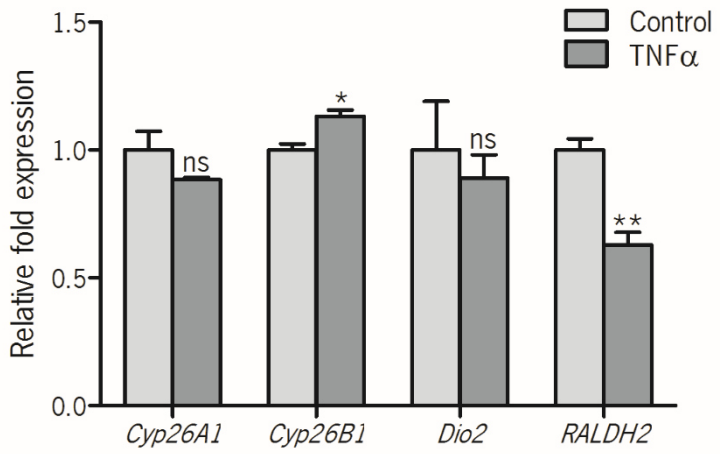

Figure 24 | TNFα reduces the cellular levels of retinoic acid by inhibiting the expression of RA anabolic enzymes and inducing the expression of RA catabolic enzymes. GT1-7 cells were treated with TNFα (1 ng/mL) for 3 hours and the expression of *Cyp26A1*, *Cyp26B1*, *RALDH2* and *Dio2* were evaluated by qPCR. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference

Tanycytes treated with LPS (1 µg/mL) for 24 hours showed increased expression of *Cyp26B1* and *RALDH2*, whereas *Cyp26A1* did not change its expression. LPS stimulation also resulted in a 12-fold induction of *Dio2* expression (**Figure 25**)

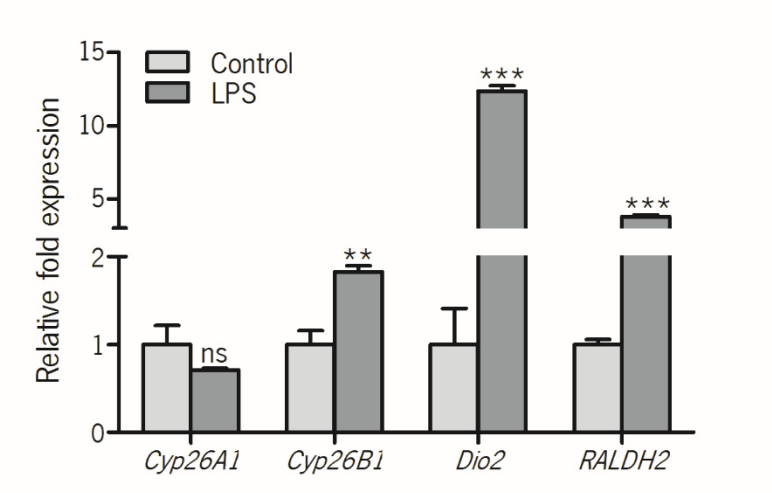

Figure 25 | LPS upregulates the expression of enzymes involved in RA and T3 metabolism in cultured tanycytes. Primary tanycytes cells were treated with LPS (1 µg/mL) for 24 hours and the expression of *Cyp26A1*, *Cyp26B1*, *RALDH2* and *Dio2* were evaluated by qPCR. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference

Macrophages are a type of immune cells formed from monocytes in response to an infection or presence of dead cells. These types of cells are very similar to microglia, the resident immune cells of the central nervous system, playing many of the same functions and expressing many of the same markers. Thus, macrophages stimulated with LPS (1 µg/mL) for 2 hours were used to study the possible function of RA signaling pathway in the inflammatory response in the brain. LPS rapidly induced *Cyp26B1*, *RALDH1* and *RALDH2* but did not affect *Cyp26A1* expression (**Figure 26**).

Organotypic slice cultures were further used as a method to explore the effect of inflammatory modulators on hypothalamic function. This is an *in vitro* method with an excellent compromise between single cell cultures and complete animal studies, allowing the maintenance of normal physiology and function of tissues, and thus to obtain results closer to those expected in *in vivo* systems. In organotypic slice cultures of hypothalamus, LPS induced the expression of *NFKB1*, *PTP1B* and *DIO2*, and repressed *RALDH1* gene expression. No changes were evident in *HDAC4* gene expression (**Figure 27**).

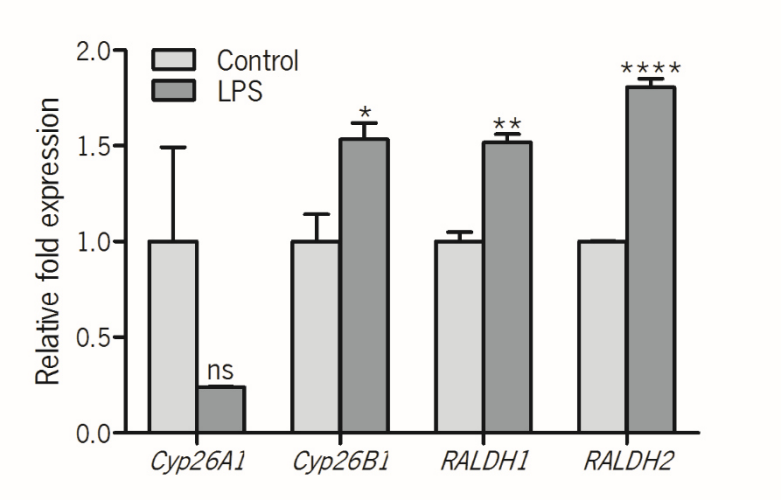

Figure 26 | Responsiveness of retinoic acid signaling pathway to LPS in macrophages. Macrophages were treated with LPS (1 µg/mL) for 2 hours and the expression of *Cyp26A1*, *Cyp26B1*, *RALDH1* and *RALDH2* were evaluated by qPCR. The relative expression levels were obtained after normalization with the expression of the reference gene *hActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value < 0.05, ** P-value < 0.01, *** P-value < 0.001, ****P-value < 0.0001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference

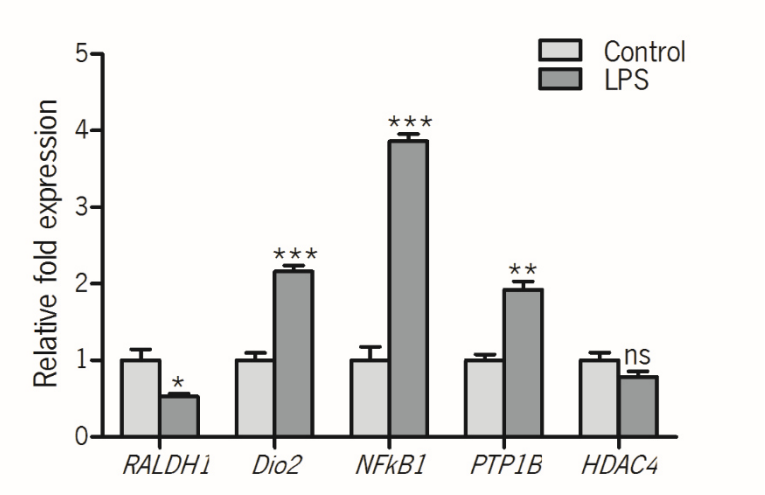

Figure 27 | LPS induces *Dio2*, *NFkB1* and *PTP1B*, and represses *RALDH1* expression in hypothalamic slices cultures. Hypothalamic organotypic slices were treated with LPS (1 µg/mL) for 24 hours and the expression of *RALDH1*, *Cyp26A1*, *Dio2*, *NFkB1*, *PTP1B* and *HDAC4* was evaluated by qPCR. The relative expression levels were obtained after normalization with the expression of the reference gene *mActb*. Values are mean ± S.E.M. of at least 3 independent experiments. * P-value <0.05, ** P-value < 0.01, *** P-value < 0.001. Statistical analysis was done using the Students' t-test. ns, no statistically significant difference

CHAPTER IV

DISCUSSION

This study highlights the involvement of epigenetic modifications in the hypothalamic regulation of the shift in feeding and energy balance in response to photoperiod, focusing on the role of HDACs on inflammatory responses and gene expression regulation. Also studied was the possible role of the retinoic acid signalling pathway in controlling inflammatory processes in the hypothalamus.

