The seasonal regulation of prolactin in the pituitary gland

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Abbreviations

AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ARNT	aryl hydrocarbon receptor nuclear translocator
AVP	arginine vasopressin
BAC	bacterial artificial chromosome
bFGF	basic fibroblast growth factor
BMAL	brain and muscle ARNT
BSA	bovine serum albumin
CA_2	cornu Ammonis field 2
cAMP	cyclic adenosine mono-phosphate
CGA	common glycoprotein alpha subunit (also alpha-GSU)
CPM	counts per minute
CPu	caudate putamen
CREB	cAMP response element binding protein
CRH	corticotrophin-releasing hormone
Cry	cryptochrome
CSF	cerebrospinal fluid
D	dark
DAB	diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
Dio2	deiodinase type II
Dio3	deiodinase type III
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
Dxt	dextran
EDTA	ethylenediaminetetraacetic acid
EL	ependymal layer
ELISA	enzyme linked immunosorbent assay
Eya3	eyes absent 3
F344	Fischer 344

FBS	fetal bovine serum
FC	fasciola cinereum
FGF2	fibroblast growth factor 2
FSH	follicle-stimulating hormone
FSK	forskolin
GABA	gamma aminobutyric acid
GH	growth hormone
GnRH	gonadotrophin releasing hormone
GSU	glycoprotein subunit
h	hour
HBSS	Hank's buffered saline solution
HIOMT	hydroxyindole-O-methyltransferase
HPD	hypothalamo-pituitary disconnected
HSD	Harlan Sprague-Dawley
ICER	inducible cAMP-response element early repressor
IG	indusium griseum
IHC	immunohistochemistry
IL-6	interleukin 6
IMEL	iodomelatonin
IMS	industrial methylated spirit
ISH	in situ hybridization
L	light
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LP	long photoperiod
Luc	luciferase
LV	lateral ventricle
MBH	mediobasal hypothalamus
Mel	melatonin
MH	medial habenula
min	minute
m/s	meters per second
MSH	melanocyte-stimulating hormone
MT1	melatonin receptor type 1
MT2	melatonin receptor type 2

NA	numerical aperture
NAT	N-acetyltransferase
NGS	normal goat serum
NIDDK	National Institute of Diabetes, Digestive and Kidney Disease
NKA	neurokinin A
NKB	neurokinin B
NKR	neurokinin receptor
NK1R	neurokinin receptor 1
NK2R	neurokinin receptor 2
NK3R	neurokinin receptor 3
NPG	neuropeptide gamma
NPK	neuropeptide K
N-POMC	N-terminal fragment of proopiomelanocortin
opt	optic tract
PACAP	pituitary adenylyl cyclase-activating polypeptide
PAS	Per-ARNT-SIM domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	pars distalis
Per	period
PFA	paraformaldehyde
PI	pars intermedia
PMT	photomultiplier tubes
PN	pars nervosa
POMC	proopiomelanocortin
PRL	prolactin
PRL-R	prolactin receptor
PT	pars tuberalis
PVN	paraventricular nucleus
RIA	radioimmunossay
RLU	relative luminescence units
ROD	relative optical density
rpm	revolutions per minute
SCN	suprachiasmatic nuclei
SDS	sodium dodecyl sulphate

SE	standard error
SIM	single-minded protein
SIX	sine oculis homeobox homolog
SP	short photoperiod
SSC	saline sodium citrate
SubP	substance P
T_m	melting temperature
TAC1	tachykinin, precursor 1
TBS	tris-buffered saline
TEA	triethanolamine
TEF	thyrotroph embryonic factor
TH	thyroid hormone
TNFα	tumour necrosis factor alpha
TRH	thyrotrophin-releasing hormone
TRIS	tris(hydroxymethyl)aminomethane
TSA	tyramide signal amplification
TSH	thyroid-stimulating hormone (or thyrotrophin)
TSHβ	thyrotrophin beta
VIP	vasoactive intestinal polypeptide
VMHVL	ventromedial hypothalamus ventrolateral division
ZT	zeitgeber time
ψ	psi, timing interval
3V	third ventricle

Abstract

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For many species, adaptation of various physiological characteristics to the seasonally changing environment is vital in their survival and reproductive fecundity. Such seasonal alterations in physiology can affect growth, food intake, energy balance and gonadal activity, as well as the secretion of the pituitary hormone prolactin. The current suggested model of seasonal prolactin regulation dictates that the melatonin-responsive pars tuberalis (PT) of the anterior pituitary gland translates the photoperiodic input (measurement of day length) into a seasonal signal, which controls prolactin release. The primary aims of this study were to investigate the downstream decoding mechanisms of the melatonin signal and the currently uncharacterized factors, named 'tuberalins', which are considered to be released by the PT and involved in the seasonal entrainment process. The most likely candidates believed to have a key role in the seasonal regulation of prolactin, as the PT-derived tuberalins, are Substance P and Neurokinin A, members of a group of bioactive peptides known as neurokinins. These molecules, which have previously been shown to have an effect on prolactin release, are encoded by the *TAC1* (tachykinin, precursor 1) gene, which was recently identified as being strongly activated by 'summer like' long photoperiods (LP) within the ovine PT.

In the present study, the Fischer 344/NHsd (F344/NHsd) rat, a photoperiod-sensitive laboratory strain that responds to 'winter like' short photoperiods (SP) by decreasing its body weight, food intake, testes weight and the secretion of pituitary prolactin, was used as a model species to investigate the potential involvement of neurokinins in the seasonal regulation of prolactin. The action of the neurokinins Substance P and Neurokinin A was tested with the help of a transgenic F344 rat, which expresses the reporter gene luciferase under the control of the prolactin promoter, providing a tool for studying prolactin regulation. An *in vitro* reporter system was set up using transgenic F344 tissue, and this revealed no effect of the neurokinins on prolactin promoter activity. *In situ* hybridization studies revealed a lack of *TAC1* mRNA expression in the PT region of the F344/NHsd rat. These results do not support a role for neurokinins in the seasonal regulation of prolactin in the rat, suggesting a possible divergence in the seasonal pathway of prolactin regulation between species. Nonetheless, the expression of *TAC1* was found to be regulated by photoperiod in other key areas of the brain, including the ependymal layer of the third ventricle of the hypothalamus, which has previously been associated with seasonal adaptations in body weight and reproduction. Furthermore, the *in situ* hybridization studies reveal the LP-activated expression of *Eyes absent 3 (Eya3),* a transcription factor-encoding gene, in the PT of the F344/NHsd rat, and this finding is similarly revealed in the Syrian hamster, a strongly seasonal species. Recent studies in seasonal birds and mammals have identified *Eya3* as the first common strongly activated gene in the PT, following exposure to LP and suggested that it may have a key role as a master regulator of seasonal responses. The novel finding of *Eya3* up-regulation in the rat under LP further highlights the conserved nature of *Eya3* expression in photoperiodic species and presents the F344/NHsd laboratory rat as a valuable rodent model in the study of photoperiodism.

Declaration

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CHAPTER 1: General Introduction

1.1 Endogenous clocks and biological rhythms

It has long been known that living creatures, including bacteria, fungi, flies, birds, plants and human beings, all possess an internal time-keeping system referred to as the 'biological clock'. Each day, their cells anticipate the predictable changes in the environment and function on the basis of a 24-hour cycle, the time it takes the Earth to rotate on its axis. Adaptation to this circadian (daily) rhythm and to a subsequent circannual (yearly) rhythm is vital in the survival and functioning of every species. Two types of long-term timing mechanisms exist that allow organisms to predict events, months or even years in advance, and are extremely useful in regulating the seasonal variations in physiology and behaviour found in a broad spectrum of mammals including humans. The first mechanism is known as 'photoperiodism', which is the organism's response to the seasonal changes in day length (Tamarkin *et al*., 1985), whilst the second is 'circannual rhythm generation', a prominent characteristic in long-lived animals that express seasonal variations in breeding and can be found in mammals across different latitudes (Lincoln *et al*., 2003a).

Organisms are considered photoperiodic if changes in day length are sufficient to cause modifications in at least one of their traits. When the number of hours of light per day begins to fall below a critical minimum during the late summer, some rodent species cease to breed. This evidence suggests that mammals are able to cue reproductive events based on changes in annual day length, ensuring that their offspring are born during periods when food is abundant and climatic conditions are favourable. The endogenous oscillations that persist in the animals are entrained under the constant environmental conditions that have a periodicity of approximately 12 months, thus driving circannual physiological rhythms such as metabolism, reproduction, social behaviour, migration and hibernation.

These prominent events are all under the control of the master circadian clock in the mammalian brain, which resides deep in the area of the anterior hypothalamus known as the suprachiasmatic nuclei (SCN). The clock genes in the SCN generating circadian rhythms have been well investigated and studies have shown that the core mechanism for its pacemaker function resides in the interacting positive and negative transcriptional/translational feedback loops (Shearman *et al*., 2000). The positive elements of the clock are two basic helix-loop-helix, PAS-domain-containing

transcription factors, CLOCK and BMAL1 (Gekakis *et al*., 1998; Hogenesch *et al*., 1998; Takahata *et al*., 1998). When these proteins heterodimerize they control the transcription of other clock genes, three *Period* genes (*Per1, Per2, Per3*) and two *Cryptochrome* genes (*Cry1, Cry2*) (Gekakis *et al*., 1998; Jin *et al*., 1999; Kume *et al*., 1999). These in turn act as the negative feedback components, shutting down the positive *Clock/Bmal1* force and completing the cycle. Delays in the feedback loops are regulated by factors such as phosphorylation and proteolysis, which determine the interactions and stability of the clock proteins, thus maintaining the 24-h cyclicity (Shearman *et al*., 2000).

The neurones making up the SCN network are positioned to receive light stimuli through non-visual photoreceptors in the retina. The periodic exposure to light stimuli every 24-h entrains the circadian clock genes of the SCN neurones, which are ultimately responsible for timing diurnal rhythms of activity/sleep, feeding behaviour, body temperature and pituitary hormone secretion, including the nocturnal (nightly) release of melatonin, the main hormonal product of the pineal gland (Tamarkin *et al*., 1985; Stehle *et al*., 2001). Pineal melatonin release is also directly inhibited by light, irrespective of circadian time, via a retinal-hypothalamic-sympathetic innervation and the control of the rate-limiting enzyme for melatonin synthesis, *N*- acetyltransferase (NAT) (Klein and Weller, 1970). These control mechanisms dictate that melatonin is secreted only at night and that its duration of release varies quantitatively with night length and therefore day length. Consequently, through melatonin, the mammalian pineal gland acts as a neuroendocrine transducer, processing information that encodes both time of day and time of year.

1.2 Melatonin: a photoperiodic signal

Melatonin is an indoleamine synthesized from a second pineal hormone, serotonin, through the action of the enzymes NAT and hydroxyindole-*O*-methyltransferase (HIOMT) (Tamarkin *et al*., 1985). During the day the concentration of serotonin in the pineal was found to be high whilst the concentration of melatonin was low, and vice versa during the night, suggesting that the decline in serotonin was due to the increased melatonin production (Tamarkin *et al*., 1985). Once synthesized, lipophilic melatonin is secreted into the peripheral blood and cerebrospinal fluid (Malpaux *et al*., 2001).

In photoperiodic mammals, melatonin has a key role in mediating seasonal adaptations in physiology, including reproductive status, breeding behaviour, hibernation and coat condition (Reiter, 1973; Kennaway *et al*., 1987; Bartness and Goldman, 1989; Morgan *et al*., 1994a; Dardente *et al*., 2003). Given that melatonin affects several physiological functions, it has not been easy to determine whether it is acting on one single site, which then regulates many downstream seasonal functions, or whether it is acting at multiple locations, each involved in a single seasonal effect. Following initial experiments demonstrating that melatonin acted in the brain of the white-footed mouse (Glass and Lynch, 1982), autoradiographic studies using the melatonin probe, 2-^{125}I-iodomelatonin, allowed for the identification of putative melatonin-binding sites in various brain regions, depending on the species (Bittman, 1993). Intriguingly, in photoperiodic mammals the tissue expressing the highest concentration of melatonin-binding sites appears to be the pars tuberalis (PT) of the pituitary gland (Morgan *et al*., 1994a).

1.2.1 Melatonin and the Pars Tuberalis

Located at the base of the brain, the pituitary gland is a major endocrine gland linked functionally to the hypothalamus, and responsible for secreting a variety of hormones, including prolactin (PRL), adrenocorticotrophic hormone (ACTH), growth hormone (GH), thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Structurally, it is subdivided into an anterior region, a posterior region and an intermediate lobe. The mammalian adenohypophysis (anterior pituitary) consists of three distinct regions, the pars distalis (PD), the pars intermedia (PI) and the less functionally understood PT (Morgan and Williams, 1996) (Figure 1.1). Although originally thought to be a supporting gland for the PD in supplementing endocrine output, the localization of melatonin receptors to the PT of all mammals studied except for human, suggested an alternative role, connected to photoperiodism and the melatonin signal, and one distinct from that of the PD (Morgan *et al*., 1994a).

The PT is anatomically positioned between the median eminence of the brain and the PD, and can be characterized by three main cell types: PT-specific secretory cells, PD-like cells and follicular cells (Morgan *et al*., 1991; Wittkowski *et al*., 1992). The PT-specific secretory cells are the most abundant of the cell types; they exhibit characteristics of peptide-secreting cells and are distinct from the secretory cells found in the PD (Wittkowski *et al*., 1998). The PT-specific cells are characterized by rough

endoplasmic reticulum and Golgi bodies, suggesting peptide or protein secretion, but have very few dense-core storage granules. Factors that regulate the secretory activity of the PT-specific cells include melatonin, adenosine and pituitary adenylyl cyclase-activating peptide (PACAP) (Barret *et al.,* 2002; von Gall *et al*., 2002; von Gall *et al*., 2005; Schuster, 2007). The PD-like cells contain rough endoplasmic reticulum and Golgi bodies, yet there appears to be an abundance of dense-core granules. These cells make up around 10-15% of the PT cells and are most commonly a mixture of gonadotrophs and thyrotrophs, proportion depending on species, and can also be found in the transition zone connecting the PT with the PD (Gross, 1984). The third type are follicular cells which are generally smaller than either of the two aforementioned types, they are inter-dispersed throughout the gland and do not appear to have a secretory function (Dellmann *et al*., 1974). The expression of growth factors from within these cells however, might suggest a trophic action on endocrine cells or perhaps even an involvement in the maintenance of the pituitary microvasculature (Inoue *et al*., 1999).

1.2.2 Melatonin Receptors in PT 'calendar cells'

The appearance of melatonin receptors very early on in the development of fetal pituitaries, even before the existence of a functional hypothalamic-pituitary unit, suggests an important function for the PT in the transduction of the melatonin signal (Morgan and Williams, 1996). These melatonin-binding sites have been found to be membrane-bound receptors of the G protein-coupled superfamily of proteins (Morgan *et al*., 1994a). Molecular cloning of the melatonin receptors in sheep, human and mouse have reported three initial subtypes, melatonin receptor types 1a, 1b and 1c (Mel1a, Mel1b, Mel1c) (Reppert *et al*., 1994). The first two were later re-designated melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) respectively, and have now both been identified in mammals (Reppert *et al*., 1994; Barrett *et al*., 1997; Cogé *et al*., 2009). These two subtypes have high structural resemblance and similar pharmacological characteristics (Browning *et al*., 2000). The third Mel1c receptor is not found in mammals; however, studies have identified the receptor G protein-coupled receptor 50 (GPR50) to be the mammalian ortholog of Mel1c (Dufourny *et al*., 2012). Initial 'knockout' experiments in mice, targeting the MT1 receptor, demonstrated the involvement of this subtype in the regulation of circadian rhythms generated by the master clock in the SCN, whilst a number of observations point towards a functional importance of MT1 in seasonal responses (Weaver *et al*., 1996; Liu *et al*., 1997).

The specialised melatonin target cells found in the brain, pituitary and perhaps the periphery, are responsible for registering and decoding the melatonin signal and must therefore express high affinity melatonin receptors. These cells are also thought to be melatonin 'signal duration sensors' because they can distinguish between short (6-10h) and long (12-16h) photoperiodic exposure to melatonin and generate the appropriate long-term physiological and/or behavioural response (Lincoln *et al.*, 2003b). The ability of these 'calendar cells' to do so, is assumed to be attributed to a clock gene-based mechanism (Lincoln *et al.*, 2003b) (see section 1.3).

So far, the best-characterized melatonin 'signal-duration-sensing' calendar cells are considered to be the secretory cells of the PT of the pituitary, which present high levels of MT1 (Klosen *et al.*, 2002; von Gall *et al.*, 2002). Whilst these cells express many of the subunits of PD hormones (Gross, 1984; Böckers *et al.*, 1996; Wittkowski *et al.*, 1998; Eagle and Tortonese, 2000), the expression of the PD thyroid stimulating hormone (TSH) subunits, {thyroid-stimulating hormone beta subunit (TSHβ) and glycoprotein hormone alpha polypeptide (CGA)}, at both the protein and mRNA level, appears to be a common feature in various mammalian species (Stoeckel *et al.*, 1994; Böckers *et al.*, 1996; Morgan and Williams, 1996; Klosen *et al.*, 2002). However, the PT-specific cells expressing these TSH heterodimer-forming subunits, do not resemble PD thyrotrophs in their secretory activity (Wittkowski *et al.*, 1998) or in the way that they are regulated, with only the PT-specific cells being sensitive to both photoperiod and melatonin (Wittkowski *et al.*, 1988; Bockmann *et al.*, 1996; Dardente *et al.*, 2003; Arai and Kameda, 2004; Lincoln *et al.*, 2005; Hanon *et al.*, 2010; Yasuo *et al.*, 2010a). These findings suggest a specialized role for this PT-specific cell type, different to that of PD thyrotrophs, and associated with the transduction of the melatonin signal.

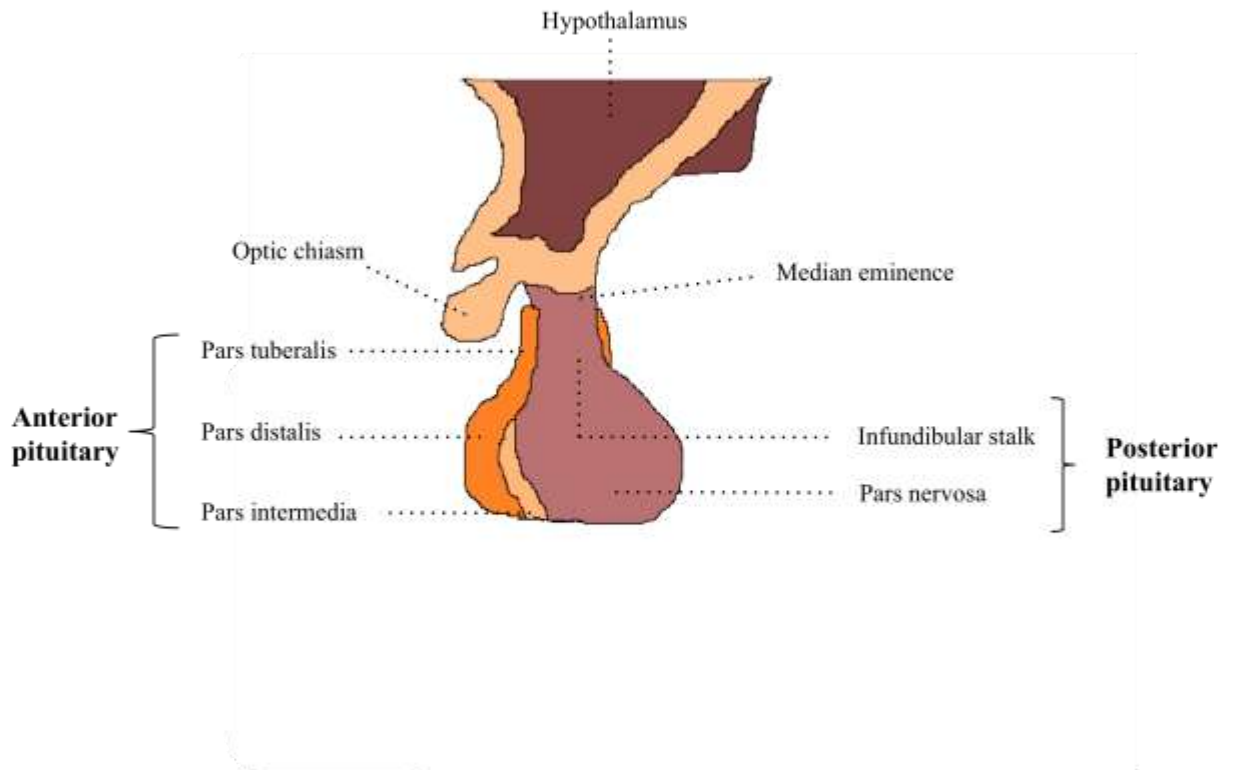


Figure 1.1: The pituitary gland.

The pituitary gland is composed of the anterior and posterior lobes, and located at the base of the brain, beneath the hypothalamus and the median eminence. The anterior lobe consists of three regions: the pars tuberalis, pars distalis and pars intermedia, whilst the infundibular stalk and pars nervosa make up the posterior lobe. Figure by the author.

1.3 Decoding of the melatonin signal in the PT

Signal transduction pathways triggered by melatonin receptors involve the activation of G proteins, with both MT1 and MT2 preferentially coupling to G_i proteins to inhibit forskolin-stimulated cyclic AMP (cAMP) formation. In an attempt to identify the mechanism underlying the decoding of the melatonin signal in the pituitary, studies have shown that melatonin binding to its receptors causes a suppression of intracellular cAMP production in the PT of sheep, hamsters, rats and mice (Morgan, *et al*., 1994a). This suppression results from the inhibition of adenylyl cyclase (AC) and the associated block of various cAMP-dependent processes, including protein kinase activation, phosphorylation of the cAMP-response element binding protein (CREB), production of inducible cAMP early repressor (ICER) and the induction of the transcription factor, c-fos (McNulty *et al*., 1996; Ross *et al*., 1996). These data suggest that cAMP-dependent pathways, thought to be triggered by an unknown factor (named Stimulus X), are responsible for the activation of gene transcription in the ovine PT, and that melatonin is functioning to prevent cellular activation by any other external stimulus to the PT cells, thus rendering the cells in a functionally inhibited state (Morgan, 2000). In other words, decoding of the melatonin signal into an output response may be regulated through a cAMP sensitization/desensitization mechanism (Hazlerigg *et al*., 1993; von Gall, *et al*., 2002).

This assumption has been helpful in predicting the diurnal functioning of the pituitary PT, which in accordance with the nocturnal melatonin peak, should be in a lowered activity state during the night and highly active during the daytime. More specific information on the regulation by melatonin was provided by *in situ* hybridization studies in sheep, Syrian and Siberian hamsters, where the 24-h rhythmic expression of two acutely inducible genes, *Per1* and *ICER*, was measured in the PT and SCN (Morgan *et al*., 1998; Messager *et al*., 1999). The results showed peak expression for both *Per1* and *ICER*, during the early light phase [zeitgeber time (ZT) = 3-7, where ZT0= time of lights on], following the decline in melatonin secretion.

Furthermore, the maximal expression of these genes was affected by photoperiod, with a higher peak seen in long photoperiod days compared to short photoperiod days (Messager *et al*., 2000). Other studies involving pinealectomised animals and timed injections of melatonin have shown that the melatonin rhythm intimately regulates the

mRNA expression profiles of *Per1* and *ICER* in the PT (Messager *et al*., 2000; Messager *et al*., 2001). When melatonin was injected right before lights-on it appeared to block or delay the morning expression of both genes (Messager *et al*., 2000), whilst pinealectomy in the Syrian hamster abolished their expression (Messager *et al*., 2001). It is therefore assumed likely that the daytime peak in *Per1* and *ICER* expression is a result of disinhibition due to the reduction in melatonin and the subsequent free-running of the cAMP signalling pathways. Melatonin has also been shown to sensitize the PT cell to the stimulatory effect of adenosine, which positively regulates cAMP-associated responses during the light phase (von Gall *et al*., 2002). Taken together, these data show that melatonin duration is decoded into a change in amplitude of expression of various acutely inducible genes, by either inhibiting or stimulating cAMP signalling, and ultimately regulating the secretory function of the PT cell (Messager *et al*., 1999). The short daily melatonin signal (long photoperiod) induces an active summer phenotype for the PT whilst a long melatonin signal (short photoperiod) promotes an inactive winter phenotype.

Major clock genes whose expression profiles have been determined for the ovine PT of both long and short photoperiods, are *Bmal1, Clock, Per1, Per2, Cry1*, *Cry2* and *Ck1ε* (Lincoln *et al*., 2002). All the selected clock genes were rhythmically expressed in the PT, but unlike the situation in the SCN, the timing of PT clock gene expression was affected by photoperiod. Peak expression of *Per1* and *Per2* occurred in the first half of the light phase (ZT 3-7), whilst the peak in expression of *Cry1* and *Cry2* occurred in the early dark phase (ZT 11-15 under short days and ZT 19 under long days). The differential regulation of the *Per* and *Cry* sets of genes; *Per* being activated at dawn and *Cry* being activated at dusk, is closely related to the diurnal rhythm in blood concentrations of melatonin, with *Per1* expression linked to the off-set of melatonin secretion and *Cry1* and *Cry2* linked to the on-set of its release. This suggests that the melatonin decoding mechanism depends on the differential expression of these clock genes and that the *Per-Cry* interval between peak expressions, termed ψ (psi), varies directly with the length of photoperiod, thus operating as an internal coincidence timer (Lincoln *et al*., 2002). It is therefore assumed that the duration of the melatonin signal regulating the ψ interval in the PT, determines the concentration of the heterodimeric protein products of these genes (CRYs and PERs) in the cytoplasm. Ultimately, upon nuclear entry, the formation of PER/CRY protein complexes can potentially regulate the transcription of other target clock genes (Kume *et al*., 1999). This suggests that through

the phasing of clock gene expression in the PT, the melatonin signal provides a local indication of photoperiod, thus inducing downstream events, which lead to the regulation of seasonal physiology (Lincoln *et al.*, 2003a; Dupré, 2011). The internal coincidence timing model was originally proposed by Pittendrigh and Bruce (1959), in an attempt to explain the role of circadian clocks in photoperiodism. Whilst their theory suggests that photoperiodism involves an interaction between two circadian oscillators, one following dawn and the other following dusk, an alternative model, known as the Bünning hypothesis (Bünning, 1936), proposes that photoperiodism involves an interaction between an external stimulus (light), and an internal rhythm of sensitivity to light, which is driven by a circadian clock. During this circadian rhythm of photoperiodic sensitivity, most of the night-phase is sensitive to light, whilst the day-phase is photoinsensitive, such that as the day gets longer in spring, the night-phase is steadily illuminated by light, thus triggering physiological or behavioural responses.

1.4 Integration of the melatonin signal: downstream molecular events and the regulation of seasonal physiology

1.4.1 Induction of photoperiod-associated genes in the PT

Whilst it is not yet fully understood how the melatonin signal is integrated in the PT of photoperiodic animals to affect seasonal physiology, recent studies utilising genomic microarray technology in both birds and mammals, have provided key evidence of photoperiod-regulated molecular events in the PT, suggesting potential transduction pathways downstream of melatonin action in PT-specific cells.

Studies in the highly seasonal Japanese quail, which displays robust changes in luteinizing hormone (LH) secretion, have identified key molecular events considered to occur early in the photoperiod-response pathway to coincide with the seasonal regulation of LH (Nakao *et al.*, 2008). Microarray analysis of gene expression revealed the *TSH beta subunit (TSHβ)* and the transcription factor-encoding *eyes absent 3 (Eya3)* genes, as being acutely induced in the PT during the first day of long photoperiod (LP) exposure. Similar microarray analysis of sheep PT tissue taken from animals that had been exposed to chronic (8-week) short photoperiods (SP) and then switched to long photoperiod (LP) exposure for 1 day, also revealed immediate activation of *Eya3* gene expression following the switch to LP exposure (Dupré *et al.*, 2010). Furthermore, LP-activated *TSHβ* expression was demonstrated in the PT of sheep (Hanon *et al.*, 2008)

and the European hamster (Hanon *et al*., 2010), two species displaying robust photoperiodic regulation of physiological functions, including pituitary hormone release and reproduction. Collectively, these results suggest that *TSHβ* and *Eya3* expression in the PT of these seasonally breeding species, may be involved in the conversion of the melatonin signal into a photoperiodic output regulating seasonal physiological responses (Hanon *et al*., 2008; Hanon *et al*., 2010; Dupré *et al*., 2010; Dupré, 2011) (Figure 1.2). Whilst the exact mechanisms underlying melatonin signal decoding in the PT-specific cells remain to be deciphered, signalling pathways downstream of the PT are thought to convey photoperiodic information to the brain to regulate the seasonal physiology of reproduction (Hazlerigg and Loudon, 2008).

1.4.2 Relaying photoperiodic information to the brain to control seasonal reproduction

Melatonin is considered to regulate seasonal changes of the reproductive axis by inhibiting the release of the gonadotrophin-releasing hormone (GnRH) from the hypothalamus, which consequently reduces the secretion of the reproductive hormone gonadotrophin, from the anterior pituitary (Malpaux *et al*., 1999). However, GnRH neurones do not co-localize with melatonin receptors (Bittman, 1993), suggesting an indirect action of melatonin on GnRH release.

Early studies investigating the site of action of the melatonin signal in relation to the seasonal secretion of gonadotrophin, suggested that melatonin was likely to act within and/or around the mediobasal hypothalamus (MBH) of the brain to exert its actions on the reproductive axis (Hastings *et al*., 1988; Lincoln and Maeda, 1992; Malpaux *et al*., 1993). Further investigations, aimed at pinpointing the exact site of melatonin action, utilised lesioning studies during which surgical incisions were made in key areas in and around the MBH region, including the paraventricular nucleus (PVN) and dorsomedial hypothalamus (DMH) (Badura and Goldman, 1992a; Maywood and Hastings 1995, Maywood *et al*., 1996). As a result, the melatonin-induced effects on the gonadotrophic response were blocked, suggesting a key involvement of the MBH in mediating the photoperiodic regulation of gonadotrophin.

However, this early hypothesis has recently come into question, with the hypothalamus no longer being considered the likely target site of melatonin action, and attention

turning to the melatonin-responsive PT-specific cells as the key mediators of the photoperiodic signal to the brain to regulate the reproductive axis (Hazlerigg and Loudon, 2008). The hypothalamus is considered to receive photoperiodic information through a retrograde signalling pathway originating from the PT, resulting in altered gene expression in the hypothalamic regions.

As previously mentioned (section 1.2.2), PT specific-cells display a thyrotrophic nature, expressing both the alpha (CGA) and beta (TSHβ) subunits of TSH in a photoperiod-regulated manner (Dardente *et al*., 2003). Furthermore, *TSHβ* expression in the PT appears to be one of the first molecular responses to altered photoperiod in seasonally-breeding animals, including the Japanese quail (Nakao *et al*., 2008), Soay sheep (Hanon *et al*., 2008) and European hamster (Hanon *et al*., 2010). These findings have implicated PT thyrotrophs with the release of a photoperiod-regulated TSH signal, considered to be associated with the photoperiod-regulated expression of *deiodinase enzyme type II* and *type III* genes (*Dio2* and *Dio3*) in the hypothalamic region (Nakao *et al*., 2008; Dardente *et al*., 2010). The Dio2 enzyme is responsible for converting the inactive prohormone thyroxin (T4) into the active 3,5,3' triiodothyronine (T3), a circulating form of the thyroid hormone (TH), whilst Dio3 is the primary inactivating enzyme, responsible for degrading T4 and T3 (Lechan and Fekete, 1995). The co-ordinated actions of the deiodinase enzymes control local thyroid hormone availability within the brain, which is linked to the seasonal regulation of reproduction (Barrett *et al*., 2007; Bechtold and Loudon, 2007).

As well as regulating seasonal responses of the reproductive axis via retrograde signalling to the brain, melatonin signal decoding in the PT is considered to be involved in a separate anterograde signalling pathway, regulating the seasonal release of prolactin from the lactotroph cells of the distal anterior pituitary (Hazlerigg and Loudon, 2008) (Figure 1.3).

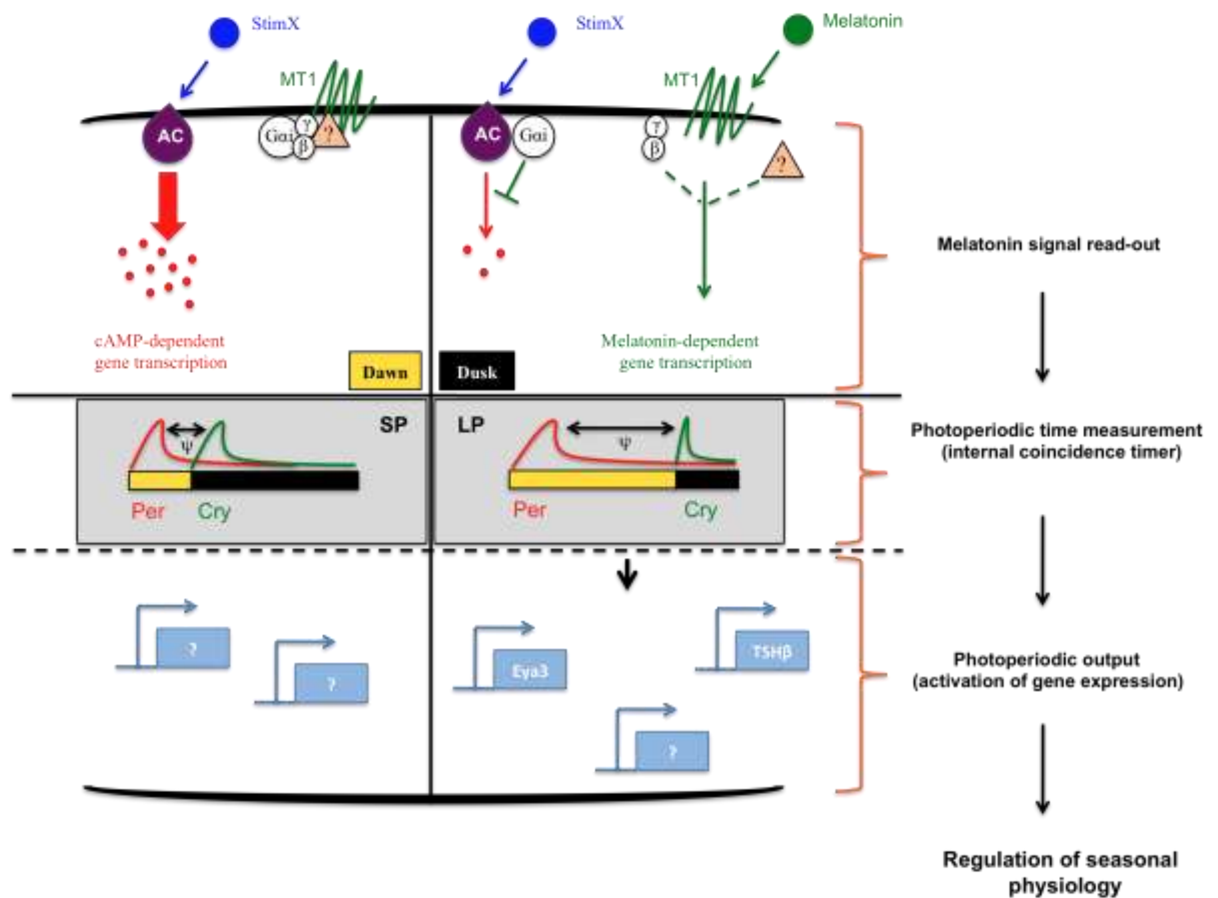


Figure 1.2: Integration of the photoperiodic melatonin signal in PT-specific cells.

Adenylyl cyclase (AC) is stimulated by an unknown factor (StimX), leading to increased intracellular cAMP and the induction of cAMP-dependent gene transcription at dawn. At the onset of dusk, melatonin binds to its receptor MT1, triggering the release of its coupled Gαi subunit and the inhibition of AC. Upon melatonin activation, unknown events are considered to occur, presumably via a cAMP-independent pathway, leading to the induction of melatonin-dependent gene transcription. The phasing of clock gene expression (*Per* being activated at dawn and *Cry* being activated at dusk) and the Per-Cry interval between peak expressions, termed ψ, provides a local indication of photoperiod (LP or SP). A short daily melatonin signal (long ψ) reflects LP whilst a long melatonin signal (short ψ) reflects SP. This is thought to trigger downstream events, including the expression of the *Eya3* and *TSHβ* genes in LP, and the activation of pathways regulating seasonal physiology. **AC**: adenylyl cyclase; **Cry**: cryptochrome; **Eya3**: Eyes absent 3; **LP**: long photoperiod; **MT1**: melatonin receptor 1; **Per**: period; **SP**: short photoperiod; **TSHβ**: thyrotrophin β; **ψ**: psi interval. Figure modified from Dupré (2011).

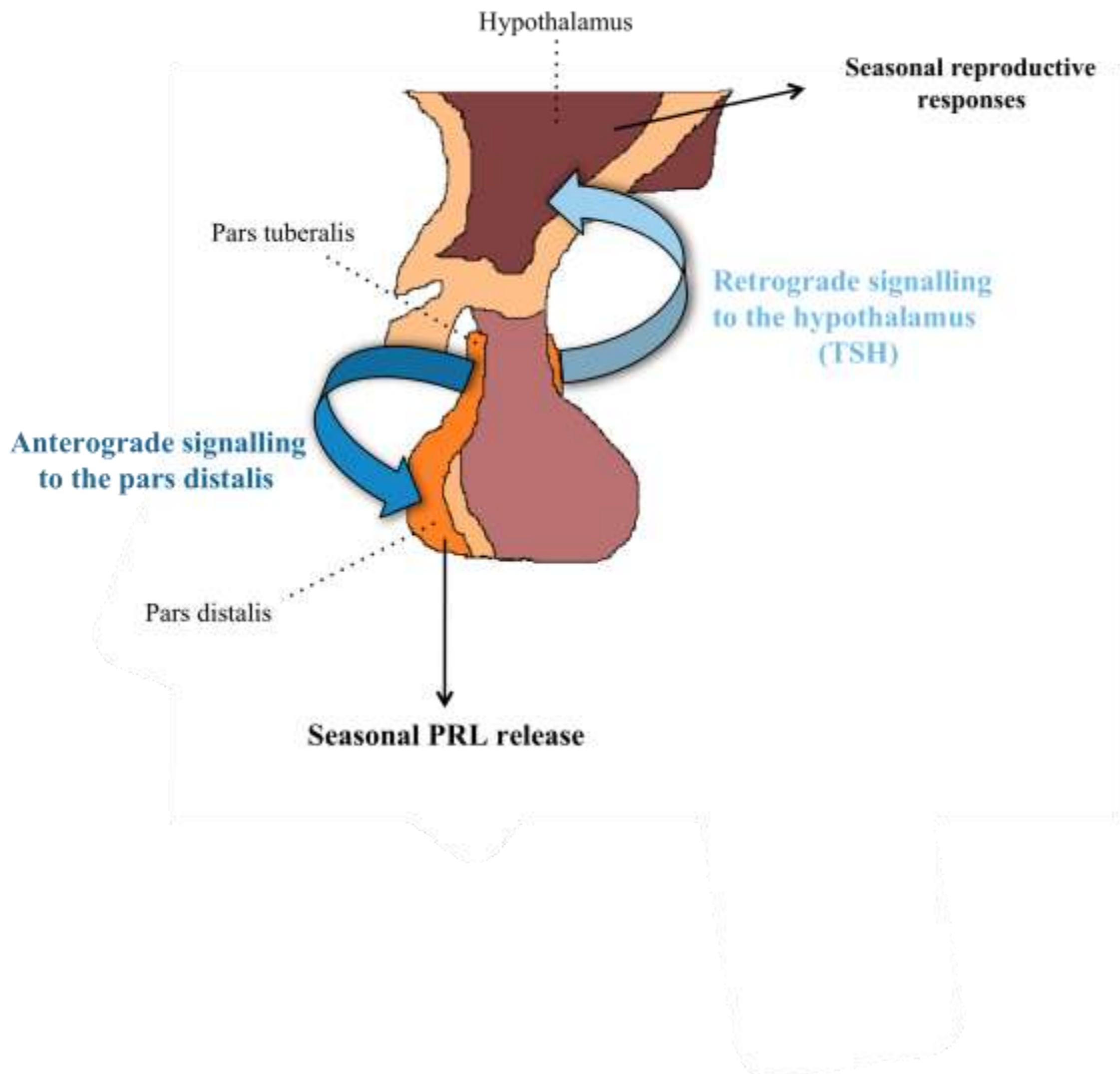


Figure 1.3: Bi-directional signalling from the PT to the hypothalamus and distal anterior pituitary.

The melatonin-responsive pars tuberalis is considered to convey photoperiodic information in a bi-directional manner to regulate seasonal physiology (Hazlerigg and Loudon, 2008). Retrograde signalling (TSH) to the hypothalamus controls seasonal reproductive responses, whilst anterograde signalling to the pars distalis regulates the seasonal release of the pituitary hormone prolactin. **PRL**: prolactin; **TSH**: thyrotrophin. Figure by the author.

1.5 The seasonal regulation of prolactin

1.5.1 Prolactin

Prolactin is a polypeptide hormone discovered more than 80 years ago, whose functionality is mainly related to lactation (Stricker and Grueter, 1928). Although originally isolated based on its stimulatory ability on mammary gland development and lactation, since then more than 300 functions or molecules have been shown to be activated through the prolactin receptor (PRL-R), unveiling a much wider spectrum of biological activity for this hormone (Bole-Feysot *et al*., 1998). Some of the functions attributed to prolactin involve the regulation of behaviour, bone homeostasis, male fertility, metabolism and the immune system (Freeman *et al*., 2000; Dorshkind and Horseman, 2001; Goffin *et al*., 2002; Foitzik *et al*., 2008). The gene encoding prolactin is found in all vertebrates; in humans it is located on chromosome 6 (Owerbach *et al*., 1981), whilst the prolactin gene family members in the rat have been localized to chromosome 17 (Cooke *et al*., 1986; Deb *et al*., 1991; Roby *et al*., 1993; Dai *et al*., 1996; Shah *et al*., 1998). The genes encoding prolactin, growth hormone, and placental lactogen have all evolved from a common ancestral gene by gene duplication (Niall *et al*., 1971), with divergence of the lineages occurring ~400 million years ago (Cooke *et al*., 1980; Cooke *et al*., 1981). The prolactin gene is 10kb in size, composed of 5 exons and 4 introns (Cooke *et al*., 1981; Truong *et al*., 1984) and regulated by two separate promoter regions. The human and rodent PRL loci appear significantly different, with the human PRL locus surrounded solely by non-coding DNA (Venter, 2003) whilst the rodent PRL genes are found as a large family of 26 closely related genes arising from gene duplication (Soares, 2004). Prolactin variants have been characterized in many mammals including humans, and can be the result of alternative splicing of the primary mRNA transcript, proteolytic cleavage and other post-translational modifications including phosphorylation, glycosylation and deamination (Freeman *et al*., 2000).

The secretion of prolactin was initially believed to be restricted to the lactotrophs of the anterior pituitary gland; however we now know that other organs and tissues are also capable of both synthesis and secretion of this hormone (Freeman *et al*., 2000). Some of these sites include the brain, placenta, amnion, decidua, uterus, mammary gland, milk, as well as cells of the immune system (Freeman *et al*., 2000). Light microscopy and staining allowed the original description to be made, of the cells in the pituitary responsible for prolactin release (Herlant, 1964). These cells were designated

lactotrophs or mammotrophs and comprise a significant percentage (20-50%) of the cellular component of the anterior pituitary (Freeman *et al*., 2000). The morphology and distribution of lactotrophs have best been described in the rat (Tougard and Tixier-Vidal, 1994) where they are found as a band adjacent to the intermediate lobe (Nakane, 1970).

Lactotroph cells appear to be heterogenous both functionally and morphologically (De Paul *et al*., 1997), displaying either an angular, polyhedral shape or at times rounded. Furthermore there appears to be functional heterogeneity with regard to their location within the anterior pituitary (Mukherjee *et al*., 1991) and also to their responsiveness to prolactin-releasing agents (Boockfor and Frawley, 1987). For example, the response to hypothalamic thyrotrophin-releasing hormone (TRH), a major secretagogue of prolactin, appears to be much greater in lactotrophs found in the outer region of the anterior pituitary compared to those found on the inner zone (Boockfor and Frawley, 1987), whilst dopamine-responsive lactotrophs are more commonly found in the inner zone (Arita *et al*., 1991). Dopamine, released by the hypothalamus, exerts a tonic inhibition of prolactin secretion (Lamberts and MacLeod, 1990).

1.5.2 Circadian patterns

In photoperiodic animals, light is an important regulator of prolactin secretion (Freeman *et al*., 2000). Studies in rats have shown that when the light schedule is shifted, there is a consequential shift in the proestrous surge of prolactin (Blake, 1976), the oestrogen-induced proestrous-like surge, as well as the mating-induced surges of prolactin (Pieper and Gala, 1979). Furthermore, under conditions of constant light, rats become acyclic (Hoffman and Cullin, 1975). Lesion studies later revealed the importance of an intact SCN in the generation of diurnal and nocturnal prolactin surges, suggesting that prolactin secretion is endogenously regulated by circadian rhythms, but is also influenced by the external light regimen (Bethea and Neill, 1980).

1.5.3 Seasonal patterns

Across all seasonal mammalian species studied so far, long photoperiods stimulate, and short photoperiods inhibit prolactin secretion, irrespective of whether animals are long or short-day breeders (Curlewis, 1992). High prolactin secretion during the summer in a wide variety of mammals is associated with the seasonal regulation of several

physiological characteristics including growth, food intake, energy metabolism, pelage quality, gonadal activity, pregnancy, lactation and/or delayed implantation, as well as the regulation of behavioural traits (Lincoln, 1989). In sheep, goats and Siberian hamsters, prolactin is primarily responsible for inducing the seasonal changes in coat condition (Badura and Goldman, 1992b; Curlewis, 1992) and has very little role in reproduction, whilst in species such as the skunk and mink, prolactin has a luteotrophic hormonal function that is required for implantation of the blastocyst (Curlewis, 1992). This clearly demonstrates how the physiological importance of the seasonal changes in prolactin varies amongst different species.

1.5.4 The melatonin-responsive PT: a key mediator of seasonal PRL release

Studies investigating the hypothalamus as a potential site of melatonin action for the photoperiodic regulation of prolactin, demonstrated that lesioning of the hypothalamic region did not influence the seasonal rhythms of prolactin secretion in Syrian hamsters (Maywood and Hastings, 1995). This suggested that the regulation of seasonal prolactin release was operating through a hypothalamus-independent intra-pituitary mechanism. These findings, coupled with the evidence of a high density of melatonin receptors in the PT of many species but not the PD (Morgan *et al*., 1994a), suggested that the PT of the pituitary might be the key melatonin target-tissue mediating the seasonal regulation of the lactotrophic axis. A direct action of melatonin on lactotroph cells was further discouraged following *in situ* hybridization studies in the ovine pituitary, which revealed no co-localization of melatonin receptor mRNA with prolactin mRNA, the former being expressed in the PT and the latter being primarily distributed in the PD region (Williams *et al*., 1997).

The strongest evidence however, suggesting the key involvement of the PT in seasonal prolactin release, came from studies using hypothalamo-pituitary disconnected (HPD) Soay rams, in which the pituitary was surgically disconnected from the hypothalamus (Lincoln and Clarke, 1994). When the animals were kept in alternating conditions of long (16 h light: 8 h dark) and short (8 h light: 16 h dark) photoperiod, the photoperiod-sensitive changes in prolactin release from the pituitary were unaffected. Furthermore, rams kept under long photoperiod and given constant-release implants of melatonin, showed a marked decrease in the concentration of serum prolactin (Lincoln and Clarke, 1994). It was concluded that photoperiodically-induced cycles in prolactin secretion via

melatonin, can occur via an intra-pituitary signalling pathway, independently of the hypothalamus.