The HDACs are typically associated with transcriptional repression by deacetylating the histones residues and thereby promoting chromatin condensation. Over the last several years, it has been shown that HDACs may modulate gene expression not only by modifying chromatin structure but also by deacetylating transcription factors and thereby modulating their transcriptional activity; some of these transcription factors are involved in inflammatory processes. For instance, HDAC1 and HDAC2 repress gene transcription by interacting with the transcription factors MyoD (Mal *et al.* 2001) and YY1 (Yao *et al.* 2001), respectively. The inflammatory modulator NF-κB activity is also regulated by deacetylation of RelA by HDAC3 (Chen Lf *et al.* 2001). Although the specific function of HDACs in the hypothalamus is still unknown, it is well known that hypothalamic inflammation is the basis of many hypothalamic functions, such as energy homeostasis, and related disorders, being associated with diet-induced metabolic disease (Valdearcos *et al.* 2015). In addition, it also known that HDAC4 is associated with obesity-related inflammation. In human obesity there is a downregulation of HDAC4 (Abu-Farha *et al.* 2013) and its haploinsufficiency is associated with this disorder (Burns *et al.* 2010; Williams *et al.* 2010).

HDAC4 activity has been associated with the NF-κB pathway in diverse physiologic processes, including ageing (Zhang *et al.* 2013) and regulation of inflammation via cAMP in macrophages (Luan *et al.* 2014). The NF-κB pathway is a central mediator of inflammatory responses that may be involved in obesity. A study by Carlsen *et al.* (2009) showed that rats fed a high-fat diet have higher NF-κB activity than rats fed with a low-fat diet. In addition, mouse models in which NF-κB p65 is over-expressed, resulting in elevated NF-κB activity, were resistant to diet induced obesity by inhibition of adipogenesis (Tang *et al.* 2010). Moreover, mice with genetic or dietary obesity have an increase in NIK activation, the NF-κB–inducing kinase of the non-canonical pathway, which promotes the gluconeogenesis and glycogenolysis (Sheng *et al.* 2012). Previous results from our group showed that *HDAC4* expression is higher in the hypothalamus of rats under long-day photoperiod relative to short-day conditions, whereas the expression of the NF-κB target gene *NFKB1* was increased in F344 rats under short-day conditions (unpublished). Propelled by these

intriguing results, this study investigated hypothalamic neurons as modelled by the GT1-7 immortalized hypothalamic neuronal cell line, and primary tanycyte culture, the two cells types where HDAC4 was shown to be expressed by immunohistochemical analysis, to evaluate if HDAC4 regulates NF-κB signalling in hypothalamic neurons and tanycytes (unpublished).

The possible role of HDAC4 in regulating the NF-κB signalling pathway was studied using two chemical inhibitors, since there is no specific inhibitor for HDAC4, one of them selective for HDAC4 and 5 and perhaps HDAC6, and other selective for HDAC6. The results pointed to a possible role of HDACs in preventing excessive inflammatory responses by repressing the action of molecules that elicit strong immune responses, such as the cell signalling protein TNFα and the endotoxin LPS, and thereby, acting as negative regulators of proinflammatory genes involved in several signalling pathways including AP1, protein kinase c and the phosphatase PTP1B (**Figure 11 and 12**). The HDACs are already suggested to be involved in inflammatory processes, given that broad-spectrum inhibitors of HDACs have anti-inflammatory effects on macrophages *in vitro* (Adcock 2007; Blanchard & Chipoy 2005). In murine bone marrow-derived macrophages (BMM), LPS regulated several members of the histones deacetylase family including HDAC6, and treatment of BMM with an HDACs inhibitor increased LPS-inducible genes (Aung *et al.* 2006). However, the mechanisms by which HDACs act in these systems remain unexplained. Moreover, the results using the specific HDAC6 inhibitor Tubastatin A suggested an important role of this HDAC. HDAC6 was shown to be photoperiodically regulated in the hypothalamus of F344 rats, a suitable model to study hypothalamic regulation of homeostasis in response to laboratory controlled alterations in day length (Heideman & Sylvester 1997). This enzyme was shown to be expressed to a greater degree in the hypothalamus of rats under summer-like than winter-like conditions (**Figure 18**). Another epigenetic modification relevant to seasonal photoperiod regulation is DNA methylation. In the adult rat brain, reversible DNA methylation is photoperiodically regulated and plays an important role in regulating the production and release of some hormones, namely thyroid hormone (Stevenson & Prendergast 2013). Thus the expression of *Dnmt1*, a gene encoding a DNA methyltransferase responsible for methylation of cytosine residues in hemimethylated DNA, was also measured in the hypothalamus of F344 rats under different day length conditions. No changes were found in the expression of *Dnmt1*. Given the new thinking about the epigenetic control of energy balance (Gali Ramamoorthy *et al.* 2015) these results can suggest a specific role of histone deacetylation as part of the mechanism involved in the seasonal switch in feeding behaviour in the hypothalamus of F344 rats. The distribution of the HDAC6 protein in the brain has not previously

been investigated. Thus, immunohistochemistry approaches were used to study the distribution of HDAC6 in the rat brain. However, HDAC6 was not detected in rat brain sections (*Supplementary Data* - Figure S1). In fact, high expression levels of HDAC6 mRNA have been detected in mouse (Fukada *et al.* 2012) but not rat (Broide *et al.* 2007) brain.

HDAC6 is a histone deacetylase belonging to the class IIb family and it is the only HDAC containing two functional catalytic domains (Seidel & Diederich 2015). This HDAC is mainly localized to the cytoplasm where it plays crucial roles deacetylating and thereby regulating others proteins, such as α-tubulin (Hubbert *et al.* 2002) and the heat shock protein (HSP) 90 (Kovacs *et al.* 2005). However, as for HDAC4, HDAC6 can be shuttled to the nucleus where it not only deacetylates histone proteins but also regulates the activity of some nuclear factors and nuclear receptors. For instance, HDAC6 interacts with NF-κB to repress the transcription of the H^+-K^+-ATPase α2-subunit gene (Zhang & Kone 2002) and also interacts with the vitamin D receptor to down-regulate the human *MYC* gene (Toropainen *et al.* 2010). Over the past few years HDAC6 has also been associated with the inflammatory response. In astrocytes, HDAC6 enhances inflammatory tolerance to LPS (Beurel 2011) and in fibroblasts this enzyme is required for expression of virus-induced *IFN*-β (Nusinzon & Horvath 2006). Until now however, the involvement of HDAC6 in photoperiod physiology has not been reported in any species.

Although shuttling of HDAC4 and HDAC6 between the nucleus and cytoplasm suggested a role of this protein in regulating cytoplasmic proteins, the inhibition of HDAC4 and 5 did not result in an increase in phosphorylation and consequently activation of NF-κB (**Figure 13**). Moreover, by immunocytochemistry these two enzymes are localized mainly to the nucleus of neurons (**Figure 20 and 21**). Taken together, these results suggest that HDAC4 and HDAC6 can induce a number of genes downstream of NF-κB in the hypothalamus but their effects are independent of molecular NF-κB pathway regulation and probably is at a nuclear level via traditional function of HDACs in remodelling chromatin structure by histone deacetylation. Other signalling pathways closely involved in inflammatory processes, the MAPK/ERK pathways, were also studied to evaluate a possible cytoplasmic role of HDAC4 and HDAC6. The HDAC4 and 5 inhibitor LMK235 reduced the capacity of TNFα to activate p38 kinase, but not p44/p42 (**Figure 14**). The blocking of p38 signalling pathway by HDACs inhibitors has been reported previously in the literature. Inhibition of HDACs increases MAPK phosphatase 1 (MKP-1) acetylation and consequently blocks p38 signalling (Choo *et al.* 2013; Cao *et al.* 2008) – this is brought about because, when acetylated,

MKP-1 interacts with p38 leading to its dephosphorylation and inactivation. In addition, it was also shown that HDAC6 knockdown results in a decrease in phosphorylation of p38 (Kästle *et al.* 2012). Despite the western blotting results, a p38 inhibitor did not affect the ability of HDAC inhibitors to promote the action of TNFα and LPS. In fact, the opposite action was verified with *Jun* expression. This may be because of an additional action of th SB202190 p38 inhibitor which is used to activate JNK which then activates Jun (Muniyappa & Das 2008). This would likely lead to an increase in *Jun* mRNA as Jun regulates its own gene expression via AP-1 (Angel *et al.* 1988).