There is now a general consensus that melatonin acting on the melatonin receptors of the PT induces the release of a factor from the PT cells, which then relays the effects to the lactotrophs in the PD, thereby promoting prolactin secretion (Figure 1.4). This factor, originally characterized as a low molecular weight peptide (<1 kDa), has been termed ‘tuberalin’ and there is currently substantial evidence to support its role as a PT-derived peptide secretagogue (Hazlerigg *et al*., 1996; Morgan *et al*., 1996; Graham *et al*., 2002). *In vitro* co-cultures of sheep PT and PD cells have demonstrated increased prolactin synthesis in response to such a PT-derived factor (Hazlerigg *et al*., 1996). The secretagogue hypothesis was further supported by the demonstration of tuberalin secretion from bovine (Lafarque *et al*., 1998) and hamster (Stirland *et al*., 2001) PT cells, indicating a conserved function amongst species.

Furthermore, there is convincing evidence to suggest that cAMP signalling pathways are important mediators in the regulation of this PT-derived factor; with *in vitro* studies showing that through its inhibitory action on forskolin and subsequently adenylyl cyclase activity, melatonin acts to decrease secretion of tuberalin-like compounds (Morgan *et al*., 1996; Stirland *et al*., 2001; Graham *et al*., 2002). Although prior photoperiodic exposure is important in determining ‘tuberalin’ secretion (Stirland *et al*., 2001; Johnston *et al*., 2003), the mechanisms by which melatonin signal duration regulates PT physiology are not yet known. As mentioned above (section 1.3), prolonged incubation of PT cell cultures with melatonin has been shown to sensitize subsequent stimulation of cAMP (Hazlerigg *et al*., 1993; von Gall *et al*., 2002), a finding which then fuelled the idea that melatonin signal duration was probably modulating the induction of cAMP by stimulatory factors such as pituitary adenylyl cyclase-activating polypeptide (PACAP) or adenosine (Barrett *et al*., 2002; von Gall *et al*., 2002). There is however a paradox, whereby the decreased *in vivo* ‘tuberalin’ secretion and amplitude of cAMP-responsive gene expression observed on short days is suggestive of a situation where long melatonin signals result in decreased cAMP concentration; something which is not in accordance with the increased sensitization of PT cells to the stimulatory effect of adenosine following a long duration of melatonin exposure, which is observed *in vitro* (Hazlerigg *et al*., 1993). This process remains to be fully characterized.

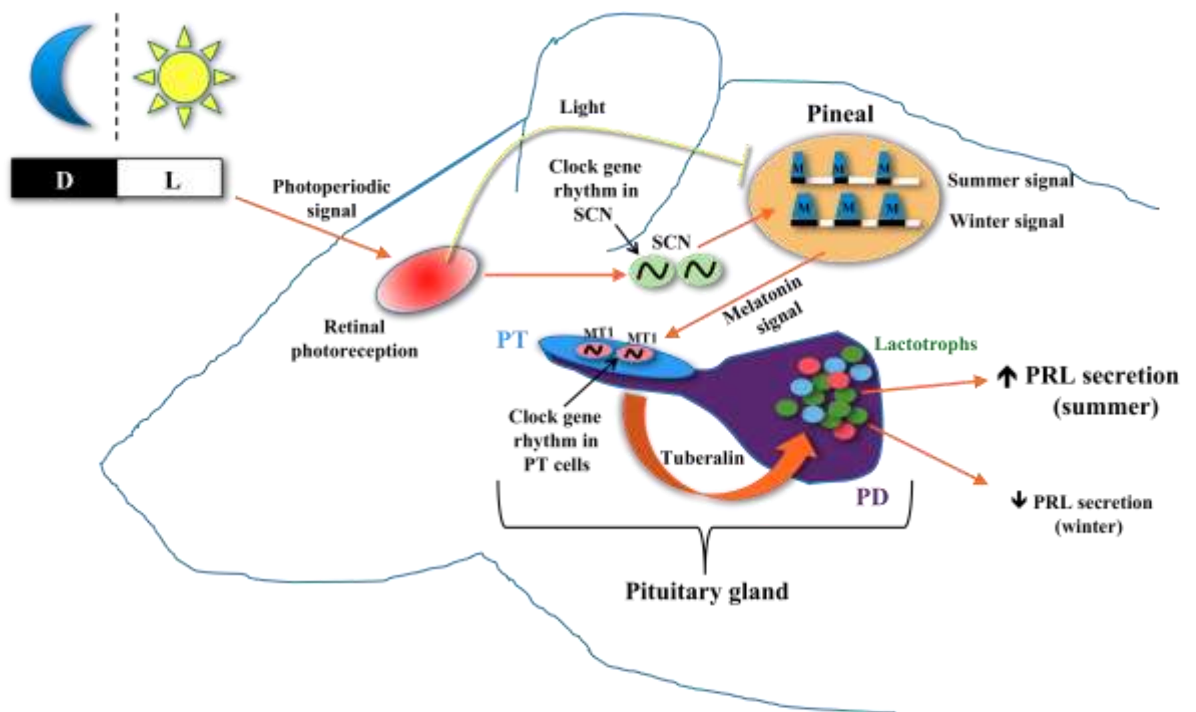


Figure 1.4: Speculative model whereby the photoperiodic signal is integrated to regulate the secretion of prolactin.

The photoperiodic signal perceived by the retina of the eye entrains the circadian clock genes of the hypothalamic SCN neurones, whose output, together with the direct inhibitory action of light (signalling through the eyes and acting on the pineal), control the rhythmic activity of the pineal gland and ultimately melatonin signal duration, which is short in summer and long in winter. Melatonin released by the pineal binds to MT1, a G-coupled receptor strongly expressed in the PT of the pituitary, and is thought to regulate the PT-expression of clock genes, whose phasing depends on melatonin signal duration, and may have an effect on downstream targets. Therefore, through an unknown as yet mechanism, the PT cells are thought to secrete a regulating signal (tuberalin), which acts on the PD lactotrophs to stimulate the seasonal release of the hormone prolactin, which is high in the summer and low in the winter. **D**: dark; **L**: light; **M**: melatonin; **MT1**: melatonin receptor 1; **PD**: pars distalis; **PRL**: prolactin; **PT**: pars tuberalis; **SCN**: suprachiasmatic nuclei. Figure by the author.

1.6 Neurokinins as candidate regulators of seasonal prolactin release

Recent studies have proposed that members of the neurokinin family may act as 'tuberalins'. Anatomical investigations by Skinner and colleagues have reported the expression of Substance P (SubP) and Neurokinin A (NKA), two members of the neurokinin family, in the ovine PT and suggested that these peptides could act as prolactin secretagogues (Skinner *et al.*, 2009; Skinner, 2009). The *in vitro* prolactin-regulating ability of neurokinins was demonstrated in ovine pituitary primary cultures, whilst the *TAC1* (tachykinin, precursor 1) gene, which encodes for the neurokinins SubP and NKA, was identified as being strongly activated within the ovine PT by long photoperiods (Dupré *et al.*, 2010). Collectively these results support the hypothesis that the neurokinin peptides are serving as 'tuberalins' in the seasonal regulation of prolactin.

Traditionally classified as neurotransmitters, neurokinins are a group of closely related bioactive peptides which are widely distributed within the mammalian peripheral and central nervous systems; and can be found in the pituitary gland and in areas of the brain controlling pituitary hormone secretion (Maggio, 1988). They are known to evoke a variety of biological responses ranging from neurone excitation, smooth muscle contraction and vasodilatation, to behavioural responses and actions on the hypothalamo-pituitary-gonadal axis as possible regulators of reproductive functions (Maggio, 1988; Debeljuk and Lasaga, 2006). The most important neurokinins known to exist in mammals are SubP, NKA, Neuropeptide K (NPK), Neuropeptide γ (NPG), and Neurokinin B (NKB) (Maggio, 1988). There are three types of neurokinin receptors: neurokinin receptor 1 (NK1R), neurokinin receptor 2 (NK2R), and neurokinin receptor 3 (NK3R), and whilst all neurokinins can bind all three receptors without there being a requirement for absolute specificity, there is a degree of preferential binding. SubP binds preferentially the NK1R, NKB prefers NK3R, whilst NKA, NPK and NPG preferentially bind NK2R (Nakanishi, 1991; Liu and Burcher, 2005).

Studies by Kato and colleagues (Kato *et al.*, 1976) were the first to report that SubP significantly induced prolactin release when injected into anaesthetised male rats. Extensive work was later carried out by different groups, which reinforced this observation (Rivier *et al.*, 1977; Chihara *et al.*, 1978; Vijayan and McCann, 1979; Eckstein *et al.*, 1980; Henriksen *et al.*, 1995). The evidence is therefore in support of

neurokinins and in these cases SubP, having a direct effect on the anterior pituitary gland to promote prolactin release. On the other hand, it has also been reported that under some circumstances and particularly when acting at the hypothalamic level, neurokinins may also be inhibiting prolactin release (Arisawa *et al*., 1990).

It is now widely accepted that prolactin secretion from the PD of the pituitary is both positively and negatively regulated; however, it is mainly controlled by inhibitory factors derived from the hypothalamus, the most important being dopamine (Lamberts and Macleod, 1990). Neurokinins have been shown to regulate a whole range of hypothalamic factors including dopamine, glutamate, oxytocin, vasopressin, serotonin, gamma amino butyric acid (GABA), vasoactive intestinal polypeptide (VIP) and thyrotrophin-releasing hormone (TRH), which in turn serve to control prolactin secretion (Debeljuk and Lasaga, 2006) (Figure 1.5).

Reports on the effects of NKA on prolactin secretion indicate a stimulatory outcome with several groups showing that injection of an antiserum to NKA, which can also cross react with NPK and NPG, results in a significant decrease in prolactin concentration in both rats and Syrian hamsters (Pisera *et al*., 1991). The effects of this endogenous blockade of NKA, NPK and NPG, therefore suggest a role in the induction of prolactin by these substances, with a likely involvement of neurokinins in the prolactin surge during proestrus (Pisera *et al*., 1991). Mau and colleagues (Mau *et al*., 1990), report that NKA induced receptor-mediated hydrolysis of phosphoinositides in the rat pituitary gland, which may be associated with the release of anterior pituitary hormones such as prolactin. Furthermore, intracerebroventricular injections of NKA in male rats showed a 48% increase in proopiomelanocortin (POMC) mRNA expression in the arcuate nucleus (Magoul and Tramu, 1997), and given the prolactin-stimulant effect of opioids (Leadem and Kalra, 1985), it is possible that activation of POMC neurones by NKA is a mechanism by which neurokinins increase prolactin release. There is sufficient evidence therefore to suggest a relationship between prolactin and neurokinins within the pituitary (Debeljuk and Lasaga, 2006). The potential seasonal nature of this relationship will be examined in this thesis.

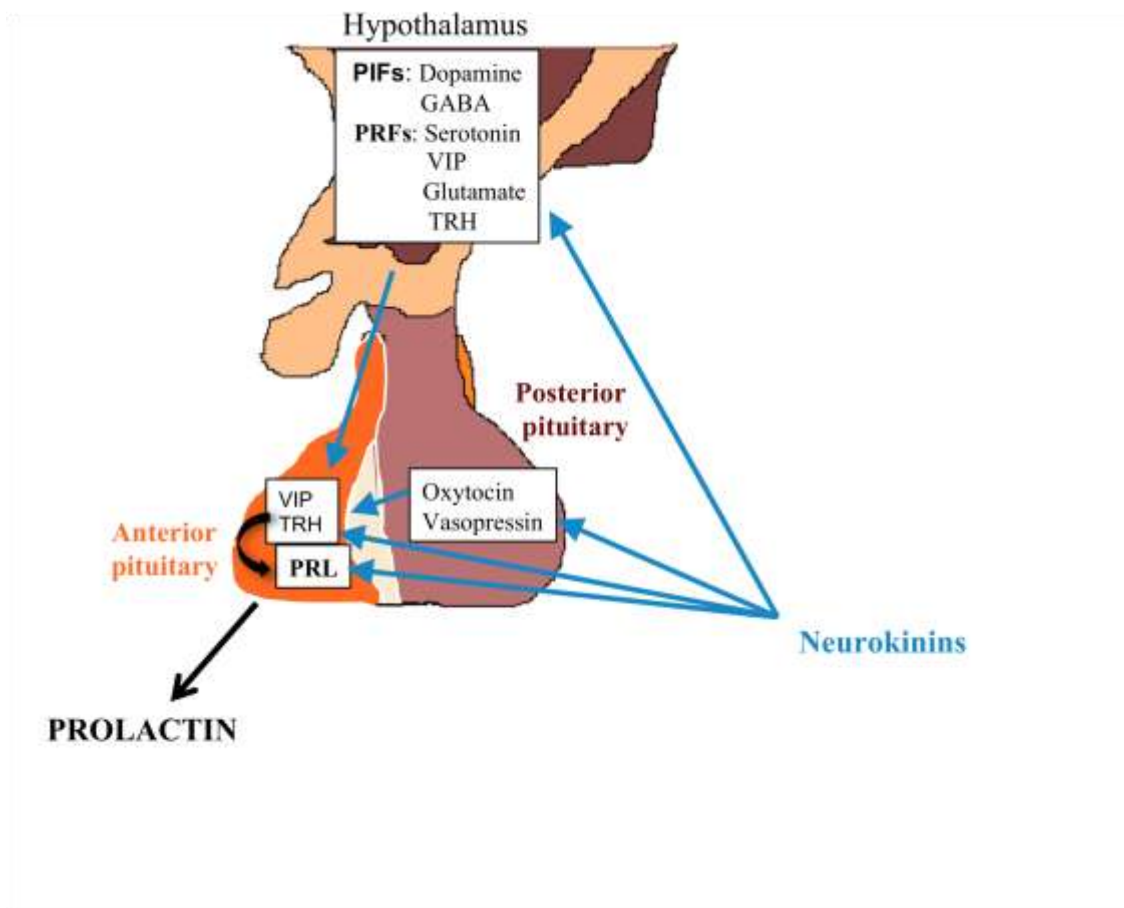


Figure 1.5: Direct and indirect actions of neurokinins on prolactin release.

Prolactin secretion from the anterior pituitary is regulated by several factors, including neuroendocrine signals from the hypothalamus (dopamine, GABA, VIP, serotonin, glutamate, TRH) and posterior pituitary (oxytocin, vasopressin), and paracrine factors (VIP, TRH) originating in the anterior pituitary itself. Neurokinins can act directly on the anterior pituitary to influence prolactin release or indirectly through regulating the release of hypothalamic prolactin-inhibiting factors (PIFs), prolactin-releasing factors (PRFs), and posterior pituitary factors. **GABA**: gamma amino butyric acid; **PIF**s: prolactin-inhibiting factors; **PRF**s: prolactin-releasing factors; **PRL**: prolactin; **TRH**: thyrotrophin-releasing hormone; **VIP**: vasoactive intestinal polypeptide. Figure by the author.

1.7 Photoperiodism in a laboratory animal model

1.7.1 The use of laboratory rats

It would be extremely valuable if one could develop a laboratory animal model for seasonal photoperiodism. The hamster displays robust seasonal changes but is limited in its use as the genome is poorly described and the small size of the pituitary gland precludes detailed *in vitro* studies. Rats on the other hand, are proving to be a very useful model system in the study of photo-responsiveness, with their physiology and neuroendocrinology becoming increasingly better known than that of other model species such as hamsters and field mice. Gene-sequence information on rats is rapidly accumulating and rat genetics have been studied across a wide spectrum of outbred and inbred strains, indicating that this type of species may prove to be extremely helpful in unmasking the mechanisms and genes involved in the phenomenon of photoperiodism. Further to this, transgenic rat models are currently available, an option not yet provided in the case of the seasonal hamster.

As previously mentioned, for species living in the wild, annual changes in photoperiod can have significant effects on several physiological parameters including reproduction, in order to maximise survival of the offspring. Variability in photo-responsiveness is clearly based on genetic differences, with several studies in wild species providing evidence that this trait is probably controlled by multiple loci (Heideman and Bronson, 1991; Wichman and Lynch, 1991). On the other hand, in laboratory animals such as rats, which have been bred for several generations under constant conditions of temperature, light and food supply to ensure maximal reproductive efficiency, the ability of reproductively responding to photoperiod appears to have been lost (Reiter, 1980). Consequently, not all species are photo-responsive, despite the elements of the neuroendocrine system regulating reproductive photo-responsiveness being highly conserved amongst eutherian mammals (Heideman and Bronson, 1990; Nelson, 1990; Bernard and Hall, 1995; Jackson and Bernard, 1999).

This is indeed true for the Wistar and Sprague-Dawley inbred rat strains, which are generally considered to be non-seasonal breeders (Reiter *et al*., 1968; Wallen and Turek, 1981; Nelson *et al*., 1994). However, some strains, including the outbred Harlan Sprague-Dawley (HSD) rats, have been shown to display reproductive photo-responsiveness but only when subjected to mild food restriction (Wallen *et al*., 1987). A

distinct example of rats exhibiting marked photo-responsiveness without manipulation are the Fischer 344 (F344) rats which display suppressed reproductive maturation, food intake and somatic growth under short photoperiods (Heideman and Sylvester, 1997; Heideman *et al*., 2000; Heideman *et al*., 2001; Shoemaker and Heideman, 2002; Ross *et al*., 2009). Interestingly, iodomelatonin (IMEL) binding studies in F344 and HSD rats demonstrated that differences in melatonin receptor location or binding are likely to be the cause of differences in responses to photoperiod observed between strains (Heideman *et al*., 2001). Whilst other inbred strains have also been shown to display photoperiodic responsiveness, this was only shown in young males (Francisco *et al*., 2004). On the other hand, F344 rats have indicated an extension of their photo-responsiveness into adulthood (Shoemaker and Heideman, 2002) suggesting that even amongst photo-responsive strains, genetic variation exists in the presence and strength of this trait. This view is further supported by the finding that photo-responsiveness varies between the different sub-strains of F344 rats, with the USA-sourced F344 sub-strain displaying a more robust response to photoperiod compared to the European-sourced sub-strain (Ross *et al*., 2009).

A considerable amount of research on the F344 rats has shown that exogenous melatonin treatment can mediate reproductive repression under long photoperiods, but also that continuously released melatonin is not interpreted as a short photoperiod and therefore the rats do not respond to it (Heideman *et al*., 2001). This highlights the importance of melatonin signal duration, in interpreting the external photoperiod. Furthermore, animals are said to acquire a photoperiodic history, which can have a significant effect on determining how they will respond to melatonin. Evidently, in Syrian hamsters and other photoperiodic, long day-breeding rodents, reproduction does not appear to be permanently suppressed when these animals are held continuously under short days; rather, after several months in these short-day conditions, they become 'photorefractory', acquiring an insensitivity towards the short photoperiod and restoring their reproductive activity (Karsch *et al*., 1984). Hence the inhibition on reproduction normally induced by short days, is abolished during the refractory phase.

1.7.2 Syrian hamsters as a model photoperiodic species

The Syrian hamster (*Mesocricetus auratus*) has been widely used in the study of seasonal biological rhythms as it undergoes a variety of physiological and behavioural

adaptations in response to alternating photoperiods, including changes in body weight, metabolism, pelage status, the reproductive system, aggressive behaviour and the regulation of pituitary prolactin (Bartness and Wade, 1984; Curlewis, 1992; Stirland *et al*., 2001; Jasnow *et al*., 2002; Johnston, 2002; Paul *et al*., 2007). Nonetheless, the use of Syrian hamsters in the study of the molecular events underlying photoperiodic responses has been limited due to the poorly described genome and lack of genetic tools. In the present study, the Syrian hamster, a 'truly' seasonal rodent, is used as a comparative model species, providing measurable markers of photoperiod-responsiveness in physiology.

1.8 The use of a transgenic F344 rat in the study of prolactin regulation

The development of double-reporter transgenic F344 rat lines for whole-body *in vivo* imaging has provided a key founding step in studying endocrine gene expression and also, most relevant to this project, the regulation of the prolactin hormone (Semprini *et al*., 2009). Bacterial artificial chromosome (BAC) recombineering was employed in order to generate a humanized double-transgenic F344 rat, which expresses two key reporter genes, luciferase (Luc) and destabilized enhanced green fluorescent protein (d2eGFP), both under the control of the entire human prolactin locus. The resultant rat allows imaging of the dynamic gene expression of the prolactin promoter *in vivo* and also through *ex vivo* analysis of tissues. This model is extremely useful for studying the physiological and pathological regulation of prolactin. Four lines of BAC PRL-Luc and two lines of BAC PRL-d2eGFP transgenics have been generated; however for the purposes of this study the BAC PRL luciferase line 49 (F344-Luc49) has been selected due to the high copy expression of the transgene in this line (transgene inserted on autosome). Characterisation of the reporter transgene has confirmed that it is expressed in the correct cell type (lactotroph cells) and responds to a range of PRL regulators, and therefore reflects the endogenous transcriptional activity of the PRL gene (Semprini *et al*., 2009; Harper *et al*., 2010). The activity of the reporter transgene is measured through the expression of the luciferase enzyme, which catalyzes the production of light (Figure 1.6).

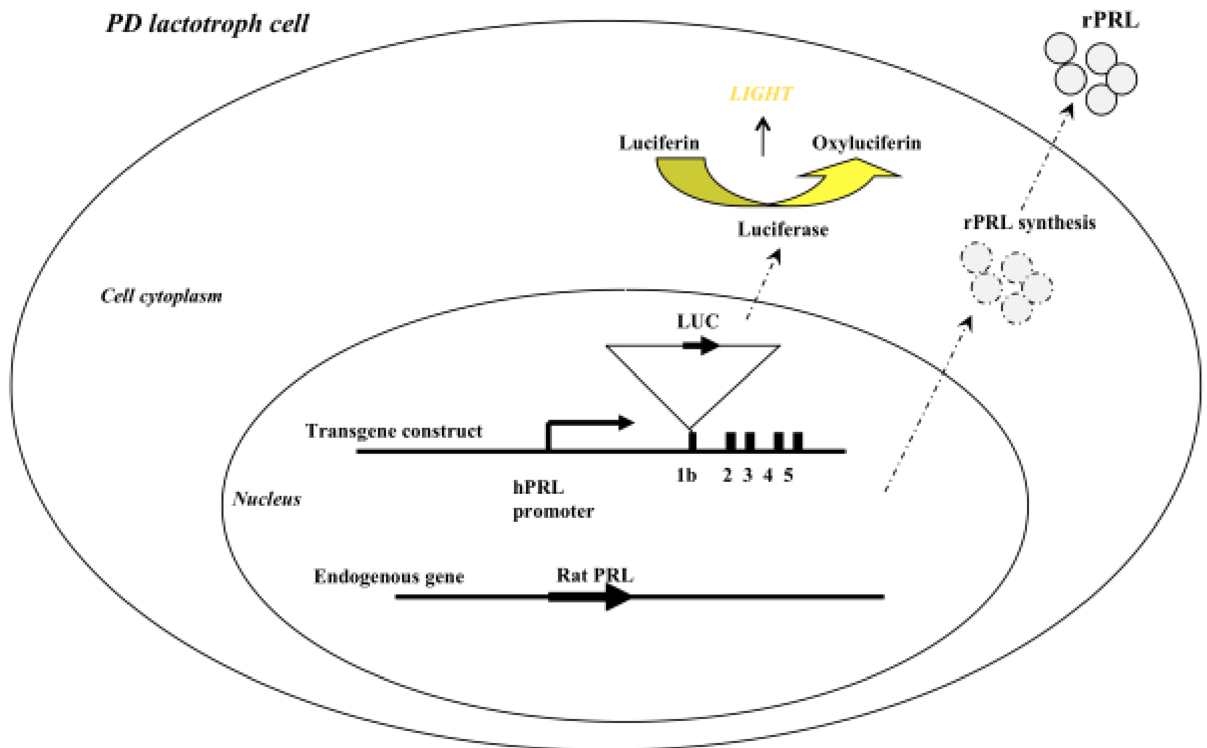


Figure 1.6: Diagram showing the expression of the reporter gene luciferase, under the control of the human prolactin promoter, in the transgenic rat lactotroph cell.

BAC recombineering was used to insert the luciferase (LUC) gene into the human prolactin (hPRL) exon 1b of the transgene construct, thus removing expression of human prolactin (Semprini *et al*., 2009). Luciferase is an enzyme, which catalyzes the oxidation of the luciferin substrate to oxyluciferin, a reaction which produces light, providing a useful marker of prolactin promoter activity. The endogenous rat prolactin (rPRL) gene is still present in the genome and is responsible for the synthesis of rat prolactin, which is ultimately released from the lactotroph. The reporter transgene is considered to faithfully reflect the activity of the endogenous gene. **hPRL**: human prolactin; **LUC**: luciferase; **rPRL**: rat prolactin. Figure by the author.