The possible role of HDAC in controlling gene expression through chromatin remodelling, thereby interfering with accessibility of nuclear receptors to DNA, was also studied. The availability of retinoic acid and thyroid hormone in the hypothalamus regulates seasonal changes in energy intake and expenditure through the nuclear receptors RAR and THR; the availability of these ligands being highest in summer compared to winter (Ebling & Barrett 2008). RAR is also involved in inflammatory pathways (Hall *et al.* 2011). HDACs and HATs bind to numerous RAREs in proximal promotor or enhancer regions of RA and T3-regulated genes. In fact, both RAR and THR are strongly affected by chromatin condensation, and their actions are dependent on an exchange of HDAC-containing versus HAT-containing complexes (Lan *et al.* 1999). Concordant with that, inhibition of HDAC4, 5 and 6 resulted in an increase of T3's capacity to induce the TH regulated gene, neurogranin (Morte *et al.* 1997). On the other hand, the upregulation induced by retinoic acid of the RA-regulated gene *Cyp26A1* (Loudig *et al.* 2000) was repressed by HDAC4, 5 and 6 inhibition. This is a surprising result given that HDAC inhibition has been generally associated with an increase in RARE-regulated genes expression (Menegola *et al.* 2006). However downregulation of *Cyp26A1* expression by HDACs inhibition has been reported elsewhere, for instance in P19 embryonal carcinoma cells, R1 mouse ES cells and mouse embryos (Jergil *et al.* 2011). In embryos, the downregulation of *Cyp26A1* by HDACs inhibition was accompanied by an upregulation of the *Aldh1a2*, which encodes an enzyme involved in the biosynthesis of retinoic acid during embryogenesis, being suggested by the authors to increase RA levels and ectopic signalling in the embryo (Jergil *et al.* 2009; Kultima *et al.* 2010). Finally, it was found in our study that only HDAC6 inhibition, not HDAC4 and 5, led to an increased capacity of retinoic acid to induce *RARβ* expression. This result suggests a possible specificity of HDAC6 in regulating RAR action on the RARβ RARE. In addition, it was also verified that T3 and RA can have either an additive or antagonist effect on *Nrgn* and *Cyp26A1* expression, respectively. Similar results have already been shown in pituitary GH3 cells where RA antagonizes the effect of T3 on *TRβ2* gene expression but

an additive effect on *rGH* gene repression (Davis & Lazar 1992). The DNA-binging domains of the RAR and THR are similar in their amino acid sequences (62% identical; Giguere *et al.* 1987). Consequently, some thyroid-response elements (TREs) are transcriptionally induced by both TRs and RARs, whereas others are only transcriptionally activated by TRs. For instance, rGH TRE, bind to RARs and THRs (Umesono *et al.* 1988). On the other hand, the TRE present in the promoter of cardiac myosin heavy chain α is only transcriptionally activated by THRs (Glass *et al.* 1989). The same is true with RAREs.

DNA methylation was shown in this present study to be an important epigenetic modifier in regulating the expression of *Cyp26A1* in GT1-7 cells. Normally, the expression of *Cyp26A1* is upregulated by RA. However, GT1-7 cells can spontaneously lose their ability to response to RA. It was found that demethylation by decitabine, an inhibitor of DNA methyltransferases, recovered *Cyp26A1* responsiveness to RA. These results are in agreement with those described in the literature in which promotor methylation of *Cyp26A1* is an important mechanism for regulating its expression (Pozzi *et al.* 2006) for instance in immortalized fibroblasts (Balb/c3T3 cells), methylation of CpG islands blocks *Cyp26A1* transcriptional regulation by RA (Kashyap & Gudas 2010). Moreover, in B lymphocytes, methylation of the *Cyp26A1* promoter was associated not only with a decline in its expression but also with the susceptibility to develop allergic diseases (Pascual *et al.* 2011).

Retinoic acid is known to be an important regulator of the homeostatic control of the immune system, playing a vital role in controlling the response to bacterial, viral, and protozoan diseases. Furthermore, is also known that RA plays an important role in the hypothalamic control of feeding and weight (Ebling 2015). In addition, it has been demonstrated that in the hypothalamus many genes encoding enzymes involved in RA signalling pathway are regulated by photoperiod. Further, many studies also suggest that inflammation influences energy balance (Wang & Ye 2014). Therefore, it may be hypothesized that RA regulates the hypothalamic switch in feeding and energy balance in response to photoperiod through its regulation of inflammatory processes. Thus, we studied the influence of inflammation in RA signalling pathways in GT1-7 cells, tanycytes, macrophages and organotypic slices. This study showed that, in GT1-7 cells, TNFα upregulated *Cyp26B1* and downregulated *RALDH2,* pointing to a possible decrease of cellular level of retinoic acid. Interestingly, in tanycytes, the radial glial-like cells which play a central role in the hypothalamic networks that control body weight and energy balance, LPS upregulates both anabolic and catabolic RA enzymes, as well *Dio2*. Despite these intriguing results, little is reported

in the literature about the influence of inflammation in RA and T3 metabolism. Primary mouse microglia activated by LPS showed increased protein expression of Cyp26A1 and Cyp26B1 suggesting a possible increase in RA catabolism (Hellmann-Regen *et al.* 2013). In our studies, macrophages activated by LPS showed an increase in *Cyp26B1* expression. However, was also found was an increase in *RALDH1* and *RALDH2* gene expression, these synthetic enzymes would oppose the degradation of retinoic acid which has been reported in microglia.

Collectively, this study has demonstrated for the first time that HDAC4 and HDAC6 are photoperiodically regulated in the rat hypothalamus and inhibition of HDACs seems to induce the expression of some transcripts involved in different signalling pathways by promoting the action of TNFα (in GT1-7 cells) and LPS (in tanycytes). This result also points to a potential role for HDACs in inflammatory signalling pathways in the hypothalamus, a topic that has also become of recent interest over the past several years as a mechanism that may alter energy balance (Purkayastha & Cai 2013). Further, HDACs can also control TH and RA regulated genes, suggesting a possible role of these enzymes at a nuclear level. Finally, RA metabolism seems to be under regulation by inflammatory signalling systems. Thus HDAC4 and HDAC6 may play an important role to modulate the inflammatory pathways, as well RA signalling pathway, in the hypothalamus to regulate energy balance.

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVES

Over the last several years, convincing evidence has supported the concept that hypothalamic inflammation contributes to the pathogenesis of obesity, wherein the NF-κB signalling pathway plays a pivotal role in hypothalamic neuroinflammation, being considered as the prime machinery directing overnutrition-induced metabolic dysfunction. In addition, some genes affecting energy balance and body weight are regulated by epigenetics mechanisms. Using F344 rats as animal models to study photoperiodic controlled energy balance, the present study aimed to investigate the possible role of HDAC4 and HDAC6 in modulating gene expression by regulating inflammatory pathways in the hypothalamus. This project also studied the possible involvement of HDAC4 and HDAC6 in the regulation of TH and RA signalling pathways, two pathways well known to be involved in the regulation of energy metabolism. Lastly, the influence of inflammation in RA and T3 metabolism was also evaluated.

The main conclusions drawn from this work were the following:

- *HDAC6* is photoperiodically regulated in the hypothalamus, with higher expression in rats under long-day photoperiod (summer-like), relative to short-day (winter-like).
- HDAC4 and HDAC6 inhibition increases the capacity of TNFα and LPS to induce the expression of several genes involved in various signalling pathways.
- *RARβ* and *Nrgn* gene expression is increased by HDAC4 and HDAC6 inhibition whereas *Cyp26A1* expression is decreased, pointing to possible regulation of TH and RA-regulated genes by HDAC4 and 6. The action of HDACs to regulated gene expression, as opposed to their cytoplasmic action, are corroborated by the nuclear localization of HDAC4 and 6 in GT1-7 cells and SH-SY5Y cells.
- The *Cyp26A1* promoter is under epigenetic regulation, being strongly regulated by DNA methylation.
- Some genes encoding enzymes involved in RA metabolism are influenced by inflammation.

Future research of interest will be an exploration of changes in histone acetylation associated with the promoters of genes whose expression is influenced by HDAC inhibition. Since there is no specific inhibitor for HDAC4 and the fact that the effects of HDAC6 inhibitor should be reconfirmed, the role of HDAC4 and 6 inhibtion in regulating the inflammatory pathways in the hypothalamus

could be evaluated by using siRNA. A more detailed study of the promoter methylation status of *Cyp26A1* is also required, as well as study of the possible correlation of Cyp26A1 methylation with inflammatory processes in the hypothalamus. Of further interest will be clarification of the involvement of inflammation in RA and T3 metabolism.

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References

Abu-Farha, M. *et al.*, 2013. Proteomics Analysis of Human Obesity Reveals the Epigenetic Factor HDAC4 as a Potential Target for Obesity. *PLoS ONE*, 8(9), pp.1–17.

Adcock, I.M., 2007. HDAC inhibitors as anti-inflammatory agents. *British Journal of Pharmacology*, 150(7), pp.829–831.

Altucci, L. & Gronemeyer, H., 2001. The promise of retinoids to fight against cancer. *Nature reviews. Cancer*, 1(3), pp.181–193.

Angel, P. *et al.*, 1988. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell*, 55, pp.875–885.

De Arrieta, C.M. *et al.*, 1999. The human RC3 gene homolog, NRGN contains a thyroid hormone-responsive element located in the first intron. *Endocrinology*, 140(3), pp.335–343.

Atkins, C.M. *et al.*, 1998. The MAPK cascade is required for mammalian associative learning. *Nature*, 1(7), pp.602–609.

Aung, H.T. *et al.*, 2006. LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. *FASEB*, 20(9), pp.1315–1327.

Barneda-Zahonero, B. & Parra, M., 2012. Histone deacetylases and cancer. *Oncogene*, 6, pp.579–589.

Barrett, P. *et al.*, 2007. Hypothalamic thyroid hormone catabolism acts as a gatekeeper for the seasonal control of body weight and reproduction. *Endocrinology*, 148(8), pp.3608–3617.

Bartness, T.J., Goldman, B.D. & Bittman, E.L., 1991. SCN lesions block responses to systemic melatonin infusions in Siberian hamsters. *The American Journal of Physiology*, 260(1), pp.R102–R112.

Barton, G.M. & Kagan, J.C., 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nature reviews. Immunology*, 9(8), pp.535–542.

Bernal, J., 2014. Action of thyroid hormone in brain. *Journal of Endocrinological Investigation*, 25(3), pp.268–288.

Beurel, E., 2011. HDAC6 regulates LPS-tolerance in astrocytes. *PLoS ONE*, 6(10), p.e25804.

Blanchard, F. & Chipoy, C., 2005. Histone deacetylase inhibitors: New drugs for the treatment of inflammatory diseases? *Drug Discovery Today*, 10(3), pp.197–204.

Blomhoff, R. & Blomhoff, H.K., 2005. Overview of retinoid metabolism and function. *Journal of Neurobiology*, 66(7), pp.606–630.

Bolborea, M. & Dale, N., 2013. Hypothalamic tanycytes: Potential roles in the control of feeding and energy balance. *Trends in Neurosciences*, 36(2), pp.91–100.

Bouwmeester, T. *et al.*, 2004. A physical and functional map of the human TNF-α/NF-kB signal transduction pathway. *Nature Cell Biology*, 6(2), pp.97–105.

Broide, R.S. *et al.*, 2007. Distribution of Histone Deacetylases 1 – 11 in the Rat Brain. *Journal of Molecular Neurosciences*, 31, pp.47–58.

Burns, B. *et al.*, 2010. Rai1 haploinsufficiency causes reduced Bdnf expression resulting in hyperphagia, obesity and altered fat distribution in mice and humans with no evidence of metabolic syndrome. *Human Molecular Genetics*, 19(20), pp.4026–4042.

Butler, K. V. *et al.*, 2010. Rational design and simple chemistry yield a superior, neuroprotective HDAC6 inhibitor, tubastatin A. *Journal of the American Chemical Society*, 132(31), pp.10842–10846.

Cao, W. *et al.*, 2008. Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. *The Journal of Experimental Medicine*, 205(6), pp.1491–1503.

Carlsen, H. *et al.*, 2009. Diet-induced obesity increases NF-κB signaling in reporter mice. *Genes & Nutrition*, 4(3), pp.215–222.

Chang, L. & Karin, M., 2001. Mammalian MAP kinase signalling cascades. *Nature*, 410(6824), pp.37–40.

Chauvet, N., Privat, A. & Alonso, G., 1996. Aged median eminence glial cell cultures promote survival and neurite outgrowth of cocultured neurons. *Glia*, 18(3), pp.211–223.

Chen, L.-F. & Greene, W.C., 2004. Shaping the nuclear action of NF-κB. *Nature reviews. Molecular cell biology*, 5(5), pp.392–401.

Chen Lf *et al.*, 2001. Duration of nuclear NF-κB action regulated by reversible acetylation. *Science*, 293(5535), pp.1653–1657.

Cheshire, J.L. & Baldwin, A.S., 1997. Synergistic Activation of NF-κB by Tumor Necrosis Factor Alpha and Gamma Interferon via Enhanced IκBa Degradation and De Novo IκBb Degradation. *Molecular and Cellular Biology*, 17(11), pp.6746–6754.

Cheung, P., Allis, C.D. & Sassone-Corsi, P., 2000. Signaling to chromatin through histone modifications. *Cell*, 103(2), pp.263–271.

Choo, Q.Y. *et al.*, 2013. The histone deacetylase inhibitors MS-275 and SAHA suppress the p38 mitogen-activated protein kinase signaling pathway and chemotaxis in rheumatoid arthritic synovial fibroblastic E11 cells. *Molecules*, 18(11), pp.14085–14095.

Constantine-Paton, M., Cline, H.T. & Al, C.E.T., 1990. Patterned activity, synaptic converge, and the NMDA Receptor. *Annual Review of Neurosciences*, 13, pp.129–154.

Cui, H. *et al.*, 2013. Eating disorder predisposition is associated with ESRRA and HDAC4 mutations. *Journal of Clinical Investigation*, 123(11), pp.4706–4713.

Darcy, M.J. *et al.*, 2010. Regional and subcellular distribution of HDAC4 in mouse brain. *The Journal of Comparative Neurology*, 518(5), pp.722–740.

Davis, K.D. & Lazar, M.A., 1992. Selective Antagonism of Thyroid Hormone Action by Retinoic Acid. *The Journal of Biological Chemistry*, 267(5), pp.3185–3189.

Dawson, a. *et al.*, 2001. Photoperiodic Control of Seasonality in Birds. *Journal of Biological Rhythms*, 16(4), pp.365–380.

Dawson, M. a & Kouzarides, T., 2012. Cancer epigenetics: from mechanism to therapy. *Cell*, 150(1), pp.12–27.

Dheen, S.T. *et al.*, 2005. Retinoic acid inhibits expression of TNFα and iNOS in activated rat microglia. *Glia*, 50(1), pp.21–31.

Diano, S. *et al.*, 1998. Fasting-induced increase in type II iodothyronine deiodinase activity and messenger ribonucleic acid levels is not reversed by thyroxine in the rat hypothalamus. *Endocrinology*, 139(6), pp.2879–84.

During, A. & Harrison, E.H., 2007. Mechanisms of provitamin A (carotenoid) and vitamin A (retinol) transport into and out of intestinal Caco-2 cells. *Journal of Lipid Research*, 48(10), pp.2283–2294.