1.9 General hypothesis and aims of study

The current suggested model of seasonal prolactin regulation dictates that the melatonin-responsive pars tuberalis (PT) of the pituitary gland translates the photoperiodic input into a seasonal signal by releasing a so far uncharacterized factor (tuberalin), which is thought to act on the pars distalis (PD) region to control prolactin release. The most likely candidates considered to have a role in this circuit are Substance P (SubP) and Neurokinin A (NKA), members of a group of bioactive peptides known as neurokinins. The core aim of this thesis therefore, will be to address this hypothesis by (a) defining the photoperiod-regulated expression of the *TAC1* gene, the precursor to the neurokinins NKA and SubP, in the PT region of the photo-responsive F344 rat; (b) exploring the action of the neurokinin molecules on prolactin promoter activity and prolactin release in the same species and (c) revealing the neurokinin receptor expression profiles within the different cell-types of the rat pituitary PD region. An additional aim is to define the photoperiod-regulated PT expression of the *Eya3* gene, which is considered to be an early molecular trigger of photoperiodic responses in seasonal physiology.

The initial objective (see Chapter 3) of the work reported here was to test the photoperiodic responses of two strains of F344 rats by measuring body weight, food intake, testes weight and prolactin secretion, in response to different photoperiods, in order to select the most appropriate strain for use as a model species in the investigation of the seasonal regulation of prolactin. The secondary objective (see Chapter 4) was to define the photoperiod-regulated gene expression profiles of *TAC1* and *Eya3*, in the PT and brain of the chosen photo-responsive F344 strain, whilst also employing the seasonal Syrian hamster as a comparative model species. The third objective (see Chapter 5) was to test the action of the neurokinin molecules on prolactin promoter activity and prolactin release, with the help of a transgenic F344 rat, which expresses the reporter gene luciferase under the control of the prolactin promoter, providing a useful tool for studying prolactin regulation. The aim therefore was to establish a robust and convenient luciferase reporter system for prolactin gene expression using primary pituitary cell or tissue cultures, derived from the transgenic F344 rats. The final objective (see Chapter 6) was to reveal the neurokinin receptor expression profiles within the different cell types of the pituitary PD region, the proposed target site of tuberalin action.

CHAPTER 2: Materials and Methods

2.1 Experimental use of animals

Animal studies were undertaken under UK Home Office License, with relevant procedures licensed under the Animals (Scientific Procedures) Act, 1986. Rats (*Rattus Norvegicus*) and Syrian hamsters (*Mesocricetus Auratus*) were used in this study.

- Male wild-type Fischer 344 (F344) rats of the European F344 colony (F344/NCrHsd) were purchased from Harlan Laboratories UK, Ltd., whilst male and female wild-type F344 rats of the American colony (F344/NHsd) were purchased from Harlan Laboratories Inc., USA.
- Male and female transgenic F344-Luc49 rats (Semprini *et al*., 2009) were obtained from a breeding colony at the University of Manchester; previously established by Prof Julian Davis.
- Male and female transgenic F344 rats with an NHsd background (F344/NHsd -Luc49) were obtained by crossing transgenic male F344-Luc49 rats with wild-type F344/NHsd females over three generations.
- Male wild-type Golden Syrian hamsters (HsdHan®:AURA) were obtained from Harlan Laboratories UK Ltd.

2.1.1 Photoperiodic treatment of animals

2.1.1.a F344/NCrHsd and F344/NHsd rats (chronic experiments)

Chronic experiments were carried out using both F344/NCrHsd and F344/NHsd rats, in order to investigate photo-responsiveness in physiology and also to collect tissue for investigation of gene expression profiles. Male rats, aged 4 weeks (weaners) on arrival from the supplier, were acclimatised for 1 week under a 12:12h light/dark (LD) cycle and then separated into weight-matched groups. Rats within each group were housed in cages (Techniplast GR1800 rat IVC cages) of three. Tail vein blood samples were taken from all animals for serum preparation, at the start of the experiment, prior to placing them in the different photoperiods. One group of rats was then exposed to long day photoperiod (LP) (16:8h light/dark cycle) and the other to short day photoperiod (SP) (8:16h light/dark cycle) using fan-ventilated photoperiod-controlled chambers. Light intensity was 100-200 lux. Rats were housed in LP/SP for 5 weeks with all other environmental conditions kept constant; room temperature was 21ºC +/- 2 and room humidity 40-60%. Access to standard rodent chow (BeeKay Universal, UK) and Hydropac water pouches was provided *ad libitum*. Body weights and food intakes were measured and recorded weekly. Following 5 weeks in these photoperiods the rats were

anaesthetised using isofluorane inhalation and decapitated 4 h after lights on (ZT4). Trunk blood was collected into 1.5ml Eppendorf (Eppendorf UK Ltd) tubes, prior to centrifugation at 10 000 x *g* for 10 min for serum preparation. Brains, with the pituitary attached and the pars tuberalis (PT) region intact, were immediately removed under aseptic and RNase-free conditions, snap frozen on dry-ice (carefully wrapped in RNase-free foil; see section 2.3) and then serum and brains were stored at -80ºC. Testes and epididymal fat were dissected free from other tissues and weighed.

2.1.1.b F344/NHsd rats (acute 1LP induction experiment)

To investigate the acute LP induction of gene expression in the brain and PT, F344/NHsd male rats aged 4-5 weeks on arrival from the supplier were acclimatised for 1 week under a 12:12h LD cycle before being separated into groups of five. All animals were housed under SP for 6 weeks, after which five animals were culled at ZT3 and five at ZT11. The remaining animals were subjected to one day of LP exposure after which five animals were killed at each time point; ZT3, ZT11 and ZT19. Brains, with the pituitary attached and the PT region intact, were immediately removed under aseptic and RNase-free conditions and snap frozen on dry ice, before being stored at -80ºC.

2.1.1.c Syrian hamsters (acute 1LP induction experiment)

To investigate the acute LP induction of gene expression in the brain and PT of Syrian hamsters, HsdHan®:AURA tissue was kindly provided by Dr Sandrine Dupré and Dr Ben Saer. This tissue was collected from animals that were housed under SP for 6 weeks, following which a group of animals was culled at ZT4 (n=5) and at ZT12 (n=5). The remaining animals were subjected to one day of LP exposure after which they were killed at three different time points; ZT4 (n=5), ZT12 (n=5) and ZT20 (n=5). Brains, with the pituitary attached and the PT region intact, were immediately removed under aseptic and RNase-free conditions and snap frozen on dry ice, before being stored at -80ºC.

2.1.1.d Syrian hamsters (chronic experiment)

To investigate chronic SP and LP gene expression profiles in the brain and PT, male HsdHan®:AURA hamsters were ordered in (50-59g), acclimatised for 1 week under a 12:12h LD cycle and then set up under LP (16:8h LD) for 3 weeks until weighing

approximately 100gms. The hamsters were then separated into weight-matched groups and kept in either LP (16:8h LD) or SP (8:16h LD) for 6 weeks using fan-ventilated photoperiod-controlled chambers with all other environmental conditions constant; room temperature was 21ºC +/- 2 and room humidity 40-60%. Access to standard rodent chow (BeeKay Universal, UK) and Hydropac water pouches was provided *ad libitum*. Light intensity was 100-200 lux. Body weights and food intakes were measured and recorded weekly. Following 6 weeks in these photoperiods the hamsters were anaesthetised using isofluorane inhalation and decapitated at ZT4. Brains, with the pituitary attached and the PT region intact, were immediately removed under aseptic and RNase-free conditions, snap frozen on dry-ice and then stored at -80ºC. Testes and epididymal fat were dissected free from other tissues and weighed.

2.1.2 Genotyping of animals to identify luciferase transgene

2.1.2.a Isolation of genomic DNA for genotyping

Rat ear clippings were taken from the offspring following breeding of the transgenic rat line F344-Luc49 with wild-type F344 or wild-type F344/NHsd rats. A DNA extraction buffer containing 0.5% sodium dodecyl sulphate (SDS) (Sigma-Aldrich Co. Ltd, UK), 5mM ethylenediaminetetraacetic acid (EDTA; Applied Biosystems, Life Technologies Ltd, UK), 10mM Tris/HCl pH 8.0, and 300mM NaCl, was added to each of the ear clipping samples along with proteinase K (1:60) (Sigma-Aldrich Co. Ltd, UK), and incubated for at least 48h at 55ºC. After 5min incubation on a shaker at room temperature, NaCl solution (5M) was added to each of the samples, incubated for a further 5min and centrifuged for 10min at 16162 x *g*. Isopropanol was added to the collected supernatants, which were then incubated at -20 ºC for 1h. These were then centrifuged for 10 min at 16162 x *g*, separating out the DNA pellet which is washed with 70% ethanol and centrifuged for 10min at 16162 x *g*. The supernatant was removed and the DNA pellet resuspended in fresh sterile H_2O.

2.1.2.b Genotyping procedure

To genotype the F344 rats being bred at the University of Manchester Animal Facility, a polymerase chain reaction (PCR) was carried out using DNA isolated from the animals, in order to identify the rats heterozygous (+/-) for the luciferase transgene. BIOTAQ™ PCR kit (Bioline, London, UK) was used and the PCR reaction was set up using 5 µl of DNA, 2 µl of 10x NH_4 buffer, 0.3 µl of dNTPs (10mM), 1 µl of $MgCl_2$ (50mM), 0.4µl

of rat rennin (RR) forward primer 5′-CCTGGCAGATCACAATGAAAGG-3′, 0.4µl of rat rennin (RR) reverse primer 5′-GCATGATCAACTACAGGGAGG-3′, 0.4µl of luciferase forward primer 5′-TTGCCAAGAGGTTCCATCGT-3′, 0.4µl of luciferase reverse primer 5′-GTCCAAACTCATCAATGTATC-3′, 0.3 µl of BIOTAQ™ DNA polymerase (5u/µl; Bioline, London, UK) enzyme and 9.8 µl of nuclease-free water, making a total volume of 20 µl. The reaction mix was then placed in a PCR machine (DNA Engine Dyad, Peltier Thermal Cycler) with the following cycling conditions: 5 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 58°C and 30 sec at 72°C, followed by 5 min at 72°C and then held at 4°C. The final reaction product was analysed by running 10 µl with 1 µl of 5x DNA loading dye (Bioline, London, UK) on a 1% agarose gel containing SafeView (2 µl in 50 ml; NBS Biologicals Ltd, Cambridgeshire, UK) at 90V for 1h. DNA HyperLadder I (5µl; Bioline, London, UK) was used to check for the presence of the luciferase band in the PCR products. Once migrated for the appropriate time, the gel was viewed using a UV Transilluminator (UVItec, Cambridge, UK).

2.1.3 Synchronizing the estrous cycle of F344 female rats using LHRH

Female rats used for cell culture experiments and neurokinin treatments were treated with a 0.04mg intraperitoneal (ip) injection of luteinizing-hormone releasing-hormone (LHRH; Sigma-Aldrich Co. Ltd, UK) 4 days prior to the day of tissue collection, to synchronise their estrous cycle. Females were always culled on the day of proestrous.

2.2 Primary cell and tissue culture

Animals used for cell and tissue culture experiments were killed by exposure to carbon dioxide (CO_2) gas in a rising concentration and death was ensured by subsequent cervical dislocation. Following decapitation, the pituitary glands were dissected out from their surrounding structures under sterile conditions and washed in Hanks Buffered Salt Solution (HBSS; Sigma-Aldrich Co. Ltd, UK), containing 1% (v/v) gentamicin (Sigma-Aldrich Co. Ltd, UK) and 1% (v/v) penicillin/streptomycin (Sigma-Aldrich Co Ltd, UK), whilst kept on ice.

2.2.1 Real-time bioluminescent imaging of pituitary tissue slices in culture

Tissue from F344-Luc49 rats containing the luciferase transgene, a reporter gene for prolactin promoter transcriptional activity, was used in these experiments. Following dissection and washing, pituitaries were sliced using an Integraslice 7550M vibratome (Campden Instruments Ltd, Leicestershire, UK) to generate 400µm-thick coronal-orientated tissue slices (Figure 2.1). The tissue slices were placed in the Lumicycle system, which is equipped with photon-counting photomultiplier tubes (PMTs) allowing the measurement of bioluminescence, thus measuring transcriptional activity of the prolactin promoter. Lumicycle recordings were visualised using Lumicycle analysis software (Actimetrics).

2.2.1.a Treatment of tissue slices with forskolin

Tissue slices were cultured in the Lumicycle system in 2ml of culture medium containing Dulbecco's modified essential medium (DMEM; Invitrogen, Life Technologies Ltd, UK) at 87% (v/v), fetal bovine serum (FBS; Invitrogen, Life technologies Ltd, UK) at 10% (v/v), glutamax (Invitrogen, Life Technologies Ltd, UK) at 1% (v/v), sodium pyruvate (Sigma-Aldrich Co Ltd UK) at 1% (v/v), penicillin/streptomycin (Sigma-Aldrich Co Ltd UK) at 1% (v/v), and 1mM of luciferin substrate (Promega UK Ltd). The tissue was then stimulated with forskolin (5µM), to confirm the inducing-action of this compound on prolactin promoter transcriptional activity.

2.2.1.b Treatment of tissue slices cultured in two different types of media

In these experiments F344-Luc49 rat tissue slices were used to test the prolactin-inducing action of forskolin using two different types of FBS in the culture media. The medium was supplemented with either 10% (v/v) regular FBS or 10% (v/v) dextran charcoal-treated FBS (Dxt-FBS; Invitrogen, Life technologies Ltd, UK). The latter reduces the serum concentration of many endogenous compounds including steroid (oestradiol), peptide and thyroid hormones (Cao *et al*., 2009). The substrate luciferin (1mM) was also added to both media. The tissue slices were placed in the Lumicycle and left for 24h unstimulated, following which they were treated with either dimethyl sulfoxide (DMSO; Sigma-Aldrich Co. Ltd, UK) control (0.1%) or forskolin (5µM), in fresh 0.22µm-filtered (Millex-GP filters; Scientific Laboratory Supplies Ltd) culture

medium [DMEM 87% (v/v), the appropriate FBS at 10% (v/v), glutamax 1% (v/v), sodium pyruvate 1% (v/v), penicillin/streptomycin 1% (v/v)] and left for 72h. Following the incubation, the slices were placed in fresh unstimulated culture medium for a 24h washout period. Finally the tissue was re-activated with forskolin (5µM) in the corresponding fresh culture medium to confirm viability of the tissue.

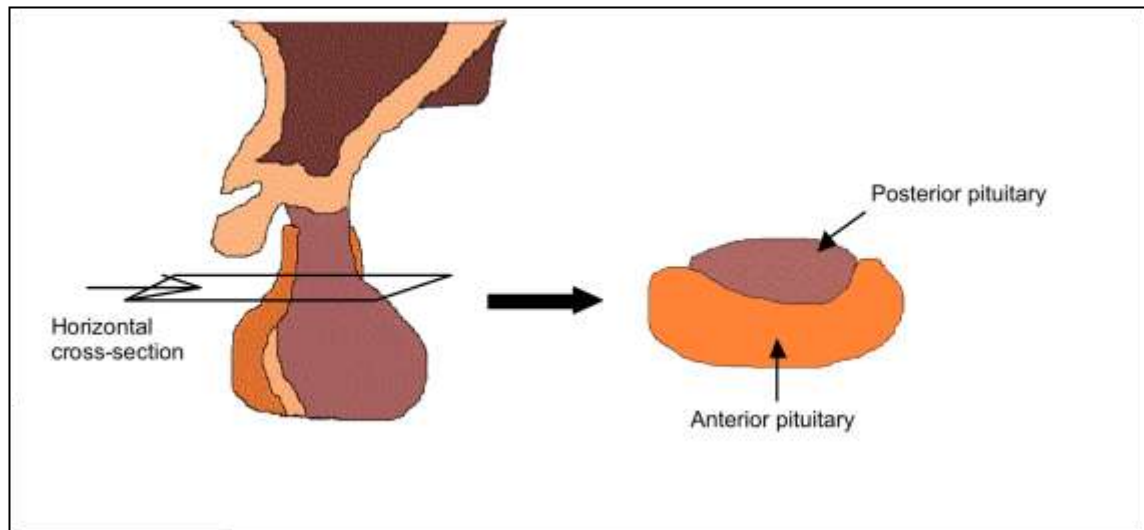


Figure 2.1: Generation of pituitary tissue slices for real-time bioluminescent imaging.

Schematic representation indicating the direction in which pituitaries, taken from transgenic rats, were sliced using a vibratome to generate 400µm-thick coronal-orientated tissue slices. Each slice incorporates both the anterior and posterior regions in a horizontal plane, as shown. Figure by the author.

2.2.2 Generating primary dispersed pituitary cell cultures

Following dissection and washing (described in section 2.2), the pituitaries were mechanically dissociated using sterile scalpels and placed in sterile bijous containing a magnetic stir bar (5 mm x 2 mm; Fisher Scientific UK Ltd) and 1ml of sterile dispersion buffer made up of 922.5µl HBSS (Sigma-Aldrich Co. Ltd, UK), 40µl of 2.5% trypsin (Sigma-Aldrich Co. Ltd, UK), 13µl of 250mg/ml bovine serum albumin (BSA; Sigma-Aldrich Co. Ltd, UK), 4.5µl of 10mg/ml DNAse 1 (Roche Diagnostics Ltd), 10µl penicillin/streptomycin (Sigma-Aldrich Co. Ltd, UK) and 10µl gentamicin (Sigma-Aldrich Co. Ltd, UK). The bijous were then placed in a 37ºC water-bath placed on a magnetic stirrer, for approximately 1.5 h, in order to fully disperse the cells, which were

then transferred into a 15-ml falcon tube, centrifuged at 250 x *g* for 6 min and then resuspended in 0.22µm-filtered (Millex-GP filters; Scientific Laboratory Supplies Ltd) DMEM (4.5g/l glucose), supplemented with sodium pyruvate 1%(v/v), penicillin/streptomycin 1%(v/v), glutamax 1%(v/v), gentamicin 1%(v/v), antibiotic-antimycotic 1%(v/v) and FBS 1%(v/v).

2.2.3 Real-time bioluminescent imaging of pituitary primary cell cultures

Following the methods described in section 2.2.2, resuspended cells were plated (1 x 10^6 cells/ dish) in cloning cylinders (6.4 mm diameter; Scienceware® Sigma-Aldrich Co. Ltd, UK) in 35-mm culture dishes (Iwaki, Scientific Laboratory Supplies Ltd, UK) (Figure 2.2A,B) and allowed 24 h to attach to the dish, whilst maintained at 37°C, in a humidified atmosphere with 5% CO_2, 95% air. The cloning cylinders were then removed and additional 10% (v/v) FBS culture medium added, including 1mM luciferin (Promega UK Ltd). The culture dishes were then sealed with 40mm sterile glass cover slips (VWR International Ltd, UK) and high vacuum grease (Dow Corning Europe SA, Belgium), and incubated under the PMTs for 72h, during which luminescent counts were recorded. Cells were then activated with either forskolin (5µM) or DMSO (0.1%) control, without changing the medium, and left for up to 48 h until the signal had stabilised. PMT recordings were collected, exported as an excel file and converted into luminescent counts per second.

2.2.4 Luciferase luminometer assay of pituitary primary cell cultures

2.2.4.a Seeding of enzymatically-dispersed pituitary cells

Following the methods described in section 2.2.2, resuspended cells were seeded onto 96-well tissue culture plates (2.5 x10^5 cells, 0.1ml/well) (Figure 2.2C,D) and cultured for 48 h in a humidified atmosphere of 5% CO_2- 95% air at 37ºC. The culture media was then replaced, 24h before treatment of the cells, with fresh DMEM 4.5g/l glucose containing the same supplements as above (section 2.2.2), except for the FBS, which was replaced with Dxt-FBS.

2.2.4.b Treatment of dispersed pituitary cell cultures with neurokinins

After a total of 72h in culture, the cells were treated by adding 11µl of the appropriate treatment, into each well of the cell culture plate. Treatment with DMSO, dissolved in media to a final concentration of 0.1% was used as a negative control and forskolin (1µM) in DMSO 0.1% was used as a positive control. Cells were also treated with 10^{-7} M NKA (Bachem, UK, Ltd) in DMSO 0.01%, 10^{-6} M NKA in DMSO 0.01%, 10^{-7} M Substance P (Bachem, UK, Ltd) in DMSO 0.01% and 10^{-6} M Substance P in DMSO 0.01%, 10^{-7} M Substance P (1-7) (Bachem, UK, Ltd) in DMSO 0.01% and 10^{-6} M Substance P (1-7) in DMSO 0.01% with each treatment being added to 100 µl of culture medium and left for either 6 h or 11.5 h.

2.2.4.c Measuring luciferase activity using a luminometer assay

Following treatment of the cells, the culture medium was aspirated, from each well of the 96-well culture plate, and stored at -80°C, to be assayed later for hormone concentrations. The cells were washed with 100 µl of phosphate buffered saline (PBS; Sigma-Aldrich Co. Ltd, UK) which was then removed, before adding 25 µl of 1x Passive Lysis Buffer (PLB; Luciferase® Reporter Assay System; Promega UK, Ltd) to each well. The plate was kept on a mini orbital shaker (SSM1, Stuart ®) at room temperature for 30 min at 90 revolutions per minute (rpm). Once the cells were lysed, 10 µl of the lysate was transferred to luminometer plates (LIA-plate, Greiner Bio-One) and mixed with 10 µl of 1x PLB, before measuring the luminescence of each well by injecting luciferin substrate into each well using an Orion L Microplate Luminometer (Berthold Detection Systems) which runs on Simplicity 4.2 software. The data was exported as an excel file and normalised to the mean DMSO control value for each experiment in order to reduce inter-assay variability.

2.3 Preparing ribonuclease-free solutions and materials

All solutions and materials used during the *in situ* hybridization (ISH) procedure (section 2.5) and all other RNA work, were RNase-free to ensure minimal RNA degradation. Laboratory solutions were either bought-in RNase- and Deoxyribonuclease- (DNase) free, or treated with 0.1% (v/v) diethyl pyrocarbonate

(DEPC; Sigma-Aldrich Co. Ltd, UK) to destroy RNases. DEPC-treated solutions were thoroughly mixed and left overnight at 37ºC, after which they were autoclaved to remove residual DEPC and then stored at room temperature. Glassware (wrapped in aluminium foil) and foil used for tissue collection were baked at 180ºC for 8 h. Bench surfaces and equipment were treated with RNase Zap® (Applied Biosystems, Warrington, UK).

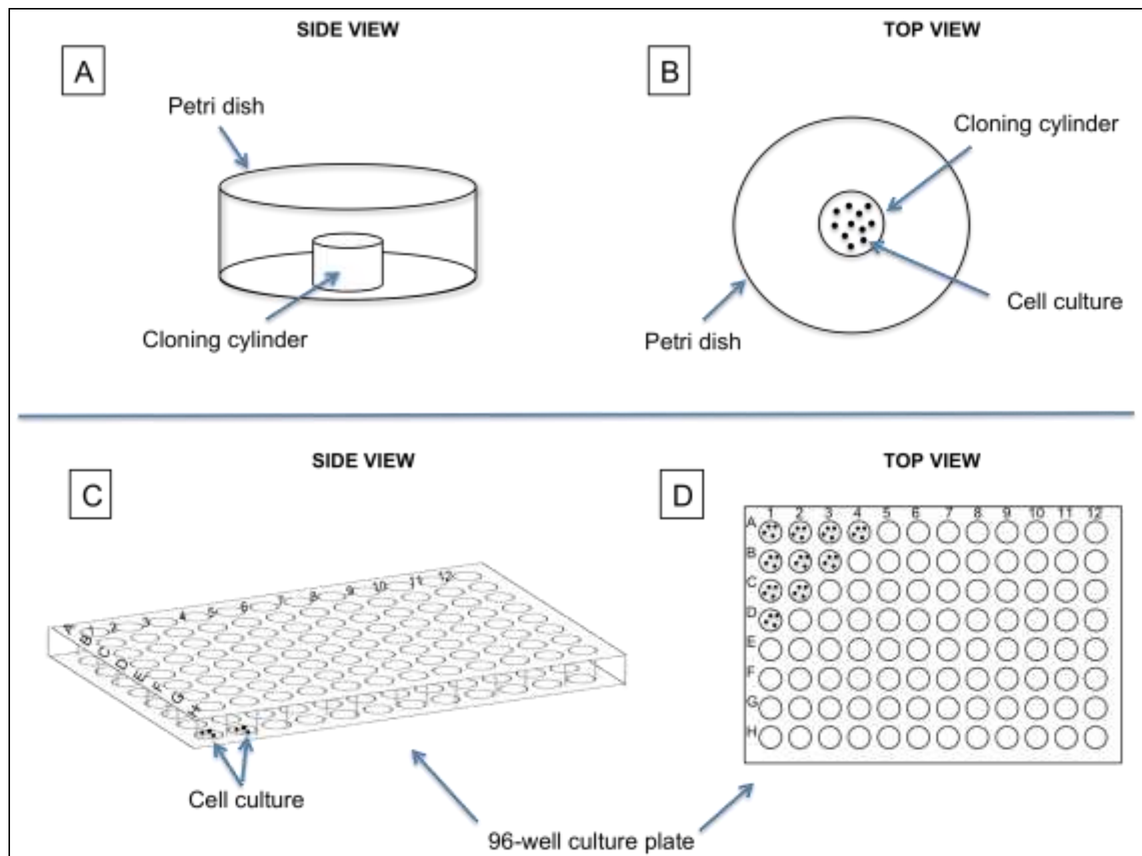


Figure 2.2: Setting up pituitary primary cell cultures.