Ebling, F.J.P., 2015. Hypothalamic control of seasonal changes in food intake and body weight. *Frontiers in Neuroendocrinology*, 37, pp.97–107.

Ebling, F.J.P. & Barrett, P., 2008. The regulation of seasonal changes in food intake and body weight. *Journal of Neuroendocrinology*, 20(6), pp.827–833.

English, J.D. & Sweatt, J.D., 1997. A Requirement for the Mitogen-activated Protein Kinase Cascade in Hippocampal Long Term Potentiation. *Journal of Biological Chemistry*, 272(31), pp.19103–19106.

Eroglu, A. & Harrison, E.H., 2013. Carotenoid metabolism in mammals, including man: formation, occurrence, and function of apocarotenoids. *Journal of Lipid Research*, 54(7), pp.1719–1730.

Fan, G. & Hutnick, L., 2005. Methyl-CpG binding proteins in the nervous system. *Cell Research*, 15(4), pp.255–261.

Feng, J., Fouse, S. & Fan, G., 2007. Epigenetic regulation of neural gene expression and neuronal function. *Pediatric Research*, 61(5), pp.58–63.

Fujioka, S. *et al.*, 2004. NF-κB and AP-1 Connection : Mechanism of NF-κB-Dependent Regulation of AP-1 Activity. *Molecular and Cellular Biology*, 24(17), pp.7806–7819.

Fukada, M. *et al.*, 2012. Loss of deacetylation activity of Hdac6 affects emotional behavior in mice. *PLoS ONE*, 7(2), p.e30924.

Funato, H. *et al.*, 2011. Fasting and high-fat diet alter histone deacetylase expression in the medial hypothalamus. *PLoS ONE*, 6(4), p.e18950.

Gali Ramamoorthy, T. *et al.*, 2015. Developmental programming of hypothalamic neuronal circuits: impact on energy balance control. *Frontiers in Neuroscience*, 9, pp.1–16.

Gao, Z. *et al.*, 2007. Inactivation of PKCθ leads to increased susceptibility to obesity and dietary insulin resistance in mice. *American Journal of Physiology.*, 292(1), pp.84–91.

García-San Frutos, M. *et al.*, 2012. Involvement of protein tyrosine phosphatases and inflammation in hypothalamic insulin resistance associated with ageing: Effect of caloric restriction. *Mechanisms of Ageing and Development*, 133(7), pp.489–497.

Gerlach, T. & Aurich, J.E., 2000. Regulation of seasonal reproductive activity in the stallion, ram and hamster. *Animal Reproduction Science*, 58(3-4), pp.197–213.

Germain, P. *et al.*, 2006. International Union of Pharmacology. LX. Retinoic Acid Receptors. *Pharmacological Reviews*, 58(4), pp.712–725.

Ghosh, S., May, M.J. & Kopp, E.B., 1998. NF-κB and REL proteins: Evolutionarily Conserved Mediators of Immune Responses. *Annual Review of Immunology*, 16, pp.225–260.

Giguere, V. *et al.*, 1987. Identification of a receptor for the morphogen retinoic acid. *Nature*, 330(6149), pp.624–629.

Glass, C.K. *et al.*, 1989. Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. *Cell*, 59(4), pp.697–708.

Goldman, B.D., 2001. Mammalian Photoperiodic System: Formal Properties and Neuroendocrine Mechanisms of Photoperiodic Time Measurement. *Journal of Biological Rhythms*, 16(4), pp.283–301.

Gregor, M.F. & Hotamisligil, G.S., 2011. Inflammatory Mechanisms in Obesity. *Annual Review of Immunology*, 29, pp.415–445.

Gregory, A.P. *et al.*, 2012. TNF receptor 1 genetic risk mirrors outcome of anti-TNF therapy in multiple sclerosis. *Nature*, 488(7412), pp.508–11.

Grosse, J. *et al.*, 1993. Testicular Regression in Pinealectomized Syrian Hamsters following Infusions of Melatonin Delivered on Non-Circadian Schedules. *Biology of Reproduction*, 49(4), pp.666–674.

Grozinger, C.M. & Schreiber, S.L., 2002. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. *Chemistry & Biology*, 9(1), pp.3–16.

Hall, J. a. *et al.*, 2011. The role of retinoic acid in tolerance and immunity. *Cell*, 35(1), pp.13–22.

Handel, A.E., Ebers, G.C. & Ramagopalan, S. V, 2010. Epigenetics: molecular mechanisms and implications for disease. *Trends in Molecular Medicine*, 16(1), pp.7–16.

Hayden, M.S. & Ghosh, S., 2008. Shared principles in NF-κB signaling. *Cell*, 132(3), pp.344–362.

Hayden, M.S. & Ghosh, S., 2004. Signaling to NF-κB. *Genes & Development*, 18(18), pp.2195–2224.

Heideman, P.D. & Sylvester, C.J., 1997. Reproductive photoresponsiveness in unmanipulated male Fischer 344 laboratory rats. *Biology of Reproduction*, 57(1), pp.134–138.

Hellmann-Regen, J. *et al.*, 2013. Accelerated degradation of retinoic acid by activated microglia. *Journal of Neuroimmunology*, 253(1-2), pp.1–6.

Hsieh, J. & Gage, F.H., 2005. Chromatin remodeling in neural development and plasticity. *Current Opinion in Cell Biology*, 17(6), pp.664–671.

Huang, W. *et al.*, 2012. Protein kinase C deficiency attenuates obesity syndrome of ob/ob mice by promoting white adipose tissue remodeling. *The Journal of Lipid Research*, 53(3), pp.368–378.

Hubbert, C. *et al.*, 2002. HDAC6 is a microtubule-associated deacetylase. *Nature*, 417(6887), pp.455–458.

Hunter, T., 1995. Protein kinases and phosphatases: The Yin and Yang of protein phosphorylation and signaling. *Cell*, 80(2), pp.225–236.

Impey, S., Obrietan, K. & Storm, D.R., 1999. Making New Connections : Role of ERK / MAP Kinase Signaling in Neuronal Plasticity. *Neuron*, 23(1), pp.11–14.

Israel, a & Kroemer, G., 2006. NF-κB in life/death decisions: an introduction. *Cell Death and Differentiation*, 13(5), pp.685–686.

Itani, S.I. *et al.*, 2000. Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. *Diabetes*, 49(8), pp.1353–1358.

Jergil, M. *et al.*, 2011. Short-time gene expression response to valproic Acid and valproic Acid analogs in mouse embryonic stem cells. *Toxicological Sciences*, 121(2), pp.328–342.

Jergil, M. *et al.*, 2009. Valproic acid-induced deregulation In vitro of genes associated in vivo with neural tube defects. *Toxicological Sciences*, 108(1), pp.132–148.

Johnson, T.O., Ermolieff, J. & Jirousek, M.R., 2002. Protein tyrosine phosphatase 1B inhibitors for diabetes. *Nature reviews. Drug discovery*, 1(9), pp.696–709.

Kalra, S.P. *et al.*, 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine Reviews*, 20(1), pp.68–100.

Karin, M. & Ben-neriah, Y., 2000. Phosphorylation Meets Ubiquitination: The Control of NF-κB Activity. *Annual Review of Immunology*, 18, pp.621–663.

Kashyap, V. & Gudas, L.J., 2010. Epigenetic regulatory mechanisms distinguish retinoic acid-mediated transcriptional responses in stem cells and fibroblasts. *Journal of Biological Chemistry*, 285(19), pp.14534–14548.

Kästle, M., Woschee, E. & Grune, T., 2012. Histone deacetylase 6 (HDAC6) plays a crucial role in p38 MAPK-dependent induction of heme oxygenase-1 (HO-1) in response to proteasome inhibition. *Free Radical Biology and Medicine*, 53(11), pp.2092–2101.

Katsuki, H. *et al.*, 2009. Retinoic acid receptor stimulation protects midbrain dopaminergic neurons from inflammatory degeneration via BDNF-mediated signaling. *Journal of Neurochemistry*, 110(2), pp.707–718.

Kawaguchi, R. *et al.*, 2007. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. *Science*, 315(5813), pp.820–825.

Khillan, J.S., 2014. Vitamin A/retinol and maintenance of pluripotency of stem cells. *Nutrients*, 6(3), pp.1209–1222.

Klingenspor, M., Niggemann, H. & Heldmaier, G., 2000. Modulation of leptin sensitivity by short photoperiod acclimation in the Djungarian hamster, Phodopus sungorus. *Journal of Comparative Physiology B*, 170(1), pp.37–43.

Klok, M.D., Jakobsdottir, S. & Drent, M.L., 2007. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obesity reviews*, 8(1), pp.21–34.

Klug, W.S. *et al.*, 2009. *Essential of genetics* Seventh Ed., United States of America: Pearson Education, Inc.

Kovacs, J.J. *et al.*, 2005. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Molecular Cell*, 18(5), pp.601–607.

Krezel, W. *et al.*, 1998. Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. *Science*, 279(5352), pp.863–867.

Krezel, W., Kastner, P. & Chambon, P., 1999. Differential expression of retinoid receptors in the adult mouse central nervous system. *Neuroscience*, 89(4), pp.1291–1300.

Kultima, K. *et al.*, 2010. Early transcriptional responses in mouse embryos as a basis for selection of molecular markers predictive of valproic acid teratogenicity. *Reproductive Toxicology*, 30(3), pp.457–468.

Lan, X., Glass, C.K. & Rosenfeld, M.G., 1999. Coactivator and corepressor complexes in nuclear receptor function. *Current Opinion in Genetics and Development*, 9(2), pp.140–147.

Lane, M. a & Bailey, S.J., 2005. Role of retinoid signalling in the adult brain. *Progress in Neurobiology*, 75(4), pp.275–293.

Lerner, A.J. *et al.*, 2012. Retinoids for treatment of Alzheimer's disease. *BioFactors*, 38(2), pp.84–89.

Liang, Y., Zhou, Y. & Shen, P., 2004. NF-κB and Its Regulation on the Immune System. *Cellular & Molecular Immunology*, 1(5), pp.343–350.

Lohnes, D. *et al.*, 1994. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development*, 120(10), pp.2723–2748.

Loudig, O. *et al.*, 2000. Cytochrome P450RAI (CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism. *Molecular Endocrinology*, 14(9), pp.1483–1497.

Loudig, O. *et al.*, 2005. Transcriptional co-operativity between distant retinoic acid response elements in regulation of Cyp26A1 inducibility. *The Biochemical Journal*, 392(1), pp.241–248.

Lu, Y.-C., Yeh, W.-C. & Ohashi, P.S., 2008. LPS/TLR4 signal transduction pathway. *Cytokine*, 42(2), pp.145–151.

Maden, M., 2007. Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nature reviews. Neuroscience*, 8(10), pp.755–765.

Mal, A. *et al.*, 2001. A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: Inhibition of the myogenic program. *EMBO Journal*, 20(7), pp.1739–1753.

Marek, L. *et al.*, 2013. Histone deacetylase (HDAC) inhibitors with a novel connecting unit linker region reveal a selectivity profile for HDAC4 and HDAC5 with improved activity against chemoresistant cancer cells. *Journal of Medicinal Chemistry*, 56(2), pp.427–436.

Marill, J. *et al.*, 2003. Retinoic acid metabolism and mechanism of action: a review. *Current Drug Metabolism*, 4(1), pp.1–10.

MArk Glover, J.N. & Harrison, S.C., 1995. Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. *Nature*, 373(6511), pp.257–261.

Matsushita, H. *et al.*, 2001. A retinoic acid receptor agonist Am80 rescues neurons, attenuates inflammatory reactions, and improves behavioral recovery after intracerebral hemorrhage in mice. *Journal of Cerebral Blood Flow & Metabolism*, 31(1), pp.222–234.

McKinsey, T. a, Zhang, C.L. & Olson, E.N., 2002. MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends in Biochemical Sciences*, 27(1), pp.40–47.

Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. *Nature*, 449(7164), pp.819–826.

Mellon, P.L. *et al.*, 1990. Immortalization of Hypothalamic GnRH Neurons by Genetically Targeted Tumorigenesis. *Neuron*, 5(1), pp.1–10.

Meloche, S. & Pouysségur, J., 2007. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene*, 26(22), pp.3227–3239.

Menegola, E. *et al.*, 2006. Inhibition of histone deacetylase as a new mechanism of teratogenesis. *Birth Defects Research Part C - Embryo Today: Reviews*, 78(4), pp.345–353.

Mielcarek, M. *et al.*, 2015. HDAC4 as a potential therapeutic target in neurodegenerative diseases: a summary of recent achievements. *Frontiers in Cellular Neuroscience*, 9(42), pp.1–9.

Mielcarek, M., Seredenina, T., *et al.*, 2013. HDAC4 does not act as a protein deacetylase in the postnatal murine brain in vivo. *PLOS one*, 8(11), pp.1–10.

Mielcarek, M., Landles, C., *et al.*, 2013. HDAC4 reduction: a novel therapeutic strategy to target cytoplasmic huntingtin and ameliorate neurodegeneration. *PLOS Biology*, 11(11), pp.1–16.

Miller, S.I., Ernst, R.K. & Bader, M.W., 2005. LPS, TLR4 and infectious disease diversity. *Nature reviews. Microbiology*, 3(1), pp.36–46.

Miscia, S. *et al.*, 2002. Tumor Necrosis Factor α (TNF-α) Activates Jak1 / Stat3-Stat5B Signaling through TNFR-1 in Human B Cells. *Cell growth & Differentiation*, 13(1), pp.13–18.

Mongan, N.P. & Gudas, L.J., 2007. Diverse actions of retinoid receptors in cancer prevention and treatment. *Differentiation*, 75(9), pp.853–870.

Morgan, P.J. *et al.*, 1994. Melatonin receptors: localization, molecular pharmacology and physiological significance. *Neurochemistry International*, 24(2), pp.101–146.

Morgan, P.J. *et al.*, 2003. Photoperiodic programming of body weight through the neuroendocrine hypothalamus. *Journal of Endocrinology*, 177(1), pp.27–34.

Morte, B. *et al.*, 1997. Thyroid Hormone-Regulated Expression of RC3/Neurogranin in the Immortalized Hypothalamic Cell Line GT1-7. *Journal of Neurochemistry*, 69(3), pp.902–909.

Muniyappa, H. & Das, K.C., 2008. Activation of c-Jun N-Terminal Kinase (JNK) by widely used specific p38 MAPK inhibitor SB202190 and SB203580: A MLK-3- MKK7-dependent mechanism. *Cell Signalling*, 20(6), pp.675–683.

Muñoz, A. *et al.*, 1991. Effects of neonatal hypothyroidism on rat brain gene expression. *Molecular Endocrinology*, 5(2), pp.273–280.

Nagy, L. *et al.*, 1997. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell*, 89(3), pp.373–380.

Napoli, J.L., 1996. Retinoic acid biosynthesis and metabolism. *FASEB Journal*, 10(9), pp.993–1001.

Neerven, S. van *et al.*, 2010. Inflammatory cytokine release of astrocytes in vitro is reduced by all-trans retinoic acid. *Journal of Neuroimmunology*, 229(1-2), pp.169–179.

Nelson, D.L. & Cox, M.M., 2013. *Lehninger - Principles of Biochemistry* Sixth Edit., New York: Macmillan Higher Education.

Ng, T.B. & Wong, C.M., 1986. Effects of pineal indoles and arginine vasotocin on lipolysis and lipogenesis in isolated adipocytes. *Journal of Pineal Research*, 3(1), pp.55–66.

Nusinzon, I. & Horvath, C.M., 2006. Positive and Negative Regulation of the Innate Antiviral Response and Beta Interferon Gene Expression by Deacetylation. *Molecular and Cellular Biology*, 26(8), pp.3106–3113.

O'Byrne, S.M. & Blaner, W.S., 2013. Retinol and retinyl esters: biochemistry and physiology. *Journal of Lipid Research*, 54(7), pp.1731–1743.

Oeckinghaus, A. & Ghosh, S., 2009. The NF-κB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology*, 1(4), pp.1–14.