Primary cell cultures were set up using either (A,B) 35-mm culture dishes containing cloning cylinders or (C,D) 96-well cell culture plates. Figure by the author.

2.4 Cloning of riboprobes

Rn_Eya3, Rn_TAC1, Ma_Eya3 and Ma_TAC1 riboprobes were cloned for use in the *in situ* hybridization (ISH) experiments described in section 2.5 of this chapter.

2.4.1 RNA extraction

2.4.1.a Tissue homogenisation

Rat and hamster tissue samples were homogenised by adding 1ml of TRIzol® reagent (Invitrogen Ltd, UK) to lysing matrix D tubes (MP Biomedicals) followed by 100mg-blocks of frozen tissue. This procedure was carried out under the fume hood, whilst maintaining RNase-free conditions. The samples were then placed into a FastPrep-24 system (MP Biochemicals, Cambridge, UK), which homogenized the tissue at 6m/s (meters per second) for 4x30 sec, whilst utilizing a 'cryohead' to maintain a temperature of 4ºC.

2.4.1.b RNA extraction and purification

Homogenised samples were incubated at room temperature for 5 min before adding 200 μl of chloroform (Sigma-Aldrich Co. Ltd, UK) under a fume hood. The tubes were vigorously agitated, then incubated at room temperature for 3 min and centrifuged at 12000 x *g* for 15 min at 4°C. The resultant solution separated into 3 different phases, with total RNA residing in the upper aqueous phase. The 500μl RNA-containing phase was then transferred to a microcentrifuge tube with 500 μl of isopropyl alcohol (Fisher Scientific Ltd) and incubated for 10 min (4°C) to precipitate the RNA. Once precipitated, the samples were centrifuged at 12000 x *g* for 10 min (4°C). The isopropyl alcohol was removed and the pellet was washed with 1 ml of 75% ethanol (prepared using nuclease-free water) before being centrifuged at 12000 x *g* for 5 min (4°C). The ethanol was then removed and the RNA pellet left to air-dry before being resuspended in 100 μl of nuclease-free water. RNA purification was carried out using the RNeasy Mini Kit (Qiagen Ltd) according to the manufacturer's protocol. Prior to using this kit, 10 μl of β-mercaptoethanol (Sigma-Aldrich Co. Ltd, UK) was added per 1ml of kit RLT buffer. To each 100 μl sample, 350 μl of RLT buffer was added, followed by 250 μl of 100 % ethanol. Once thoroughly mixed, the sample was applied to a column and centrifuged at 10000 x *g* for 15 sec, after which the column was washed with 500 μl of kit RPE buffer and centrifuged at 1000 x *g* for 15 sec. The flow-through was discarded; the column was again washed with 500 μl of RPE buffer and then centrifuged at 10000 x *g* for 2 min. The column was transferred to a new microcentrifuge tube and the RNA was eluted using 50 μl of nuclease-free water and centrifugation at 10000 x *g* for 1 min.

The purified RNA samples were quantified using a NanoDrop 1000 spectrophotometer (ThermoScientific) and stored at -80°C.

2.4.2 Reverse-transcription (RNA to cDNA conversion)

To prepare the rat and hamster cDNA, 2 µg of RNA was added with 1 µl of RQ1 DNase (Promega Ltd) in a PCR tube (Life Technologies Ltd, Paisley, UK) and made up to a final volume of 9.6 µl using nuclease-free water (Fisher Scientific UK, Ltd). The reaction was incubated at 37°C for 30 min, after which 1 µl of RQ1 DNase Stop solution (Promega Ltd) was added for a 10-min incubation at 65°C and 1 min on ice. Next, 1 µl of Oligo(dT)$_{12-18}$ (10 µM; Invitrogen, Life Technologies Ltd) and 0.4 µl of dNTP mix (25 mM; Invitrogen, Life Technologies Ltd) were added and the tube incubated at 70°C for 10 min. Then 4 µl of 5X first-strand buffer (Invitrogen, Life Technologies Ltd), 2 µl of DTT (0.1 M; Invitrogen, Life Technologies Ltd) and 1 µl of SuperScript® II Reverse Transcriptase (Invitrogen, Life Technologies Ltd, UK) enzyme were added to the reaction tube, which was incubated at 42°C for 1 h and then at 70°C for 15 min. The resulting cDNA was stored at -20 °C.

2.4.3 Primer design

The mRNA sequences of the rat (Rn_TAC1 and Rn_Eya3) and hamster (Ma_TAC1) target genes, were retrieved from the GeneBank database (accession numbers: NM001107910, NM012666 and X80662 respectively). The sequences were used to design two PCR primers for each 350-450 bp fragment to be amplified from either rat or hamster pituitary cDNA (obtained by RNA extraction and conversion to cDNA-described above), using the following criteria: primer length between 20-22bp, GC content close to 50%, melting temperature (T_m) above 50ºC, with a maximum 1ºC difference between the T_m of each primer. Vector NTI software (Invitrogen Life Technologies Ltd, UK) was used to calculate the $T_{m.}$ The primers are shown in table 2.1. The rat primers designed for amplifying Rn_Eya3 were also used to amplify the hamster Ma_Eya3 fragment.

2.4.4 'Insert' (cDNA) amplification using RT-PCR

The rat Eya3 (Rn_Eya3; 441 bp) and TAC1 (Rn_TAC1; 358 bp) and the hamster Eya3 (Ma_Eya3 441 bp) and TAC1 (Ma_TAC1; 353 bp) fragments to be cloned were amplified by PCR using the FastStart High Fidelity PCR System dNTPack (Roche Diagnostics Ltd). Each reaction was set up using 2 µl of cDNA, 2 µl of the corresponding forward primer (Table 2.1), 2 µl of the corresponding reverse primer (Table 2.1), 5 µl of FastStart High Fidelity Reaction 10x buffer containing $MgCl_2$, 1 µl of dNTP mix (10mM), 0.5 µl of Fast Start High Fidelity Enzyme Blend (5U/µl), 1µl of DMSO; and made up to a volume of 50 µl using nuclease-free water. The reaction mix was then placed in a PCR machine (DNA Engine Dyad, Peltier Thermal Cycler) with the following cycling conditions: 10 min at 95°C; 35 cycles of 30 sec at 95°C, 30 sec at the appropriate Ta °C depending on the primers used (Table 2.1) and 30 sec at 68°C; followed by 5 min at 68°C. The final reaction product was analysed by running the total 100 µl with 10 µl of 5x DNA loading dye (Bioline, London, UK) on a 1% agarose gel containing SafeView (2 µl in 50 ml; NBS Biologicals Ltd, Cambridgeshire, UK) at 50V for 20 min. DNA HyperLadder IV (5µl; Bioline, London, UK) was used to check the band size of the PCR product. Once migrated for the appropriate time, the gel was viewed using a UV Transilluminator (UVItec, Cambridge, UK) and the DNA band was excised and then purified using a QIAquick gel extraction kit (Qiagen Ltd, West Sussex, UK). Briefly, 3 volumes of Buffer QG were added to 1 volume of excised gel, which was then incubated at 50°C for 10 min. Next, 1 'gel volume' of isopropanol (Fisher Scientific Ltd) was added to the mix and vortexed. Once dissolved, this was applied to a QIAquick spin column and centrifuged for 1 min at 13000 x *g*. The flow-through was discarded and 0.5ml of Buffer QG added to the DNA in the column, which was centrifuged for 1 min at 13000 x *g*. The DNA was then washed by adding 0.75 ml of Buffer PE and centrifuging for 1 min at 13000 x *g*. Finally, the column was transferred to a new microcentrifuge tube and the DNA was eluted with the addition of 50 µl of Buffer EB and centrifuging for 1 min at 13000 x *g*.

2.4.5 Ligation and transformation

The amplified fragment or insert (4 µl) was ligated into the pGEM®-T easy vector (1 µl; Promega Ltd) with 2 µl of 10x ligation buffer for T4 DNA ligase (Roche Diagnostics

Ltd), 0.5 μl of T4 DNA Ligase (Roche Diagnostics Ltd) and made up to a final volume of 20 μl using nuclease-free water (Applied Biosystems). The reaction was left to incubate overnight at 16°C. The ligation product was transformed into DH5α *E.coli* competent cells (Invitrogen Ltd) in order to select for positive plasmidic constructs (i.e. containing the amplified fragment). This was done by incubating 2 μl of the ligation mix with 25 μl of DH5α bacterial cells for 30 min on ice. The cells were then heat shocked for 45 sec at 42°C and incubated for a further 5 min on ice. The bacteria were then carefully resuspended in 250 μl of SOC (Super Optimal Broth) medium (Invitrogen Ltd), incubated for 30 min at 37°C and then spread on a pre-warmed lysogeny broth (LB) agar plate supplemented with 100 μg/ml of Ampicillin (Sigma-Aldrich Co. Ltd, UK). Following an overnight incubation upside down at 37°C in a humidified atmosphere, the plate was removed from the incubator and placed at 4°C to stop further bacterial growth.

2.4.6 Plasmid preparation

Several individual colonies were then selected from the incubated plate and grown separately in 2 ml of LB medium supplemented with 100 μg/ml of Ampicillin (Sigma-Aldrich Co. Ltd, UK). These were incubated at 37°C for 16 h whilst being shaken at 300 revolutions per minute (rpm). Aliquots were taken from each bacterial colony expansion and plasmid DNA was extracted from the bacteria using a Qiagen Midiprep kit (Qiagen Ltd) according to the manufacturer's protocol. In brief, a 1.5 ml aliquot of each bacterial culture was centrifuged at 3000 x *g* (15 min), the supernatant was aspirated and each pellet resuspended in 4ml of buffer P1 (4°C). Buffer P2 (4ml) was then added to lyse the bacteria and allow plasmid release. Buffer P3 (4ml) was added to precipitate the cellular debris before centrifugation at 20000 x *g* (30 min at 4°C). The supernatants containing the plasmid DNA were carefully removed and centrifuged again at 20000 x *g* for 15 min at 4°C before being transferred into a Qiagen-tip 100 column, which had been pre-equilibrated by allowing 4ml of buffer QBT to run through the column by gravity flow. Once the supernatant had entered the resin of the column, the Qiagen-tip was washed with 2 x 10ml of buffer QC. The DNA was eluted using 5 ml of buffer QF.

2.4.7 Sequencing

Plasmids extracted from each clone were then sequenced in order to determine which contained the insert of interest. First, the plasmids were PCR-amplified using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies Ltd, UK) by adding 4 µl of BigDye polymerase enzyme, 1 µl of 10 x reaction buffer and 1 µl of pUC M13 sequencing primer (10 µM; Eurofin MWG Operon, Ebersberg, Germany) to 350 ng of plasmid which was made up to 10 µl with nuclease-free water. The reaction mix was placed in a thermal cycler for 4 min at 96°C and then 35 cycles of: 30 sec at 98°C, 15 sec at 50°C and 4 min at 60°C. The amplification product was then precipitated by adding 68 µl of nuclease-free water, 11 µl of sodium acetate pH 5.2 3M (Sigma-Aldrich Co. Ltd, UK), 2 µl of EDTA 125 mM (Applied Biosystems, Life Technologies Ltd, UK), 1 µl of glycogen 20 mg/ml (Invitrogen, Life Technologies Ltd, UK) and 300 µl of 100% ethanol (Fisher Scientific Ltd, UK) and the resulting mix was centrifuged at 13000 x *g* for 30 min (4°C). The supernatant was discarded and the pellet washed with 100 µl of 70% ethanol and spun for a further 5 min. The supernatant was again removed and the pellet left to air-dry for 15 min. Samples were then sent to the DNA Sequencing Facility (University of Manchester) and the results were analyzed using the NCBI Blast database software. One bacterial clone containing the insert of interest was chosen and grown overnight in 200 ml of LB medium, supplemented with 100 µg/ml of Ampicillin. The plasmidic DNA was then recovered using a Qiagen Maxiprep kit (Qiagen Ltd), following the manufacturer’s protocol. Nucleotide concentration (ng/µl) and purity was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Ltd) and the plasmid was stored at -20°C. Glycerol stocks of the bacterial clones of interest were also prepared by using a 1:1 mix of bacterial culture and sterile 40% glycerol, and kept for storage at -80°C.

Table 2.1: Primers used for RT-PCR insert (cDNA) amplification

Primer	Sequence	Length	GC %	Tm °C
Rn_Eya3_F	CTCAAACCAGGACTATCCCACC	22	54.5	54
Rn_Eya3_R	CCTGAGCCAATTACGACTGTTG	22	50	54
Rn_TAC1_F	AATGCAGAACTACGAAAGAAGGC	23	43.5	53.8
Rn_TAC1_R	TCTCTGCCTCCAGCAGCAG	19	63.2	53.8
Ma_TAC1_F	GCAATGCAGAACTATGAAAGAAGACG	26	42.3	57.5
Ma_TAC1_R	TCATGGCAGCAGCTGTCTGAA	21	52.4	56.7

2.5 *In situ* hybridization (ISH)

In situ hybridization was used to visualise and quantify mRNA expression of *TAC1* and *Eya3* genes in the brain and PT of the F344/NHsd rat and Syrian hamster.

2.5.1 Processing of tissue for ISH

2.5.1.a Cryosectioning

Snap frozen brains were sectioned using a Leica CM3050 S cryostat (Leica Microsystems Ltd, Milton Keynes, UK). The cryostat chamber was cleaned with 70% ethanol and set at a temperature of -20°C, whilst equipment such as the blade, brushes and chuck were RNase-treated and placed inside the chamber to equilibrate to the set temperature. Brains were mounted using OCT compound medium (Leica Microsystems Ltd) and left to attain a temperature of -18°C. Rostal to caudal coronal-orientated sections of the brain attached to the PT, were taken from each animal at a thickness of 12µm and placed on polysine slides (VWR International Ltd, Lutterworth, UK). The sections were organised into sets of slides that covered the whole PT region. The slides were left to dry for 5min before being frozen at -80°C.

2.5.1.b Preparation of cryosections for ISH

Prior to the use of the cryosections for ISH the mounted slides were fixed by immersion in 4% paraformaldehyde (PFA) solution for 15 min to preserve tissue morphology and

mRNA. The PFA solution was prepared under a fume hood by adding 12g of PFA powder (Sigma-Aldrich Co. Ltd, UK) in 300 ml of 0.1M PBS, which was then dissolved using a magnetic stirrer at 55ºC. The solution was clarified by adding 3 to 5 drops of 5M sodium hydroxide pH 5.2 (NaOH; Merck Chemicals Ltd, Nottingham, UK), cooled on ice and filtered through 0.2µm Whatman filter paper (Whatman International, Kent, UK).

Following PFA fixation, the cryosections were washed twice (5min each) in 0.1M phosphate buffered saline solution (PBS; Sigma-Aldrich Co. Ltd, UK). To prevent non-specific binding during the ISH, the sections were acetylated for 10min by immersion in a triethanolamine (TEA; Sigma-Aldrich Co. Ltd, UK) and acetic anhydride (AA; Sigma-Aldrich Co. Ltd, UK) solution, whilst on a magnetic stirrer. The TEA/AA solution was freshly prepared before use for each set of slides by adding together 200 ml of 0.9% NaCl (Promega Ltd, Chilworth, UK), 3 ml of TEA and 520 µl of AA. The acetylation was followed by a 5min wash in 0.1M PBS, and sequential dehydration in increasing grades of ethanol (Fisher Scientific Ltd, UK); 70%, 80% and 90% (v/v) (5min each), followed by 95% and 100% (v/v) (10 min each). Finally the sections were immersed in chloroform (Fisher Scientific Ltd) for 5 min and then left to air-dry.

2.5.2 Preparation of radiolabelled riboprobes

2.5.2.a Plasmid Linearization and Purification

Plasmids (Rn_Eya3, Rn_TAC1, Ma_Eya3, Ma_TAC1) were linearized using the appropriate restriction enzymes, NCoI and SalI (Roche Diagnostics Ltd), to generate templates for sense (NCoI) and antisense (SalI) transcripts of each plasmid. Reactions were prepared using 20 µg of plasmid DNA, 10 µl of 10x reaction buffer (Roche Diagnostics Ltd), 10 µl of restriction enzyme (10U/µl) and nuclease-free water to make up a final volume of 100 µl. The resulting mix was vortexed and spun down before incubation at 37°C on a heat block (Labnet International Inc, Rutland, UK) for 4 h. To ensure plasmid linearization, 5 µl of the reaction product was run on a 1% (w/v) agarose (Sigma-Aldrich Co. Ltd, UK) gel using a non-linearized plasmid as a control. The digested bands could therefore by distinguished from the non-digested.

Linearized plasmids were purified using a standard phenol:chloroform (Sigma-Aldrich Co. Ltd, UK) extraction protocol, during which the enzymes and salts used during

linearization are removed and the final concentration of DNA is increased through precipitation. This was done by adding an equal volume (200 µl) of phenol:chloroform to 300 µl of nuclease-free water and 100 µl of linearized plasmid, vortexing and centrifuging at 13000 x *g* for 5 min. The upper aqueous phase was transferred to a new microcentrifuge tube with 350 µl of chloroform, then vortexed and centrifuged at 13000 x *g* for a further 5 min. The upper aqueous phase was again transferred to a new microcentrifuge tube with 1/10 volume of 3M Sodium Acetate and 1 µl of 20 mg/ml glycogen. This was vortexed and then mixed with 2x volume of 100% cold ethanol and left to precipitate at -80°C for 1h. This was followed by centrifugation at 13000 x *g* for 45 min (4°C), after which the supernatant was discarded. Residual salts were removed by adding 100 µl of 70% (v/v) ethanol and centrifuging at 13000 x *g* for 5 min. The DNA pellet was left to air-dry and then resuspended in 15 µl of nuclease-free water. Nucleotide concentration (ng/µl) and purity was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Ltd) and stored at -20°C prior to use in transcription reactions.

2.5.2.b Cold transcription

Non-radiolabelled riboprobes were first synthesized in a 'cold' transcription by using non-radiolabelled nucleotides. Sense and antisense riboprobes were transcribed using RNA Polymerases (Promega Ltd), SP6 and T7 respectively, and 1 µg of linearized template DNA, in a reaction mix shown in table 2.2. The transcription reactions were set up in RNase-free tubes and incubated in a 37°C water bath for 45 min. After transcription, template DNA was removed by adding 3 µl of RQ1 DNase (Promega Ltd) and incubating for a further 10 min. The transcriptional products were then analyzed on a 1% agarose gel to ensure riboprobe synthesis was attained, prior to proceeding to the transcription of the radiolabelled riboprobes.

2.5.2.c Synthesis and Purification of ^{33}P α-UTP Labelled Probe

Radiolabelled riboprobes were synthesized following the same protocol as for the cold transcription (Table 2.2), the only difference being that rUTP was replaced by the radiolabelled ^{33}P alpha- Uridine 5'-triphosphate (PerkinElmer LAS, UK, Ltd). All radioactive work was carried out in a designated area of the laboratory following the rules and regulations associated with ionizing radiation. Once synthesized, the

radiolabelled riboprobes were purified using ProbeQuant™ G-50 micro columns (GE Healthcare Ltd, Chalfont, UK). These were used to remove any unincorporated ^{33}P-UTP, in order to prevent unspecific binding. Before use, the column gel matrix was resuspended and centrifuged at 735 x *g* (1 min), the equilibration buffer was discarded and the column placed into a new microcentrifuge tube. The newly-transcribed riboprobes, with 27 µl of added DEPC-treated water, were then loaded into individual columns. The tubes were centrifuged at 735 x *g* (2 min), and the eluate was retained, to which 50 µl of deiodinized formamide 99.5% (Sigma-Aldrich Co. Ltd, UK) was added. The riboprobes were then placed on a heat block at 65°C for 5 min and then quenched on ice for 2 min. The radioactive count of each riboprobe was then measured using a TRI-CARB 2100CA Liquid Scintillation Analyser, by adding 1 µl of riboprobe to 4 ml of scintillation fluid (National Diagnostics Ltd, Hessle, UK). Calculations were then made and the riboprobes were diluted in the required amount of preheated to 60°C hybridization buffer, (Table 2.3), before being applied to each slide during the ISH.

Table 2.2: Reaction mix for synthesis of non-radiolabelled and radiolabelled sense and antisense riboprobes

Reagents	**Sense probe**	**Antisense probe**
DNA template	1 µg	1 µg
Nuclease-free water	20-X	20-X
DTT (100mM)	2 µl	2 µl
5x Transcription Buffer	4 µl	4 µl
rATP (10mM)	1 µl	1 µl
rCTP (10mM)	1 µl	1 µl
rGTP (10mM)	1 µl	1 µl
rUTP (10mM)	1 µl (or * 3 µl ^{33}P-UTP)	1 µl (or * 3 µl ^{33}P-UTP)
RNase inhibitor	1 µl	1 µl
SP6 RNA Polymerase	2.5 µl	-
T7 RNA Polymerase	-	1.5 µl
TOTAL VOLUME	**20 µl**	**20 µl**

X = Total volume of reagents used in reaction mix except for water.

*** ^{33}P-UTP was used for the synthesis of the radiolabelled riboprobes**

2.5.3 Hybridization and post-hybridization washes

In order to ensure an even coverage of each slide with 15 x 10^5 cpm (counts per minute) of radioactivity, 100 µl of the riboprobe / hybridization buffer mixture was applied to each slide. The tissue was then covered with a hybrid-slip (Sigma-Aldrich Co. Ltd, UK) and the slides incubated overnight at 60°C in a hybridization chamber, which was kept humid using moistened strips of filter paper. Post hybridization, the hybrid-slips were removed and slides were immersed in 2x saline sodium citrate (SSC) (Promega Ltd) buffer with 50% formamide (Sigma-Aldrich Co. Ltd, UK) (Table 2.3) at room temperature for 10 min. The solution was then replaced with 2x SSC (pre-heated to 60°C) with 50% formamide for 2 x 30 min. Next, the slides were incubated in TEN buffer (Table 2.3) containing 30 µg/ml of RNase A (Sigma-Aldrich Co. Ltd, UK), at 37 °C for 30 min, in order to degrade and remove unbound riboprobes. The slides were again placed in 2xSSC-50% formamide solution at 60°C for 2 x 15 min, followed by 0.5xSSC at 60°C for 30 min. Finally they were dehydrated by washing in increasing grades of ethanol; 70%, 95% and 100% (v/v), for 5 min each.

2.5.4 Detection of hybridization signal and quantification

The hybridised slides were then securely fixed inside development cassettes using masking tape, and covered with autoradiographic films (Kodak Biomax MR Films, Kodak, USA) to allow signal exposure. The cassettes were kept in -80 °C for either 1 week or 3 weeks (see individual result sections for duration of exposure of film) before developing the film in safe red light conditions using a Compact automatic film processor (Xograph Healthcare Ltd, Tetbury, UK). The developed film was then scanned using a CoolSNAP-Pro camera (Photometrics, Marlow, UK) while placed on a light box (Jencons Scientific Ltd, Leighton, UK). Signal intensity was analysed using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Marlow, UK), by highlighting the area of interest (AOI) and recording the mean optical density, in order to determine gene (mRNA) expression. Recordings were also taken of film background (FILM), light box (LB) without film and control area (C) on tissue that is not the AOI. The Relative Optic Density (ROD) of the regions of interest to be quantified, was then calculated using the following formula (Johnston, 2002):

$$\textbf{ROD} = (LOG(1/(AOI/LB)) - LOG(1/(FILM/LB))) - (LOG(1/(C/LB)) - LOG(1/FILM/LB)))$$

This formula takes into account the optic density of the AOI, LB and C areas, in order to deduce the ROD. The number of sections and animals used for the quantification of each gene in each area is specified in later sections.

2.5.5 Cresyl violet staining of slides post ISH

In order to visualize tissue structure of the sections post ISH, the slides were stained using a filtered 0.5% cresyl violet acetate solution made up of 5g cresyl violet acetate (Sigma-Aldrich Co. Ltd, UK), 10ml of acetic acid and 1000ml of distilled water. The staining procedure, carried out at room temperature under a fume hood, involved a successive processing of the slides in 70% ethanol (12 dips), distilled water (12 dips), cresyl violet solution (5min), distilled water (12 dips), 70% ethanol (12 dips), 95% ethanol (12 dips), 95% ethanol plus 30 drops of acetic acid (dip until differentiated), 95% ethanol (12 dips), 100% ethanol (12 dips), 100% ethanol (12 dips) and finally placed in histoclear (or xylene) (Sigma-Aldrich Co. Ltd, UK) before being covered by adding 2-3 drops of depex mounting medium (VWR International Ltd, UK) and placing a glass coverslip (VWR International Ltd, UK) on top.

2.6 Immunohistochemistry (IHC)

Immunohistochemistry was used to determine the protein expression of the hormones PRL, ACTH, LH, GH, TSH, S100, and the neurokinin receptors NK1R and NK2R in the F344/NHsd rat pituitary.

2.6.1 Tissue collection for immunohistochemical studies

F344/NHsd male rats aged 6 weeks old and weighing on average 120g, were separated into two weight-matched groups as described above in section 2.1.1.a and kept in either LP or SP for 5 weeks, before being anaesthetised and decapitated. Hypothalamic tissue blocks with the pituitary attached and the PT intact were dissected under aseptic conditions, quickly immersed in Bouin's fixative solution (Sigma-Aldrich Co. Ltd, UK) at room temperature, and left to fix overnight (approximately 12-16 h).

Table 2.3: Table of *in situ* hybridization solutions

Solution	Components
PBS DEPC treated (2L)	10 PBS tablets 2L double distilled water 2ml DEPC Homogenised and autoclaved
NaCl 0.9% DEPC treated (2L)	18g NaCl 2L double distilled water 2ml DEPC Homogenised and autoclaved
Hybridization buffer (30ml)	6ml 20x SSC pH 7 15ml Deiodinised formamide 7ml DEPC-treated water 600µl transfer RNA (tRNA) 60µl 0.5M, EDTA pH 8 Stir to homogenize solution and add the following while avoiding bubbles: 600µl 50x Denhart's solution (Sigma-Aldrich Co Ltd) 750µl 10% Sodium dodecyl sulphate solution (SDS) (Sigma-Aldrich Co Ltd) 3g Dextran sulphate (Sigma-Aldrich Co Ltd) Warm the mixture to 55°C to aid the solubilisation of the dextran sulphate and store at -20°C.
2x SSC-50% formamide (1L)	100ml 20x SSC pH 7 500ml formamide 400 ml double distilled water
0.5x SSC (1L)	25ml 20x SSC 975ml double distilled water
TEN buffer (2L)	20ml 1M TRIS HCL pH8 (Sigma-Aldrich Co Ltd) 4ml 0.5M EDTA 200ml 5M NaCl 1776ml double distilled water
0.5xTBE (1L)	54g TRIS Base (Sigma) 27.5g Orthoboric Acid (AnalaR) 20ml 0.5M EDTA pH 8 980ml double distilled water

2.6.2 Paraffin processing, embedding and sectioning of tissue for IHC

Following Bouin's fixation the tissue was transferred into 70% ethanol for a couple of washes and then rehydrated using an automatic tissue processor (Shandon Citadel 2000; ThermoShandon). The 18-h program includes processing in industrial methylated spirit (IMS), xylene and paraffin wax (table 2.4). As soon as the processing was complete, the tissue was transferred to a ThermoShandon Histocentre 2, were it was embedded in a base mould to produce a wax block. To ensure a coronal-orientated sectioning of the gland, the coronal surface was placed face down in the embedding mould and the tissue was gently pressed whilst being immersed in hot wax, which was rapidly cooled and solidified. The solid block of embedded tissue was then sectioned using a microtome (Leica RM2155, Leica Microsystems) generating 5µm sections on X-tra Adhesive Micro Slides (Leica Biosystems Peterborough Ltd), which were allowed to dry overnight at 55ºC.

Table 2.4: Automatic paraffin tissue processor 18-h programme

Reagent	Time (h)	Temperature (ºC)
70% IMS	2.5	ambient
70% IMS	1	ambient
90% IMS	1.5	ambient
95% IMS	2	ambient
100% IMS	1	ambient
100% IMS	1	ambient
100% IMS	1	ambient
Xylene	1	ambient
Xylene	1	ambient
Xylene	1	ambient
Paraffin Wax	2	60ºC
Paraffin Wax	3	60ºC

2.6.3 Diaminobenzidine (DAB) staining for PRL expression

Tissue which had been fixed, processed, embedded and sectioned onto slides was dewaxed through passage in 2 xylene baths for 5min each and then rehydrated in 100% alcohol (x2), 95% alcohol and 70% alcohol, for 20secs each, and then rinsed in tap water. Antigen retrieval was performed in Na Citrate buffer 0.01M using a pressure cooker (5min in 2L of boiling solution followed by a 20min cooling down period, off the heat for at least 20min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol, on a rocker at room temperature for 30min. Sections were then washed in tap water for 5min, followed by 2 tris-buffered saline (TBS) washes (2x5min). They were then drained and blocked in Normal Goat Serum (NGS: 1 part, TBS: 4 parts, 5%BSA) for 30min in a humidity tray. Incubation with the primary antibody (mouse anti-PRL diluted 1:100, 1:500, 1:1000, Biodesign International) was performed overnight at 4ºC in NGS/TBS/5%BSA. Sections were washed again in TBS and incubated with the secondary antibody (biotinylated goat anti-mouse, Dako) diluted 1:500 in NGS/TBS/5%BSA for 30min in a humidity tray. Streptavidin horseradish peroxidase (HRP) (Vector) was then used diluted 1:1000 in TBS, followed by further washing with TBS. For DAB detection 1 drop of DAB (Dako) in 1ml of buffer was added, colour development was monitored microscopically and the reaction was stopped by washing in water. Sections were coverslip-mounted using 1-2 drops of PermaFluor™(Thermo Fischer Scientific). Images of the tissue sections were photographed using an Olympus Provis Microscope Model AX.

2.6.4 Tyramide Signal Amplification (TSA) system for dual IHC

For fluorescent staining of the rat pituitary tissue sections, a tyramide signal amplification system (TSA) was used as a method of increasing the sensitivity of detection and reducing the concentration of antibodies utilized. Initially, the protocol follows as for DAB detection above, up until and including the blocking step in NGS, but using PBS instead of TBS for washes and for the blocking buffer. Double IHC was carried out to identify co-localization patters of PRL, LHβ (LH β-subunit), ACTH, GH, TSH and S100 antigens with NK1R and NK2R neurokinin receptors. All antibodies/reagents, including suppliers and dilutions used, are shown in table 2.5. Following initial blocking of the sections in 5% BSA in PBS (1 part NGS/ 4 parts PBS/5%BSA) for 30min, slides were incubated in a humidified tray overnight at 4°C in

first primary antibody (mouse anti-PRL; mouse anti-LHβ; mouse anti-ACTH; mouse anti-GH; guinea pig anti-TSH and rabbit anti-S100) diluted in blocking buffer. Slides were then incubated with first secondary antibodies. PRL, LH, ACTH and GH slides were incubated with goat anti-mouse Alexa Fluor 488 (green), TSH slides with goat anti-guinea pig biotinylated, followed by avidin, and S100 slides with goat anti-rabbit peroxidase IgG for 1h, then followed by TSA-Green in kit buffer. Sections were then blocked in NGS/PBS/5%BSA for 30 min and incubated overnight at 4°C in second primary antibody (polyclonal rabbit anti-NK1R; anti-NK2R) diluted in blocking buffer as follows: for PRL and LH slides anti-NK1R was used at 1:1000, and with anti-NK2R at 1:500; for ACTH, GH, TSH and S100 slides anti-NK1R was used at 1:100 and anti-NK2R at 1:500 for 1h. Sections were then incubated in second secondary antibodies. PRL, LH and S100 slides with goat anti-rabbit peroxidase IgG diluted 1:500 for 1h, followed by TSA diluted in kit buffer for 10 min in the dark. ACTH, GH and TSH slides were incubated with goat anti-rabbit Alexa Fluor 546 (red) diluted 1:200 for 1h. Slides were washed with PBS in between the different treatments. Control sections were incubated with NGS/PBS/5%BSA in place of the first primary antibody, second primary antibody or both. The slides were covered by adding 1-2 drops of PermaFluor™(Thermo Fischer Scientific) before placing a glass coverslip on top. Images of the tissue sections were photographed using a Zeiss 70SM 510 Meta Confocal microscope.

Table 2.5: Antibodies/reagents used for IHC

Antibody/Reagent	Company/origin	Dilution
Goat anti-rabbit peroxidase (GARP) IgG	Abcam, Cambridge, UK	1:500 in PBS
Goat anti-rabbit Alexa Fluor 546 IgG	Molecular Probes, Life Technologies Ltd, UK	1:200 in PBS
Goat anti-mouse Alexa Fluor 488 IgG	Molecular Probes, Life Technologies Ltd, UK	1:200 in PBS
Goat anti-guinea pig biotinylated IgG	Vector Laboratories Ltd, UK	1:500 in PBS
Avidin	Sigma-Aldrich Co Ltd, UK	1:200 in PBS
Goat anti-mouse biotinylated IgG	Dako UK, Ltd	1:500 in TBS
Streptavidin HRP(Peroxidase)	Vector Laboratories Ltd, UK	1:1000 in TBS
TSA – Plus Fluorescein System - Green	PerkinElmer LAS, UK, Ltd	1:50 in kit buffer
TSA – Plus Fluorescein System - Red	PerkinElmer LAS, UK, Ltd	1:50 in kit buffer
anti-PRL raised in mouse	Biodesign International, Meridian Life Science Inc., USA	1:2000
anti-LHβ raised in mouse	Gift from Prof Roser, University of California	1:1000
anti-ACTH raised in mouse	Novocastra, Leica Microsystems Ltd, UK	1:100
anti-GH raised in mouse	Gift from Prof Wallis, University of Sussex, Brighton	1:500
anti-TSH raised in guinea pig	NIDDK-NIH	1:50
anti-S100 raised in rabbit	Gift from Prof Heizmann, Zurich	1:200
anti-NK1R raised in rabbit	ABR, Affinity BioReagents, USA	1:100; 1:1000
anti-NK2R raised in rabbit	Santa Cruz Biotechnology Inc., Germany	1:500

2.7 Quantification of hormone concentrations

These assays were performed at Professor Alan McNeilly's laboratory in Edinburgh.

2.7.1 ELISA (Enzyme Linked Immunosorbent Assay) for PRL

Media collected from cell culture experiments (neurokinin treatments) and rat serum samples taken following photoperiodic treatments, were assayed for PRL using an in-house ELISA. ELISA plates (Nunc Biologicals, Thermo Scientific, Epsom, Surrey, UK) were coated with 100 µl of affinity purified donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc) diluted 1:200 in coating buffer (Sigma-Aldrich Co. Ltd, UK), and left overnight at 4°C. Then the plate was washed (x5) in wash buffer (Tris 60.4g; NaCl 450g; Tween 20 25ml; Deionized water 2000ml; adjusted to pH 7.5 using concentrated HCl) using 200 µl per well and blocked using 200 µl of assay buffer (Tris 6g; NaCl 9g; BSA 10g; Bovin Globulin 1g; Tween20 1ml; Thiomersalate 0.05g; Deionised water 1000ml; adjusted to pH 7.5 using concentrated HCl), at room temperature for a minimum of 1 h. The plate was then washed (x3) in wash buffer. The standards (NIDDK-Rat PRL-RP-3), unknown samples (10 µl) and quality controls (10 µl) were added to the plate, mixed in assay buffer (90 µl), with rat anti prolactin antiserum (NIDDK-anti rat Prolactin-RIA-9) to cover a 400ng/ml-1.56ng/ml range. The secondary antibody, biotinylated rat prolactin (NIDDK-rat PRL-I-6) was added using 50 µl per well at a dilution of 1:100000 and the plate incubated overnight at 4°C. The plate was then washed (x5) and 100 µl of Amedex Streptavidin-HRP (GE Healthcare, UK) added at 1:5000, before incubating for 30 min at room temperature. The plate was washed (x5) and 100 µl of 3,3′,5′,5′-tetramethylbenzidine (TMB) micro-well peroxidase blue chromogen substrate solution (Millipore UK Ltd) was added. Oxidation was stopped by adding 6% phosphoric acid. The absorbance was read using a spectrophotometer at a wavelength of λ=450 nm. The results were analysed using AssayZap (Biosoft, Cambridge, UK).

2.7.2 ELISA for LH

Media collected from cell culture treatments was also assayed for LH using an in-house ELISA. Anti-bovine LH chain MAb (2 µg/ml; 518B7; Gift by Dr J Roser, UCLA) was used as capture antibody and was diluted in buffer (50µl of 0.2M sodium bicarbonate–carbonate buffer) before being adsorbed onto plastic plates (Nunc Biologicals, Thermo

Scientific, Epsom, Surrey, UK) overnight. Plates were then washed with 200 μl of wash buffer (0.1M Tris–HCl pH 7.5) and blocked with assay buffer (Tris–HCl, pH 7.5) containing 0.001% Tween20, 1% BSA (Sigma-Aldrich Co. Ltd, UK) and 0.1% bovine-γ-globulin for 1h. After a further wash, 5 μl of LH (NIDDK AFP-7187B) standards (range 0.04–20 ng/ml), 25 μl of quality controls and either 10 μl or 25 μl of unknown samples were added to the plate in duplicate and incubated with 90μl of assay buffer (same as section 2.7.1) overnight at 4 °C. All antibodies/samples were diluted in assay buffer. The secondary antibody, biotinylated anti-human LH MAb (1 μg/ml; MedixBiochemica 5303, Kauniainen, Finland) was added and the plate incubated for a minimum of 2 h at room temperature. Amdex streptavidin-HRP conjugate (1:60 000; GE Healthcare, Chalfont St Giles, Bucks, UK) RPN4401Vq was used for signal detection. After a final wash cycle, 100 μl of TMB micro-well peroxidase blue chromogen substrate solution (Millipore) was added for 30 min. Oxidation was stopped by adding 6% phosphoric acid. The absorbance was read using a spectrophotometer at a wavelength of λ=450 nm. The results were analysed using AssayZap (Biosoft, Cambridge, UK).

2.8 Reverse transcription and PCR amplification (RT-PCR) of neurokinin receptors in the rat pituitary gland

PCR primers for NK1R, NK2R and NK3R were designed against the nucleotide sequences of the rat neurokinin receptors, which are available in the GeneBank (accession numbers: NM31477, NM_080768 and NM_017053 respectively). The isolation of total RNA from rat pituitaries and conversion into cDNA was carried out as described above in sections 2.4.1 and 2.4.2). PCR was then used to amplify the cDNA, using the FastStart High Fidelity PCR System dNTPack (Roche Diagnostics Ltd). Each reaction was set up using 3 μl of cDNA, 2 μl of the corresponding forward primer (table 2.6), 2 μl of the corresponding reverse primer (table 2.6), 5 μl of FastStart High Fidelity Reaction 10x buffer containing $MgCl_2$, 1 μl of dNTP mix (10mM), 0.5 μl of Fast Start High Fidelity Enzyme Blend (5U/μl), 1.5μl of DMSO; and made up to a volume of 50 μl using nuclease-free water. The reaction mix was then placed in a PCR machine (DNA Engine Dyad, Peltier Thermal Cycler) with the following cycling conditions: 10 min at 95°C; 39 cycles of 30 sec at 95°C, 30 sec at the appropriate Tm °C depending on the primers used (Table 2.6) and 30 sec at 68°C; followed by 5 min at 68°C. The final reaction product was analysed by running the total 10 μl with 1 μl of 5x DNA loading

dye (Bioline, London, UK) on a 1% agarose gel containing SafeView (2 μl in 50 ml; NBS Biologicals Ltd, Cambridgeshire, UK) at 90V for 1hr. DNA HyperLadder I (5μl; Bioline, London, UK) was used to check the band size of the PCR products. Once migrated for the appropriate time, the gel was viewed using a UV Transilluminator (UVItec, Cambridge, UK).

Table 2.6: Primers used for RT-PCR amplification of rat neurokinin receptors

Primer	Sequence	Length	GC %	Tm °C
Rn_NK1R_F	CCAGCTTCTACTCTAACATGCTGGC	25	52	57.6
Rn_NK1R_R	CTTCGCTGAAGAAAGACAGACGC	23	52.2	57.2
Rn_NK2R_F	CATCTGGGATGTACCCTGAATAGC	24	50	55.6
Rn_NK2R_R	TTGGCGTCAGAAACAATGGC	20	50	55.9
Rn_NK3R_F	CAAGCTGGCAACTTCTCTTCAGC	23	52.2	57.2
Rn_NK3R_R	GAGGGAAGGCAAGTAGAAATGCC	23	52.2	57.2

2.9 Statistical analysis

Data are shown as means ± standard error (SE). Statistical analysis was carried out using GraphPad PRISM 5 (GraphPad Software Inc. San Diego, CA, USA) and included the use of unpaired student's t-test, one-way analysis of variance (ANOVA) and two-way ANOVA, followed by the appropriate *post hoc* tests, either Bonferroni or Dunnett's. The statistical test used is specified in each section.

CHAPTER 3: Characterizing the photoperiodic response in two different sub-strains of the F344 rat: finding the right model for investigating the seasonal regulation of prolactin

3.1 Introduction

In seasonally breeding species, *when* to breed is as crucial to fitness as *whether* to breed (Prendergast *et al*., 2001). This evolutionary trait of predicting the most appropriate time of year to engage in reproduction allows for better survival of the offspring. In temperate zones, seasonal mammals have developed a mechanism of anticipation that is based on measuring day length, otherwise known as photoperiod, or the 'period of light'. This allows for several indispensable physiological changes, one of which is the secretion of the hormone prolactin. In short-day winter-like conditions, photoperiodic rodents commonly decrease body mass in order to conserve energy and halt sex hormone-dependent behaviours such as mating, territorial defence and aggression, which also coincide with gonadal regression (Prendergast *et al*., 2001).

Laboratory strains of rats are traditionally considered to be non-photoperiodic due to their unresponsiveness to photoperiod changes (Kinson and Robinson, 1970; Reiter *et al*., 1971; Wallen and Turek, 1981; Wallen *et al*., 1987), however there is evidence suggesting otherwise in the Fischer 344 (F344) rat. Heideman and Sylvester (Heideman and Sylvester, 1997) were the first to demonstrate that un-manipulated F344 male rats altered their physiology in response to photoperiod. Rats exposed to short photoperiods (SP) (8L:16D) had significantly lower body weight, whilst testicular growth and spermatogenesis were completely inhibited in these animals; compared to ones exposed to long photoperiods (LP) (16L:8D). These pioneering studies clearly unveiled a potential of the F344 strain to be a valuable model for studying photoperiodic responses. In light of this photoperiod-sensitive nature and the availability of a transgenic F344 prolactin-reporter rat (Semprini *et al*., 2009), the F344 strain was chosen as the most suitable rodent strain for the investigation of seasonal prolactin regulation in the present study.

The objective of the work presented in this chapter was to design experiments in order to characterize the photoperiodic response in two sub-strains of F344 rats, those of a European (F344/NCrHsd), and those of an American background (F344/NHsd), in an attempt to assess their potential suitability for use as a photoperiodic model for examining the regulation of prolactin. The parameters examined in response to different photoperiods were body weight, food intake, paired testes weight, epididymal fat and serum prolactin concentration. Photoperiodic responses were also characterized in the

Syrian hamster, which was used in these studies as a comparison model as it is known to be a strongly seasonal species.

3.2 Methods

The work described in this chapter was divided into three separate experiments. In the first experiment, 2 groups of 6 male F344/NCrHsd rats were housed on either SP (8:16h LD cycle) or LP (16:8h LD cycle) for 5 weeks, during which time body weight and food intake were measured weekly. A tail vein blood sample was taken from each animal at the beginning of the experiment, prior to housing the animals under SP or LP. After photoperiod treatment, the rats were killed 4h into the light phase (ZT4) as described previously (section 2.1.1.a). Trunk blood was collected, testes were dissected from their surrounding tissues to allow measurement of paired testes weight whilst epididymal fat was also dissected and weighed. All blood serum samples were analysed using ELISA to determine serum prolactin concentration (detailed procedure in section 2.7.1). In the second experiment all methods were repeated similarly to the first, the only difference being that male F344/NHsd rats were used instead of male F344/NCrHsd rats. Finally, in the third experiment, male Syrian hamsters were used instead of rats and all methods were repeated. Data in this chapter were analysed by either unpaired t-test or two-way ANOVA.

3.3 Results

3.3.1 Characterizing the photoperiodic response in the F344/NCrHsd and F344/NHsd rat sub-strains.

3.3.1.a Effect of photoperiod on body weight and food intake

The results show that photoperiod had a significant effect on body weight in the F344/NHsd but not the F344/NCrHsd rats, with the F344/NHsd SP animals being consistently lighter than the corresponding LP animals (* $p<0.05$; Figure 3.1). Two-way repeated measures ANOVA showed a significant effect of photoperiod on body weight gain, only in the F344/NHsd sub-strain (* $p<0.05$; Figure 3.2). By the end of each experiment, the F344/NHsd SP rats had gained approximately 14% less weight than the LP rats, whilst the F344/NCrHsd SP rats gained approximately 11% less than the LP-treated. The F344/NHsd sub-strain therefore showed a greater magnitude of response to the different photoperiods compared to the F344/NCrHsd sub-strain of rats.

Food intake measurements revealed a trend of being consistently lower in the SP rats of both sub-strains. Statistical analysis showed that photoperiod had a significant effect on food intake only in the F344/NHsd rats (*$p<0.05$; Figure 3.3). Time also had a significant effect on food intake in this strain ($p<0.05$), although the interaction between food intake and the duration of the experiment was not significant ($p=0.0518$). In the F344/NCrHsd rats, food intake did not appear to be affected by photoperiod, however time did have an increasing effect on food intake in both SP and LP treated animals ($p<0.05$). The cumulative food intake per cage of three F344/NCrHsd rats over 5 weeks was 1369g and 1516g in SP and LP respectively, with no significant difference between the two photoperiods (Figure 3.4). In the F344/NHsd rats, the cumulative food intake per cage of three rats was 1516g and 1694g in SP and LP respectively, revealing a significant effect of photoperiod (*$p<0.05$; Figure 3.4).