Orfali, N. *et al.*, 2014. Retinoid receptor signaling and autophagy in acute promyelocytic leukemia. *Experimental Cell Research*, 324(1), pp.1–12.

Pahl, H.L., 1999. Activators and target genes of Rel / NF-κB transcription factors. *Oncogene*, 18(49), pp.6853–6866.

Paik, J. *et al.*, 2014. Inhibition of Retinoic Acid Biosynthesis by WIN 18,446 Markedly Suppresses Spermatogenesis and Alters Retinoid Metabolism in Mice. *The Journal of Biological Chemistry*, 289(21), pp.15104–15117.

Pålsson-McDermott, E.M. & O'Neill, L. a J., 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*, 113(2), pp.153–162.

Park, Y.C. *et al.*, 2000. A novel mechanism of TRAF signaling revealed by structural and functional analyses of the TRADD-TRAF2 interaction. *Cell*, 101(7), pp.777–787.

Pascual, M. *et al.*, 2011. Epigenetic changes in B lymphocytes associated with house dust mite allergic asthma. *Epigenetics*, 6(9), pp.1131–1137.

Potthoff, M.J. & Olson, E.N., 2007. MEF2: a central regulator of diverse developmental programs. *Development*, 134(23), pp.4131–4140.

Pozzi, S. *et al.*, 2006. RAR-mediated epigenetic control of the cytochrome P450 Cyp26a1 in embryocarcinoma cells. *Nature*, 25(9), pp.1400–1407.

Prevot, V. *et al.*, 2003. Activation of erbB-1 Signaling in Tanycytes of the Median Eminence Stimulates Transforming Growth Factor β1 Release via Prostaglandin E2 Production and Induces Cell Plasticity. *The Journal of Neuroscience*, 23(33), pp.10622–10632.

Purkayastha, S. & Cai, D., 2013. Neuroinflammatory basis of metabolic syndrome. *Molecular Metabolism*, 2(4), pp.356–363.

Raetz, C.R.H., 1990. Biochemistry of endotoxins. *Annual Review of Biochemistry*, 59, pp.129–170.

Raetz, C.R.H. & Whitfield, C., 2008. Lipopolysaccharide Endotoxins. *Annual Review of Biochemistry*, 71, pp.635–700.

Ransom, J. *et al.*, 2013. The rhythm of retinoids in the brain. *Journal of Neurochemistry*, 129(3), pp.366–376.

Revel, F.G. *et al.*, 2006. Melatonin regulates type 2 deiodinase gene expression in the Syrian hamster. *Endocrinology*, 147(10), pp.4680–4687.

Rhinn, M. & Dollé, P., 2012. Retinoic acid signalling during development. *Development*, 139(5), pp.843–858.

Rochette-Egly, C., 2014. Retinoic acid signaling and mouse embryonic stem cell differentiation : Cross talk between genomic and non -genomic effects of RA. *Biochimica et Biophysica Acta*, 1851(1), pp.65–75.

Rodrigues, D., 2015. *Study of the retinoic acid regulated genes in the rodent hypothalamus controlling growth and appetite and the potential for the epigenetic control.*,

Ross, A.W. *et al.*, 2004. Photoperiodic regulation of hypothalamic retinoid signaling: association of retinoid X receptor gamma with body weight. *Endocrinology*, 145(1), pp.13–20.

Ross, S. a *et al.*, 2000. Retinoids in embryonal development. *Physiological Reviews*, 80(3), pp.1021–1054.

Rottach, A., Leonhardt, H. & Spada, F., 2009. DNA methylation-mediated epigenetic control. *Journal of Cellular Biochemistry*, 108(1), pp.43–51.

Roux, P.P. & Blenis, J., 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiology and molecular biology reviews*, 68(2), pp.320–344.

Sahu, A., 2003. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. *Frontiers in Neuroendocrinology*, 24(4), pp.225–253.

Schafe, G.E. *et al.*, 2001. Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends in Neurosciences*, 24(9), pp.540–546.

Schanga, A.-L. *et al.*, 2014. Failure of thyroid hormone treatment to prevent inflammation-induced white matter injury in the immature brain. *Brain, Behavior, and Immunity*, 37, pp.95–102.

Schiitze, S. *et al.*, 1992. TNF Activates Phospholipase Sphingomyelin NF-κB by Phosphatidylcholine-Specific C-Induced "Acidic" Sphingomyelin Breakdown. *Cell*, 71(5), pp.765–776.

Seidel, C. & Diederich, M., 2015. Histone deacetylase 6 in health and disease. *Epigenomics*, 7(1), pp.103–118.

Sharif, O. *et al.*, 2007. Transcriptional profiling of the LPS induced NF-κB response in macrophages. *BMC Immunology*, 8, pp.1–17.

Shatz, C.J., 1990. Impulse Activity and the Patterning of Connections during CNS Development. *Neuron*, 5(6), pp.745–756.

Sheng, L. *et al.*, 2012. NF-κB-inducing kinase (NIK) promotes hyperglycemia and glucose intolerance in obesity by augmenting glucagon action. *Nature Medicine*, 18(6), pp.943–949.

Shinomiya, A. *et al.*, 2014. Regulation of seasonal reproduction by hypothalamic activation of thyroid hormone. *Frontiers in Endocrinology*, 5, pp.1–12.

Sodhi, R.K. & Singh, N., 2014. Retinoids as potential targets for Alzheimer's disease. *Pharmacology, Biochemistry, and Behavior*, 120, pp.117–123.

Song, C.K. & Bartness, T.J., 2001. CNS sympathetic outflow neurons to white fat that express MEL receptors may mediate seasonal adiposity. *American Journal of Physiology.*, 281(2), pp.R666–R672.

Song, Y., Yao, X. & Ying, H., 2011. Thyroid hormone action in metabolic regulation. *Protein & Cell*, 2(5), pp.358–368.

Soprano, D.R., Qin, P. & Soprano, K.J., 2004. Retinoic acid receptors and cancers. *Annual Review of Nutrition*, 24, pp.201–221.

Sporn, M. *et al.*, 1976. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Federation Proceedings*, 35(6), pp.1332–1338.

Stevenson, T.J. & Prendergast, B.J., 2013. Reversible DNA methylation regulates seasonal photoperiodic time measurement. *PNAS*, 110(41), pp.16651–16656.

Stoney, P.N. *et al.*, 2015. Thyroid hormone activation of retinoic acid synthesis in hypothalamic tanycytes. *Glia*, 63(12).

Suto, R.K. *et al.*, 2000. Crystal structure of a nucleosome core particle containing the variant histone H2A.Z. *Nature*, 7, pp.1121–1124.

Suzuki, M.M. & Bird, A., 2008. DNA methylation landscapes: provocative insights from epigenomics. *Nature reviews. Genetics*, 9(6), pp.465–476.

Tang, T. *et al.*, 2010. Uncoupling of Inflammation and Insulin Resistance by NF-κB in Transgenic Mice through Elevated Energy Expenditure. *Journal of Biological Chemistry*, 285(7), pp.4637–4644.

Tang, X.-H. & Gudas, L.J., 2011. Retinoids, retinoic acid receptors, and cancer. *Annual Review of Pathology*, 6, pp.345–364.

Tanumihardjo, S.A., 2011. Vitamin A: biomarkers of nutrition for development. *The American Journal of Clinical Nutrition*, 94(2), pp.658–665.

Thé, H. de *et al.*, 1990. Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature*, 343(6254), pp.177–180.

Thiagalingam, S. *et al.*, 2003. Histone Deacetylases: Unique Players in Shaping the Epigenetic Histone Code. *Annals of the New York Academy of Sciences*, 983, pp.84–100.

Toropainen, S. *et al.*, 2010. The down-regulation of the human MYC gene by the nuclear hormone 1α,25-dihydroxyvitamin D3 is associated with cycling of corepressors and histone deacetylases. *Journal of Molecular Biology*, 400(3), pp.284–294.

Umesono, K. *et al.*, 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. *Nature*, 336(6196), pp.262–265.

Valdearcos, M., Xu, A.W. & Koliwad, S.K., 2015. Hypothalamic Inflammation in the Control of Metabolic Function. *Annual Review of Physiology*, 77, pp.131–160.

Varela, L. *et al.*, 2012. Hypothalamic mTOR pathway mediates thyroid hormone-induced hyperphagia in hyperthyroidism. *Journal of Pathology*, 227(2), pp.209–222.

Verdin, E., Dequiedt, F. & Kasler, H.G., 2003. Class II histone deacetylases: versatile regulators. *TRENDS in Genetics*, 19(5), pp.286–293.

Volmar, C.-H. & Wahlestedt, C., 2015. Histone deacetylases (HDACs) and brain function. *Neuroepigenetics*, 1, pp.20–27.

De Vries, E.M. *et al.*, 2014. NF-κB signaling is essential for the lipopolysaccharide-induced increase of type 2 deiodinase in tanycytes. *Endocrinology*, 155(5), pp.2000–2008.

Wang, A.H. *et al.*, 1999. HDAC4 , a Human Histone Deacetylase Related to Yeast HDA1 , Is a Transcriptional Corepressor. *Molecular and Cellular Biology*, 19(11), pp.7816–7827.

Wang, H. & Ye, J., 2014. Regulation of energy balance by inflammation: Common theme in physiology and pathology. *Endocrine and Metabolic Disorders*, 16(1), pp.47–54.

Wang, R.-M. *et al.*, 2005. Activation of extracellular signal-regulated kinase 5 may play a neuroprotective role in hippocampal CA3/DG region after cerebral ischemia. *Journal of Neuroscience Research*, 80(3), pp.391–399.

Wang, X. & Liu, Y., 2007. Regulation of innate immune response by MAP kinase phosphatase-1. *Cellular Signalling*, 19(7), pp.1372–1382.

Wang, Z., Qin, G. & Zhao, T.C., 2014. HDAC4: mechanism of regulation and biological functions. *Epigenomics*, 6(1), pp.139–150.

Watanabe, M. *et al.*, 2004. Photoperiodic regulation of type 2 deiodinase gene in Djungarian hamster: possible homologies between avian and mammalian photoperiodic regulation of reproduction. *Endocrinology*, 145(4), pp.1546–1549.

Watt, F. & Molloy, P.L., 1988. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. *Genes & Development*, 2(9), pp.1136–1143.

Wellen, K.E. & Hotamisligil, G.S., 2003. Obesity-induced inflammatory changes in adipose tissue. *The Journal of Clinical Investigation*, 112(12), pp.1785–1788.

Whitmarsh, a J. & Davis, R.J., 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *Journal of Molecular Medicine*, 74(10), pp.589–607.

Whitmarsh, A.J., 2007. Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. *Biochimica et Biophysica Acta*, 1773(8), pp.1285–1298.

WHO Global Health Observatory Data Repository [online database], 2013. Nutrition, Physical Activity and Obesity. *World Health Organization*. Available at: http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/country-work [Accessed April 24, 2015].

Williams, S.R. *et al.*, 2010. Haploinsufficiency of HDAC4 causes brachydactyly mental retardation syndrome, with brachydactyly type E, developmental delays, and behavioral problems. *American Journal of Human Genetics*, 87(2), pp.219–228.

Wu, C. & Morris, J.R., 2000. Genes, Genetics, and Epigenetics : a correspondence. *Science*, 293(5532), pp.1103–1105.

Yao, B. & Jin, P., 2014. Unlocking epigenetic codes in neurogenesis. *Genes & Development*, 28(12), pp.1253–1271.

Yao, Y., Yang, W. & Seto, E., 2001. Regulation of Transcription Factor YY1 by Acetylation and Deacetylation. *Molecular and Cellular Biology*, 21(17), pp.5979–5991.

Yasuo, S. *et al.*, 2005. The reciprocal switching of two thyroid hormone-activating and -inactivating enzyme genes is involved in the photoperiodic gonadal response of Japanese quail. *Endocrinology*, 146(6), pp.2551–2254.

Yoshimura, T. *et al.*, 2003. Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. *Nature*, 426(6963), pp.178–181.

Yu, I.-C. *et al.*, 2013. Neuronal androgen receptor regulates insulin sensitivity via suppression of hypothalamic NF-κB-mediated PTP1B expression. *Diabetes*, 62(2), pp.411–423.

Zhang, G. *et al.*, 2013. Hypothalamic programming of systemic ageing involving IKK-β, NF-κB and GnRH. *Nature*, 497(7448), pp.211–216.

Zhang, W. & Kone, B.C., 2002. NF-κB inhibits transcription of the H+-K+-ATPase α2-subunit gene : role of histone deacetylases. *American Journal of Physiology*, 283(5), pp.904–911.

Zhang, W. & Liu, H.T., 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research*, 12, pp.9–18.

Zhang, Y. & Dong, C., 2005. MAP Kinases in Immune Responses. *Cellular & Molecular Immunology*, 2(1), pp.20–27.

Ziouzenkova, O. *et al.*, 2007. Retinaldehyde represses adipogenesis and diet-induced obesity. *Nature Medicine*, 13(6), pp.695–702.

SUPPLEMENTARY DATA

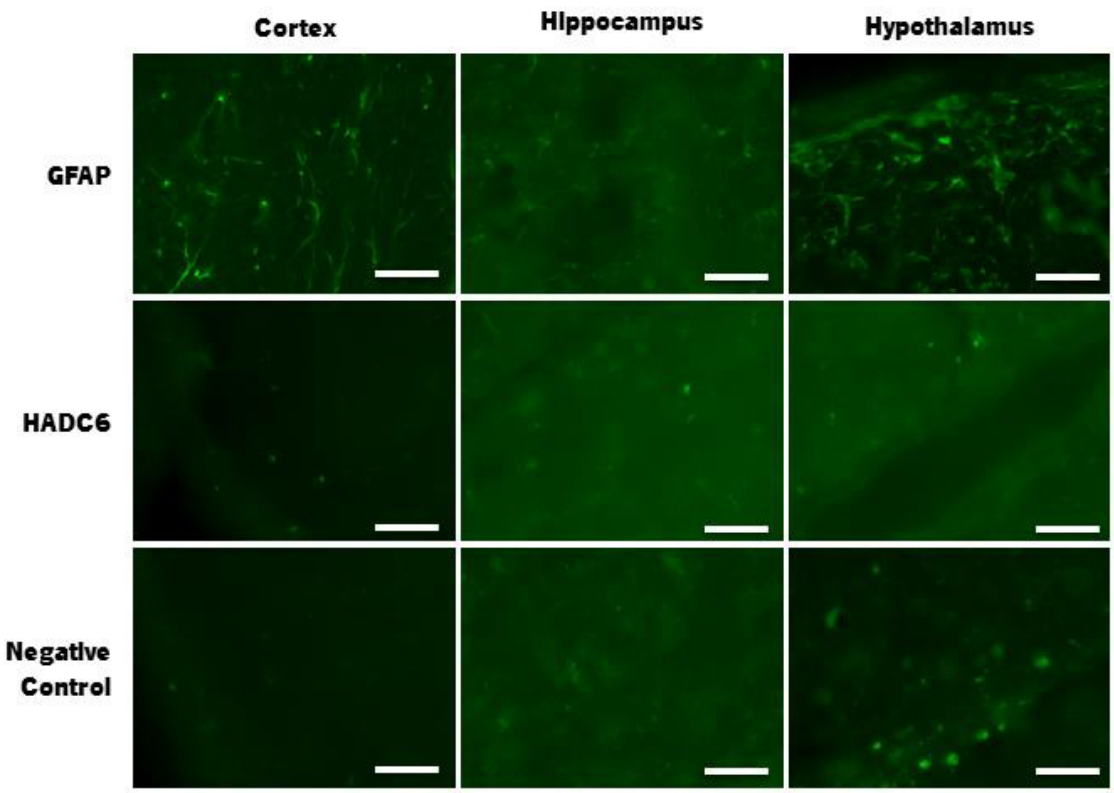

Figure S1 | Immunohistochemical analysis of HDCA6 localisation in the rat brain. GFAP, an astrocyte specific cell marker, was used as positive control. A negative control sample that was stained with the secondary antibody but not with HDAC6 antibody was also run alongside the positive samples.