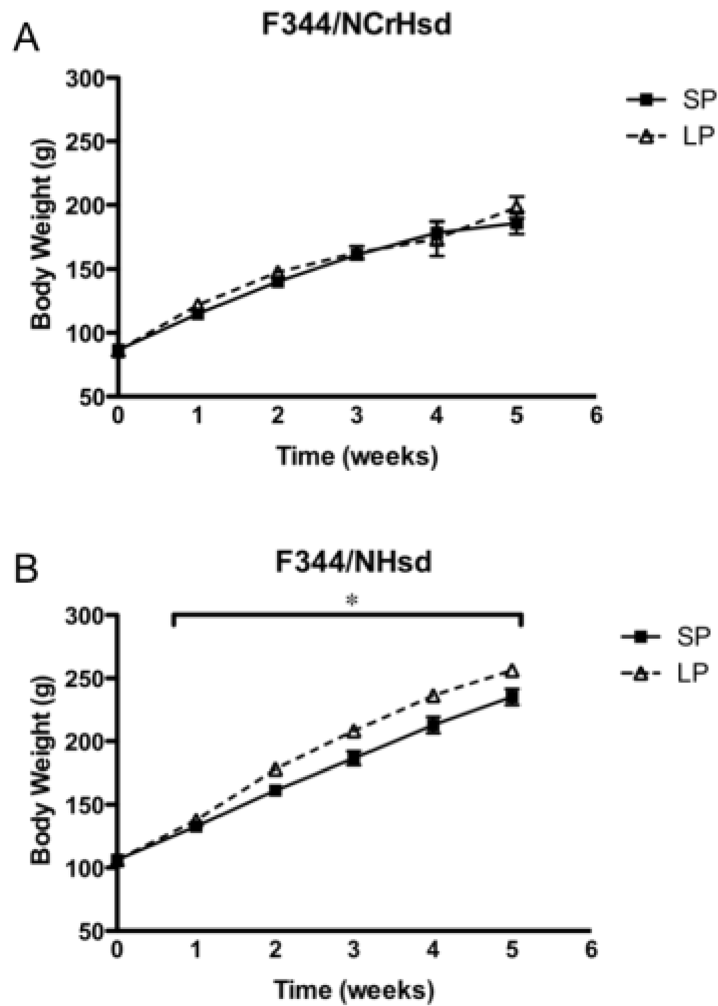


Figure 3.1: Body weight of F344/NCrHsd and F344/NHsd rats following exposure to short and long photoperiods.

(A) Body weight measurements of F344/NCrHsd rats over a 5-week exposure to either LP (n=6) or SP (n=6). **(B)** Body weight measurements of F344/NHsd rats over a 5-week exposure to either LP (n=6) or SP (n=6). The reduction in body weight gain of rats in SP relative to LP was greater in the F344/NHsd compared to the F344/NCrHsd strain. Statistical analysis showed that photoperiod caused significant differences in weight gain only in the F344/NHsd strain (*$p<0.05$; Two-way repeated measures ANOVA). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

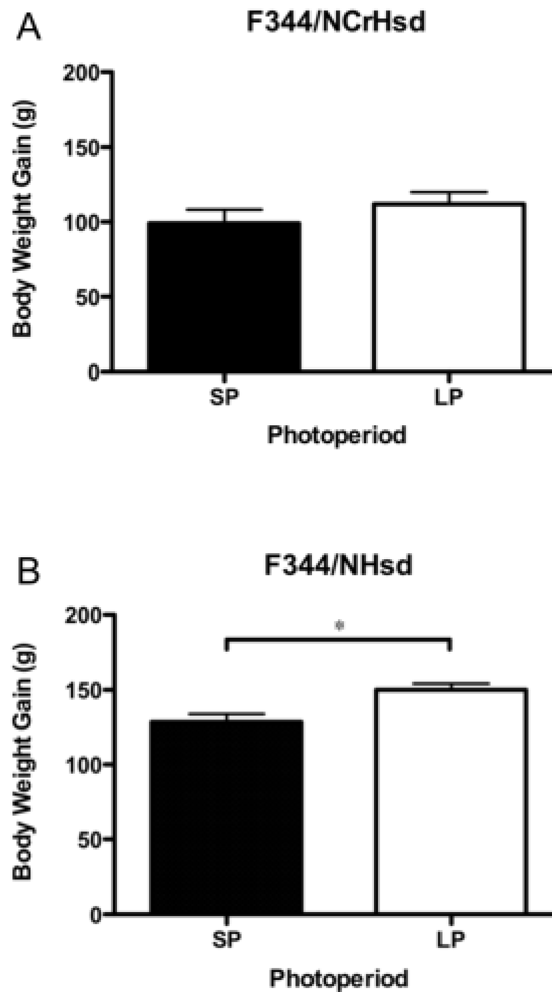


Figure 3.2: Body weight gain in F344/NCrHsd and F344/NHsd rats, following exposure to short and long photoperiods.

(A) In the F344/NCrHsd rats there was an overall weight gain of approximately 11% less in the SP group compared to the LP group, however the result was not statistically significant (p=0.32; Student's t-test). **(B)** In the F344/NHsd rats the SP group gained 14% less weight compared to the LP group and this result was statistically significant (*p<0.05; Student's t-test). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

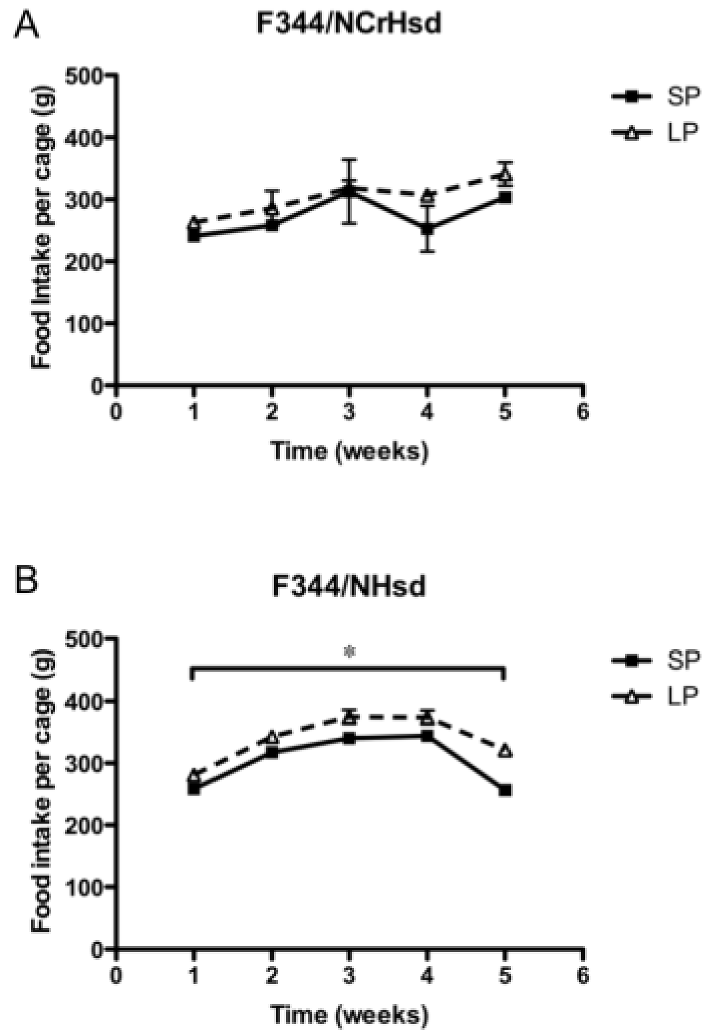


Figure 3.3: Food intake of F344/NCrHsd and F344/NHsd rats, following exposure to short and long photoperiods.

Each data point represents the average food intake of two cages, each housing three rats. **(A)** The F344/NCrHsd rats in SP had a lower food intake than the LP group throughout the course of treatment in either photoperiod; however the result was not statistically significant (p=0.27; Two-way repeated measures ANOVA) **(B)** The F344/NHsd rats showed an increasing trend in food intake during the first 4 weeks, and overall the LP animals were eating more than the SP animals (*p<0.05; Two-way repeated measures ANOVA). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

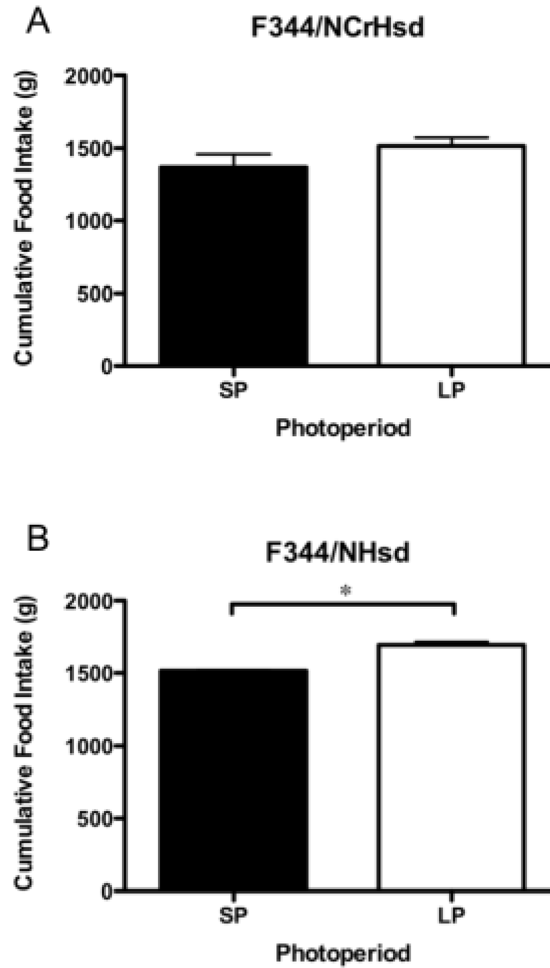


Figure 3.4: Cumulative food intake of F344/NCrHsd and F344/NHsd rats over 5 weeks following exposure to long and short photoperiods.

(A) The cumulative food intake per cage of F344/NCrHsd rats (n=3) was 9.7% lower in the SP group compared to the LP group but the difference between the two photoperiods was not statistically significant. **(B)** The cumulative food intake per cage of F344/NHsd rats (n=3) was significantly lower in the SP group by 10.5% compared to the LP group. (* $p<0.05$; Student's t-test). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

3.3.1.b Effect of photoperiod on paired testes weight and epididymal fat

Photoperiod had a significant effect on paired testes weight in the F344/NHsd but not in the F344/NCrHsd group. Exposure of F344/NHsd rats to SP treatment for 5 weeks resulted in a marked decrease in paired testes weight compared to LP treatment (Figure 3.5). Statistical analysis revealed a significant effect of photoperiod (**$p<0.01$). Conversely, paired testes weight of F344/NCrHsd rats was not affected by photoperiod with no significant difference between SP and LP treatment (Figure 3.5). Epididymal fat was also weighed in the F344/NHsd rats exhibiting a significant decrease in SP compared to LP (*$p<0.05$; Figure 3.6).

3.3.1.c Effect of photoperiod on serum prolactin concentration

Photoperiod induced marked changes in serum prolactin concentration in the F344/NHsd rats following 5 weeks of treatment, but not in the F344/NCrHsd sub-strain. At the start of the experiment and prior to photoperiod treatment serum prolactin did not differ between the groups in either the F344/NCrHsd or the F344/NHsd rats, as expected. At the end of the experiment, serum prolactin was significantly lower in the group which had been exposed to SP treatment (**$p<0.01$; Figure 3.7), but only in the F344/NHsd sub-strain and not the F344/NCrHsd. Serum prolactin concentration in the SP group of the F344/NCrHsd rats was only slightly lower than in the LP group after 5 weeks, and this difference was not significant ($p=0.5516$).

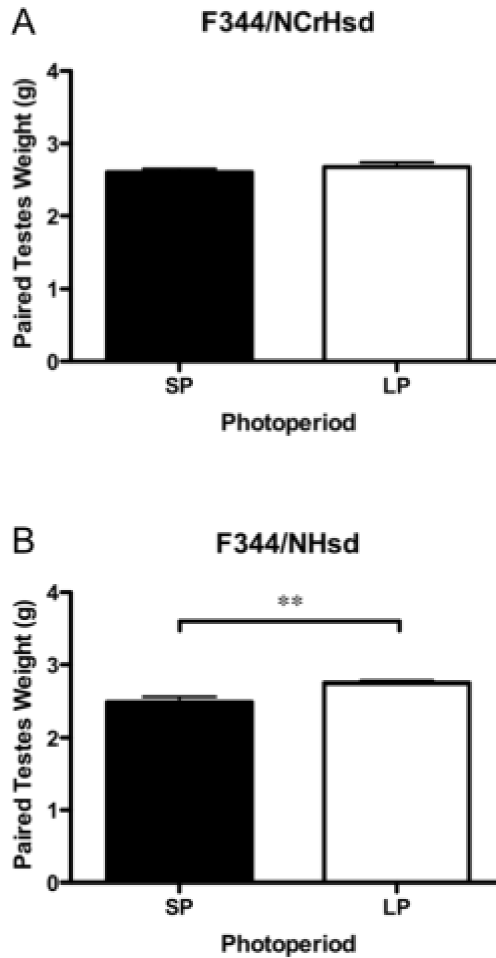


Figure 3.5: Paired testes weight of F344/NCrHsd and F344/NHsd rats following exposure to long and short photoperiods for 5 weeks.

(A) Paired testes weight of F344/NCrHsd rats did not differ between SP (n=6) and LP (n=6). **(B)** Paired testes weight of F344/NHsd rats was significantly lower in SP (n=6) compared to LP (n=6) (**$p<0.01$; Student's t-test). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

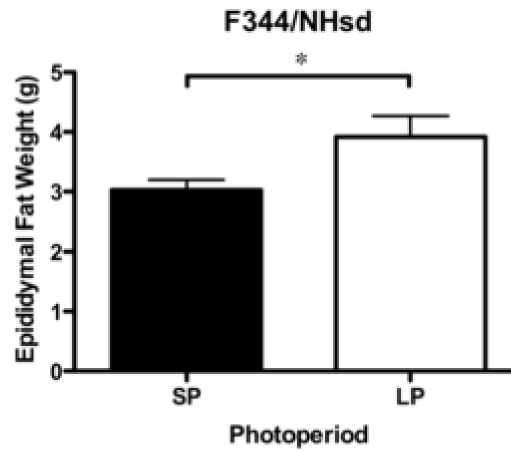


Figure 3.6: Epididymal fat weight in F344/NHsd rats following exposure to long and short photoperiods for 5 weeks.

Epididymal fat weight was significantly lower in the SP group (n=6) compared to LP (n=6) (*p<0.05; Student's t-test). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

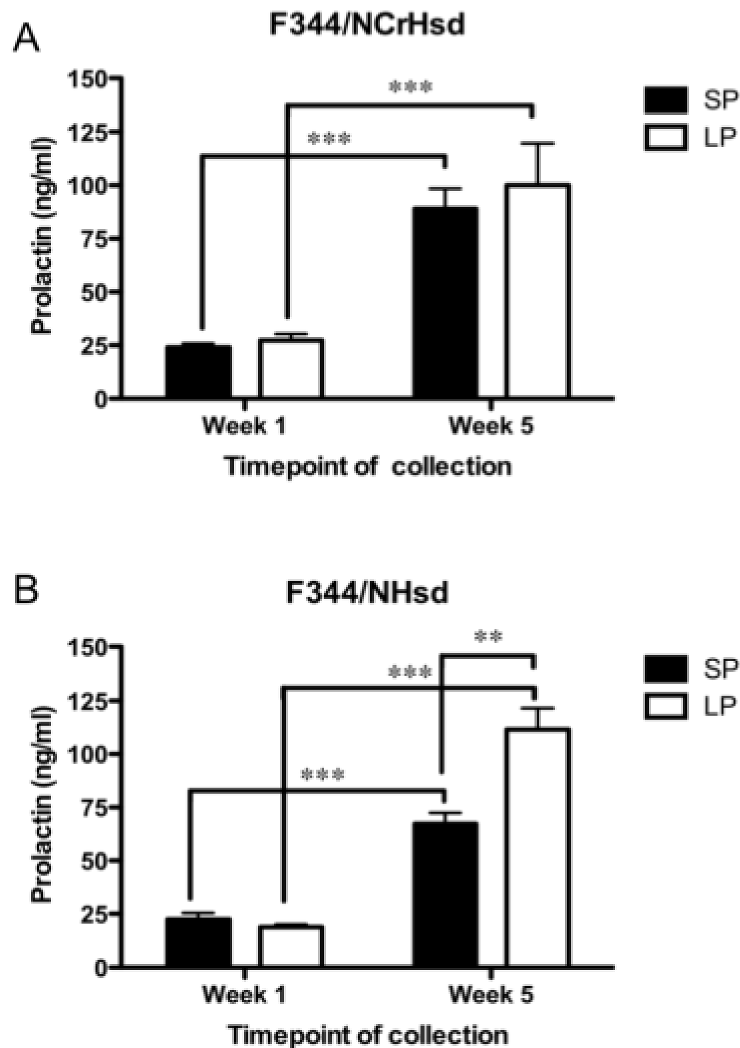


Figure 3.7: Serum prolactin concentration in F344/NCrHsd and F344/NHsd rats in long and short photoperiods, measured at two different time points.

(A) The serum prolactin of F344/NCrHsd rats was measured at the beginning (week 1) and end (week 5) of treatment under SP (n=6) and LP (n=6). Photoperiod did not have a significant effect on serum prolactin at either time point, however time did had a significant effect on both SP and LP groups (***p<0.001; Two-way ANOVA). **(B)** The same measurements were made for F344/NHsd rats (SP, n=6; LP, n=6) and serum prolactin was found to be significantly lower in the SP group at week 5 of treatment, compared to LP (**p<0.01; Two-way ANOVA) Time also had an effect on serum prolactin concentration in both SP and LP groups, with higher prolactin concentration at week 5 compared to week 1 (***p<0.001; Two-way ANOVA). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

3.3.2 Characterizing the photoperiodic response in the Syrian hamster.

3.3.2.a Effect of photoperiod on body weight and food intake

Photoperiod treatment induced changes in body weight over time, with the SP group gaining more weight than the LP group towards the end of treatment. Statistical analysis revealed a significant ($p<0.001$) interaction between photoperiod and duration of treatment. Overall body weight gain following the 6 weeks of treatment was significantly reduced in LP-treated animals compared to SP (*$p<0.05$; Figure 3.8). Food intake measurements did not reveal a significant difference between SP and LP treatment independent of time, however time did have an effect on food intake ($p<0.001$) and over the duration of treatment the interaction between photoperiod and time was significant (*$p<0.05$; Figure 3.8).

3.3.2.b Effect of photoperiod on paired testes weight and epididymal fat

Exposure of hamsters to SP or LP for 6 weeks resulted in a marked difference in paired testes weight between the two groups. Hamsters exposed to SP had significantly lower paired testes weight (**$p<0.01$; Figure 3.9), revealing a significant effect of photoperiod treatment on this physiological parameter. Epididymal fat was also measured in these animals and although appearing to be slightly greater in the LP group compared to the SP, the result was not statistically significant (Figure 3.9).

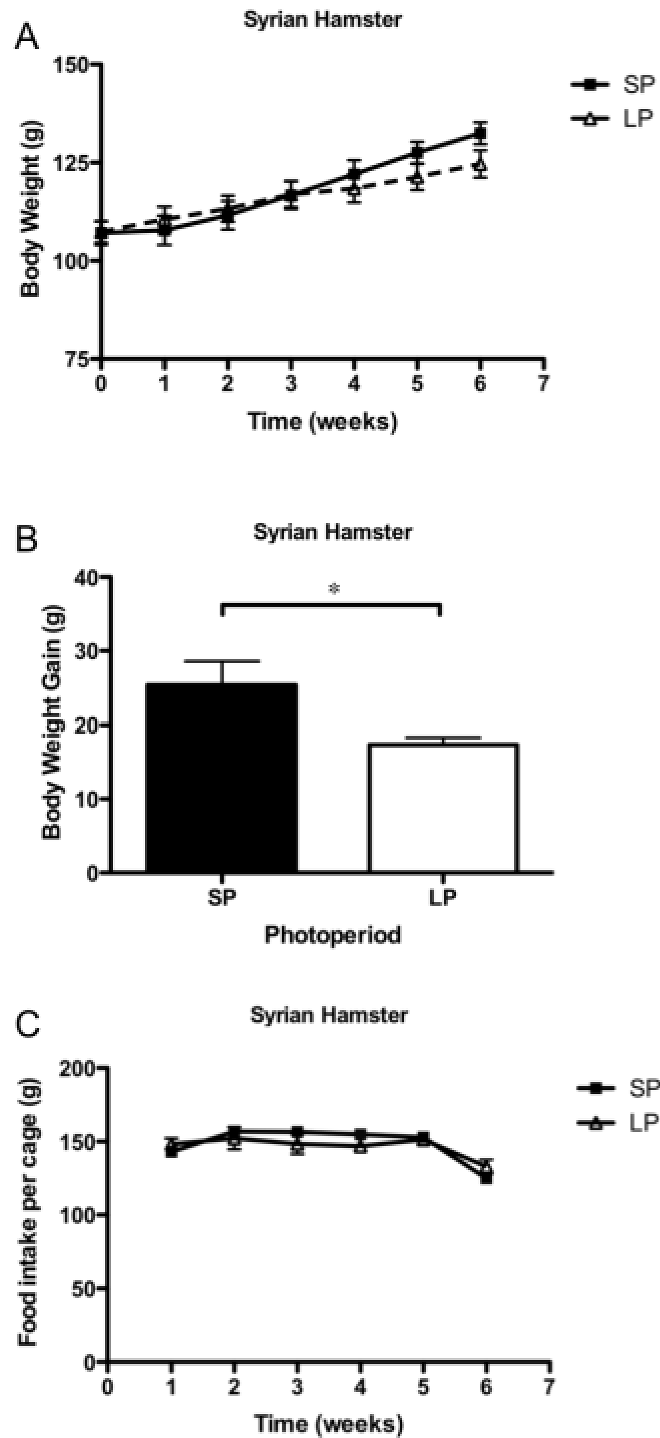


Figure 3.8: Body weight, overall body weight gain and food intake of Syrian hamsters following exposure to long and short photoperiods over 6 weeks.

(A) Body weight measurements of Syrian hamsters in SP (n=6) were higher than those in LP (n=6) over time ($p<0.0001$; interaction of time and photoperiod, two-way repeated measures ANOVA), however independent of time, photoperiod did not have a significant effect ($p=0.6922$). **(B)** Body weight gain was reduced in LP (n=6) compared to SP (n=6) by 31.6% (*$p<0.05$; Student's t-test). **(C)** Food intake was not significantly different between SP and LP, however time did have a significant effect on food intake as it gradually decreased over the 6-week experiment ($p<0.001$). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

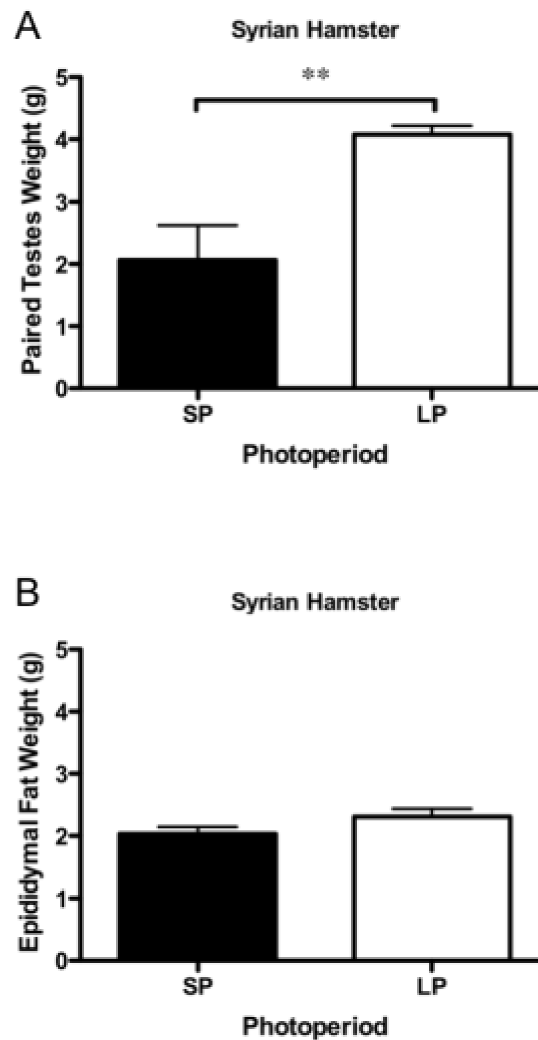


Figure 3.9: Paired testes and epididymal fat weight of Syrian hamsters following exposure to long and short photoperiods over 6 weeks.

(A) Paired testes weight of Syrian hamsters was significantly greater in LP (n=6) compared to SP (n=6) (**$p<0.01$; Student's t-test). **(B)** Epididymal fat weight of Syrian hamsters (LP, n=6; SP, n=6) was not affected by photoperiod ($p=0.1426$; Student's t-test). Data are expressed as means ± SE. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

3.4 Discussion

The results in this chapter provide strong evidence that photoperiod can have significant effects on body weight, food intake, testes weight, epididymal fat and serum prolactin concentration in the F344/NHsd but not the F344/NCrHsd sub-strain of rats.

Exposure of F344/NHsd rats to SP over a 5-week period induced a marked reduction in body weight gain compared to LP treatment, a result that is consistent with previous studies (Heideman and Sylvester, 1997; Heideman *et al*., 2000; Shoemaker and Heideman, 2002; Ross *et al*., 2009). Furthermore, over the time-course of the experiment the food intake of SP animals was consistently lower than that of animals exposed to LP, a result that has also been reported elsewhere (Shoemaker and Heideman, 2002; Ross *et al*., 2009). These changes observed in the SP group were accompanied by a reduction in paired testes weight and epididymal fat weight, indicating reproductive photo-responsiveness of the F344/NHsd strain; a finding that correlates well with earlier reports (Heideman and Sylvester, 1997; Heideman *et al*., 2000; Shoemaker and Heideman, 2002). The reduction in weight gain under SP relative to LP was not apparent in the F344/NCrHsd strain, and neither was a reduction in food intake nor paired testes weight. The body weight and food intake findings are in slight disagreement with a previous study that showed a weak but nonetheless significant effect of photoperiod on these parameters (Ross *et al*., 2009). However, the same study also showed that testes weight was not differentially affected by photoperiod in the F344/NCrHsd sub-strain (Ross *et al*., 2009), and this does coincide with our findings.

The suppression of serum prolactin concentration in the F344/NHsd SP animals following 5 weeks of exposure is consistent with an earlier report (Ross *et al*., 2009), and clearly demonstrates that photoperiod can influence prolactin in the F344/NHsd rat, in the same way that it does in many seasonal mammals (Schams and Reinhardt, 1974; Reiter, 1975; Ravault, 1976; Martinet *et al*., 1982; Loudon *et al*., 1989). By contrast, prolactin concentration was unaffected by photoperiod in the F344/NCrHsd rats. This finding is not in agreement with a previous study whereby the authors employed a slightly dissimilar methodological approach with regards to the time of culling of the animals (Ross *et al*., 2009). Whereas Ross and colleagues decapitated the animals for sample collection during the 'mid-light phase', which we estimate to be approximately ZT4 for the SP group and ZT8 for the LP group, during our study the animals of both

SP and LP groups were killed 4 hours after lights on (ZT4) and so the measurements made are directly comparable between SP and LP groups. The authors themselves discuss this caveat, stating that single time point measurements were made and therefore they could not exclude any circadian variations in prolactin. Interestingly though, the magnitude of the reduction in serum prolactin under SP compared to LP in the F344/NHsd strain, was similar between this study and the study by Ross and colleagues (Ross *et al*., 2009). Overall this chapter demonstrates that photoperiod treatment did not have a significant effect on the F344/NCrHsd sub-strain but did have a marked effect on the F344/NHsd rat physiology.

The studies carried out using the Syrian hamsters showed that relative to the LP group, the SP group of hamsters gained more weight over time, whilst food intake did not differ between the LP and SP groups. In fact whilst the hamsters gained significantly more weight under SP, this was not reflected in their food intakes, which did not increase over time, a characteristic that is typical of Syrian hamsters and suggests that their body weight changes are associated with metabolism (Bartness and Wade, 1985). Paired testes weight was differentially affected by photoperiod and was measurably reduced under SP, thus confirming the well-known reproductive responsiveness of these animals to photoperiod. Whilst epididymal fat measurements showed a trend of being slightly higher in LP relative to SP, this difference was not statistically significant.

The usefulness of these findings in the Syrian hamster is two-fold. Firstly, it validates the methods used in these photoperiodic studies which are confirmed to be robust as they verify well-established findings with regards to the Syrian hamster, a species which has long been known for its seasonal nature; and secondly, the range of physiological responses are comparable to those of the F344/NHsd rat, thus validating the use of this sub-strain as a model to study photoperiodism.

Despite the evidence demonstrating that the F344/NHsd rat provides an excellent model for studying the mechanisms behind photoperiodic responses, it is clear that this is not the most robustly seasonal species, when compared to hamsters for example, and therefore perhaps not the strongest of photoperiodic model species. However, there are several advantages to using the F344/NHsd rat and these include the vast availability of genetic tools and gene-sequence information across a broad array of rat strains, the reagents and equipment designed and already being used in rat studies, and the growing

knowledge of their neuroendocrine systems which one could argue is substantially greater than that of other common model species used in the study of photoperiodism. Nonetheless, the relatively limited robustness of the seasonal responses in the F344/NHsd rat, compared to other more seasonal species, should be considered in the interpretation of subsequent findings using this model. Of particular relevance to the present study is the photoperiod-dependent serum prolactin concentration in the F344/NHsd rat, which is perhaps the most reliable endpoint measurement, compared to the other parameters such as body weight and food intake, as the result unambiguously reveals a photoperiodic effect on the physiology of this species.

In summary, the data presented in this chapter provide an important extension of the literature and support the hypothesis that the seasonal machinery responsible for the physiological responses to photoperiod is in place in the F344/NHsd rat. Consequently, the photoresponsive F344/NHsd sub-strain was chosen as the most appropriate model to be used in further experiments designed to investigate the seasonal regulation of prolactin.

CHAPTER 4: Photoperiod-regulated gene expression in the F344/NHsd rat and Syrian hamster pars tuberalis and brain

4.1 Introduction

The hypophyseal pars tuberalis (PT) is a thin layer of cells surrounding the infundibular stalk and is located between the median eminence of the brain, the portal vessels and the hypophyseal pars distalis (PD), whilst also being linked to the third ventricle via tanycytic processes (Fitzgerald, 1979). As can be expected from its structural connections, the PT is an integral regulatory centre for the transmission of neuroendocrine and endocrine signals involved in the control of several physiological functions and behaviour, such as reproduction, metabolism, hibernation and molt. A current model proposes that the PT operates in a bi-directional signalling circuit, sending information either via a retrograde pathway to the hypothalamus or via an anterograde pathway to the distal region of the pituitary, the PD (Hazlerigg and Loudon, 2008).

Studies in seasonally breeding species have identified the PT of the pituitary as being a strongly photo-responsive tissue, able of interpreting the seasonal melatonin signal which subsequently regulates PT gene expression (Messager *et al*., 1999; Messager *et al*., 2000; Lincoln *et al*. 2002a; Hazlerigg *et al*., 2004; Johnston *et al*., 2005; Johnston *et al*., 2006; Tournier *et al*., 2007; Dupré *et al*., 2008). Furthermore, this region of the pituitary is thought to play a central role in regulating the seasonal secretion of the hormone prolactin from the PD, by releasing a so far unidentified signal.

Recent reports have proposed that products of the *TAC1* gene may be involved in the seasonal regulation of prolactin within the pituitary (Skinner *et al*., 2009; Dupré *et al*., 2010). *TAC1* is cleaved to make various different small molecular weight peptides, known as neurokinins, two of which have been shown to have endocrine-regulating action and are involved in the release of prolactin from PD lactotrophs (Skinner *et al*., 2009; Dupré *et al*., 2010). What is more, *TAC1* gene expression is induced by LP stimulation in the ovine PT and this rise matches the increase in prolactin secretion over the same time-course (Dupré *et al*., 2010). Whilst these data are in support of the hypothesis that neurokinins present as candidates for the role of the elusive PT-derived ‘tuberalin’ that regulates seasonal hormone release, the evidence is far from firm.

Studies in birds investigating the immediate molecular responses to changing photoperiod revealed the induction of *thyrotrophin-β (TSH-β)* and *eyes absent 3 (Eya3)*

gene expression in the PT upon LP exposure (Nakao *et al*., 2008). This first wave of gene expression in the photoperiodic signal transduction pathway was followed by an increase in the expression of *Dio2* in the ependymal cells of the third ventricle and the adjacent infundibular nucleus (Nakao *et al*., 2008). Dio2 and Dio3 enzymes have long been linked to thyroid hormone metabolism and the seasonal regulation of reproduction in birds (Yoshimura *et al*., 2003; Yasuo *et al*., 2005).

Seasonal changes in the deiodinase enzymes were subsequently discovered in tanycytes of several seasonal mammals, including the Syrian hamster (Revel *et al*., 2006; Barrett *et al*., 2007), the Siberian hamster (Barrett *et al*., 2007), sheep (Hanon *et al*., 2008), the European hamster (Hanon *et al*., 2010), and even the Fischer 344 strain of rats (Yasuo *et al*., 2007, Ross *et al*., 2011). However, the pattern of expression of these enzymes appears to be species-dependent. Interestingly, in the F344 rat, *Dio2* and *Dio3* are reciprocally regulated (Ross *et al*., 2011) as demonstrated in the Japanese quail (Yasuo *et al*., 2005). Given that their expression, along with that of *TSH-β,* has been associated with the seasonal regulation of reproduction in mammals by melatonin (Hanon *et al*., 2008; Hazlerigg and Loudon, 2008), there are grounds for believing that much of the photoperiodic machinery will be present in the rat PT.

Subsequent to its identification in birds in the PT as being strongly up-regulated in response to LP stimulation, *Eya3* was also found to be a strongly induced by LP's in the ovine PT (Dupré *et al*. 2010). Eya3 is a transcription co-activator (lacking direct DNA binding) thought to be involved in many developmental processes including the formation of the eye (Wawersik and Maas, 2000). It is also known to act as a tyrosine phosphatase (Rebay *et al*, 2005). Whether and how *Eya3* is involved in the seasonal regulation of prolactin is yet to be deciphered, however its significance in helping to understand the immediate molecular responses to LP stimulation is undeniable.

Despite the well-documented expression of *Eya3* and *TAC1* genes in the PT of other species, there is no information in the literature regarding either expression or the response of these genes to photoperiod changes in the rat PT. The primary goal of this chapter is therefore to specifically test whether *Eya3* and *TAC1* gene expression is present in the rat PT and whether chronic and acute LP stimulation alters that expression. Secondly, the expression of these genes was also assessed in the PT of a

highly seasonal species, the Syrian hamster, in order to compare the results to those of the F344/NHsd rat.

4.2 Methods

4.2.1 Chronic studies

Male F344/NHsd rats were housed in either LP (n=6) or SP (n=6) for five weeks as described previously (section 2.1.1.a). Following photoperiod treatment, animals were culled at ZT4 (Figure 4.1A) and whole brains were collected with the pituitary attached and PT region intact. The tissue was then sectioned, generating coronal sections to be used for *in situ* hybridization to investigate mRNA expression. Rat riboprobes for both *Eya3* and *TAC1* were cloned and then radiolabelled, ready to be hybridised on rat tissue sections. Three slides were used per animal with each slide holding four sections, giving a total of seventy-two sections per photoperiod. Quantification of mRNA expression of each gene of interest was therefore carried out for each treatment and the result represented as relative optic density (ROD). The same experiment was repeated in Syrian hamsters (Figure 4.1A) as described in section 2.1.1.d, keeping all parameters of the experiment the same in order to have a direct comparison of gene expression in the two different species.

4.2.2 Acute studies

Male F344/NHsd rats were housed in SP (n=25) for six weeks, after which five animals were culled at ZT3 and five at ZT11 (section 2.1.1.b). The remaining fifteen rats were exposed to one day of LP (1LP) treatment and then culled at ZT3 (n=5), ZT11 (n=5) and ZT19 (n=5) (Figure 4.1B). Tissue was collected and processed as described in the chronic studies described above. During the *in situ* hybridization, two slides were used per animal with each slide holding four sections, giving a total of forty sections per time-point. Tissue which had been collected from Syrian hamsters (n=22) following a similar experiment (section 2.1.1.c), was also analysed for *Eya3* and *TAC1* gene expression, the only difference being that the tissue had been collected at ZT4 and ZT12 for the SP group, and for the group exposed to one day of LP (1LP), at ZT4, ZT12 and ZT20 (Figure 4.1C).

Data in this chapter were analysed by either unpaired (Student's) t-test or one-way ANOVA, followed by post hoc analysis, as appropriate.

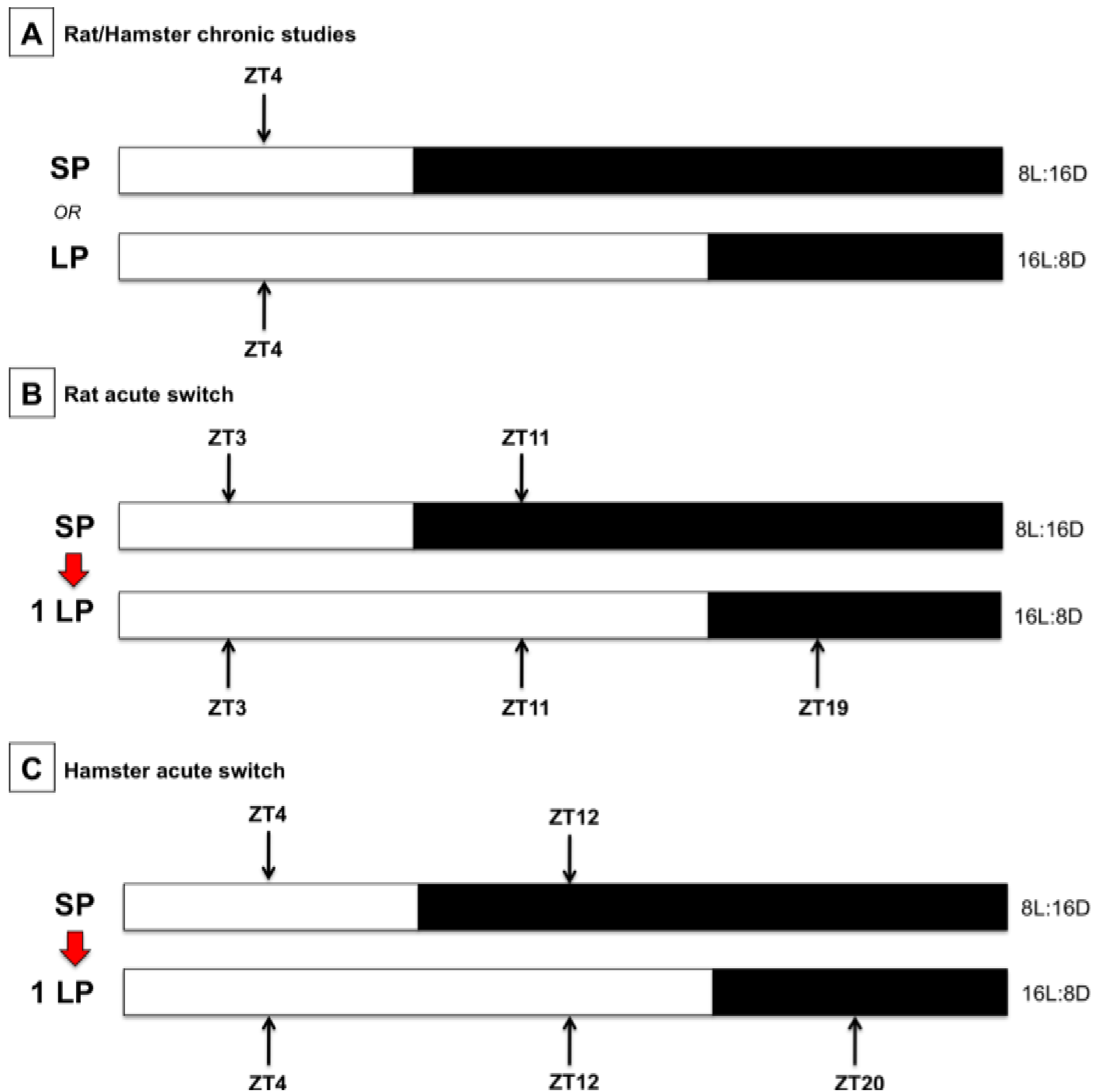


Figure 4.1: Experimental design of the chronic and acute studies in rats and hamsters.

(A) Chronic exposure of F344/NHsd rats and Syrian hamsters to either SP (8L:16D) or LP (16L:8D) followed by collection of the tissue at ZT4 in both groups. **(B)** Acute switch to one day of LP exposure (i.e. '1LP group') in prior SP-housed F344/NHsd rats (n=25). Tissue was collected at ZT3 (n=5) and ZT11 (n=5) from the SP animals, and at ZT3 (n=5), ZT11 (n=5) and ZT19 (n=5) from the LP animals. **(C)** Acute switch to one day of LP exposure (1LP) in prior SP-housed Syrian hamsters (n=22). Tissue was collected at ZT4 (n=4) and ZT12 (n=4) from the SP animals, and at ZT4 (n=5), ZT12 (n=5) and ZT20 (n=4) from the LP animals. **LP:** long photoperiod, **SP:** short photoperiod, **ZT:** zeitgeber time.

4.3 Results

4.3.1 Effect of chronic exposure to long and short photoperiods on gene expression profiles in the F344/NHsd rat

Eya3 and *TAC1* mRNA expression was detected in areas of the brain and PT of F344/NHsd rats following ISH with antisense riboprobes Rn_Eya3 and Rn_TAC1, respectively. Photoperiod had a significant effect on certain gene expression profiles following chronic exposure.

4.3.1.a Chronic Eya3 mRNA expression in the F344/NHsd rat PT and brain

Expression of *Eya3* mRNA was detected throughout the brain of the F344/NHsd rat. However a strong hybridization signal was observed in the PT of animals kept in chronic LP (Figure 4.2B,E) and culled 4h into the light phase (ZT4), whereas an extremely weak signal was observed in the PT of chronic SP animals (Figure 4.2A,D) culled at the same time point. *Eya3* was also weakly expressed in the hippocampus (HIP) of both SP and LP animals.

Eya3 signal intensity in the PT was quantified using 12 PT sections from each animal (n=6/photoperiod). The expression was significantly affected by photoperiod and was higher in LP-exposed compared to SP-exposed animals (***$p<0.001$; Figure 4.2I). Cresyl violet staining of the slides post ISH confirmed the presence of an intact PT in the tissue sections analysed (Figure 4.2G), thus eliminating the possibility of an absent signal in the SP group being due to absence of the PT structure. Furthermore, expression of *Eya3* in LP was clearly restricted to the PT, with no expression in the neighbouring hypothalamic area (Figure 4.2E).

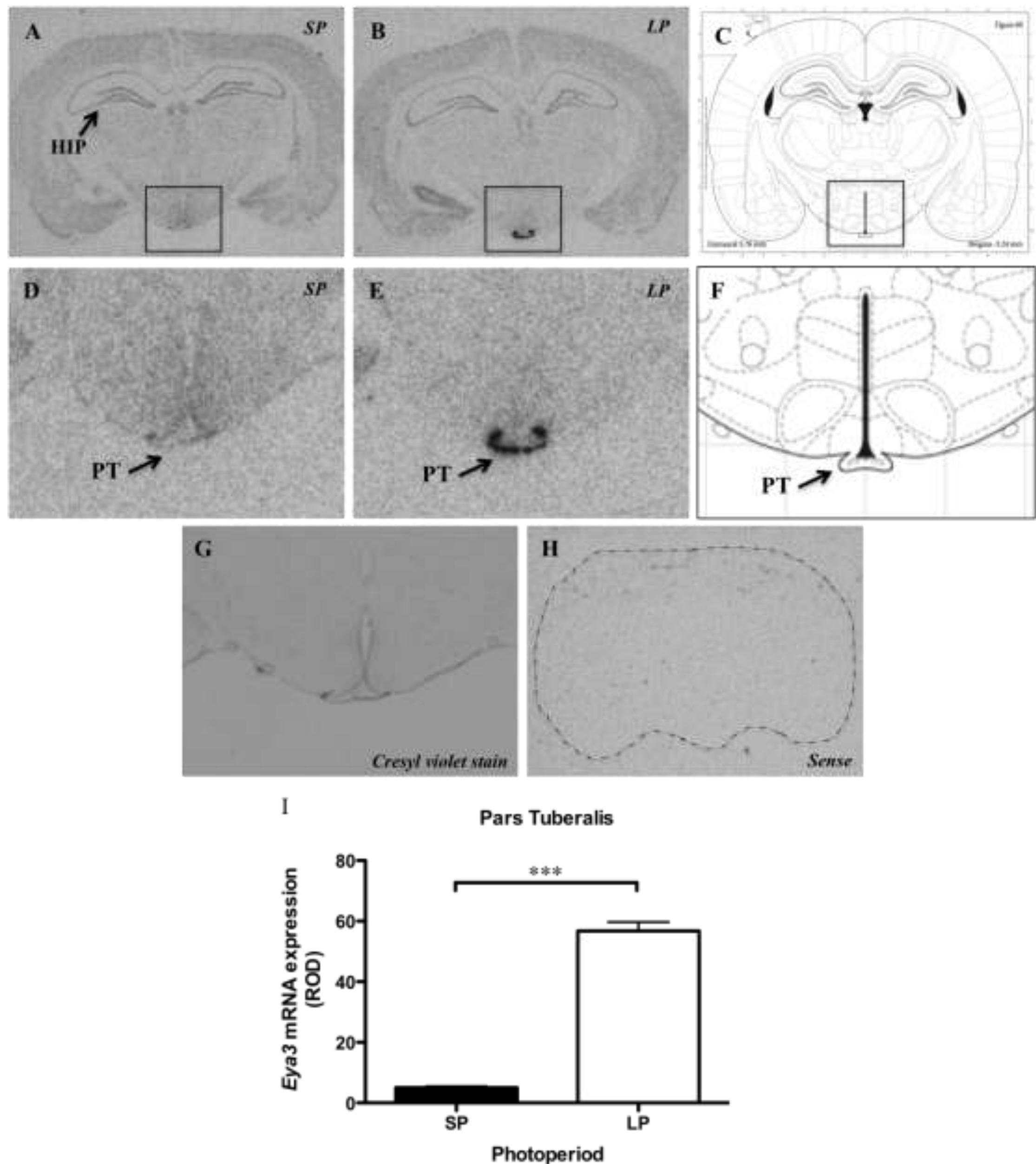


Figure 4.2: Expression of Eya3 mRNA in the pars tuberalis (PT) of the F344/NHsd rat following chronic exposure to SP and LP.

In situ hybridization autoradiographs of coronal sections from **(A)** SP and **(B)** LP F344/NHsd rats showing hybridization of antisense Eya3 riboprobes in the PT region. Arrows indicate the location of the PT and HIP. **(C)** Coronal image of the rat brain, taken from 'The Rat Brain' atlas, stereotaxic coordinates Bregma -3.24mm (Paxinos and Watson, 2005). **(D)**, **(E)** and **(F)** are magnifications of the selected areas in (A), (B) and (C) respectively. **(G)** Section stained with cresyl violet post *in situ* hybridization, confirming the intact structure of the tissue with PT attached. **(H)** Section hybridized with sense riboprobe gave no specific signal. **(I)** Quantification of the Eya3 signal showed that expression was significantly reduced in SP compared to LP (n=6 animals/group). **HIP:** hippocampus, **LP:** long photoperiod, **PT:** pars tuberalis, **ROD:** relative optic density, **SP:** short photoperiod. Data are expressed as means ± SE (***$p<0.001$; Student's t-test). Film exposed for 1 week.

4.3.1.b Chronic TAC1 mRNA expression in the F344/NHsd rat brain

Expression of *TAC1* mRNA was detected in areas of the F344/NHsd rat brain, with a strong hybridization signal observed in the medial habenula in both SP (Figure 4.3A,D) and LP (Figure 4.3B,E), in the ventromedial hypothalamus ventrolateral division (VMHVL) in both SP (Figure 4.4A,D) and LP (Figure 4.4B,E) animals, and in the hypothalamic ependymal layer around the third ventricle, in SP animals (Figure 4.4A,D) but not in LP (Figure 4.4B,E). *TAC1* was also weakly expressed in the caudate putamen (CPu) and the optic tract (opt) in both photoperiods but no expression was detected in the rat PT in either photoperiod.

TAC1 signal intensity was quantified in the medial habenula, the ependymal layer and the VMHVL, using 12 sections for each of those areas from each animal (n=6/photoperiod). The expression of *TAC1* in the medial habenula was significantly reduced in LP compared to SP (*$p<0.05$, Figure 4.3I). The *TAC1* signal was present in SP animals in the ependymal layer but absent in LP animals (Figure 4.4I). Finally, there was no significant difference in the *TAC1* signal between the two photoperiods in the VMHVL (Figure 4.4J).

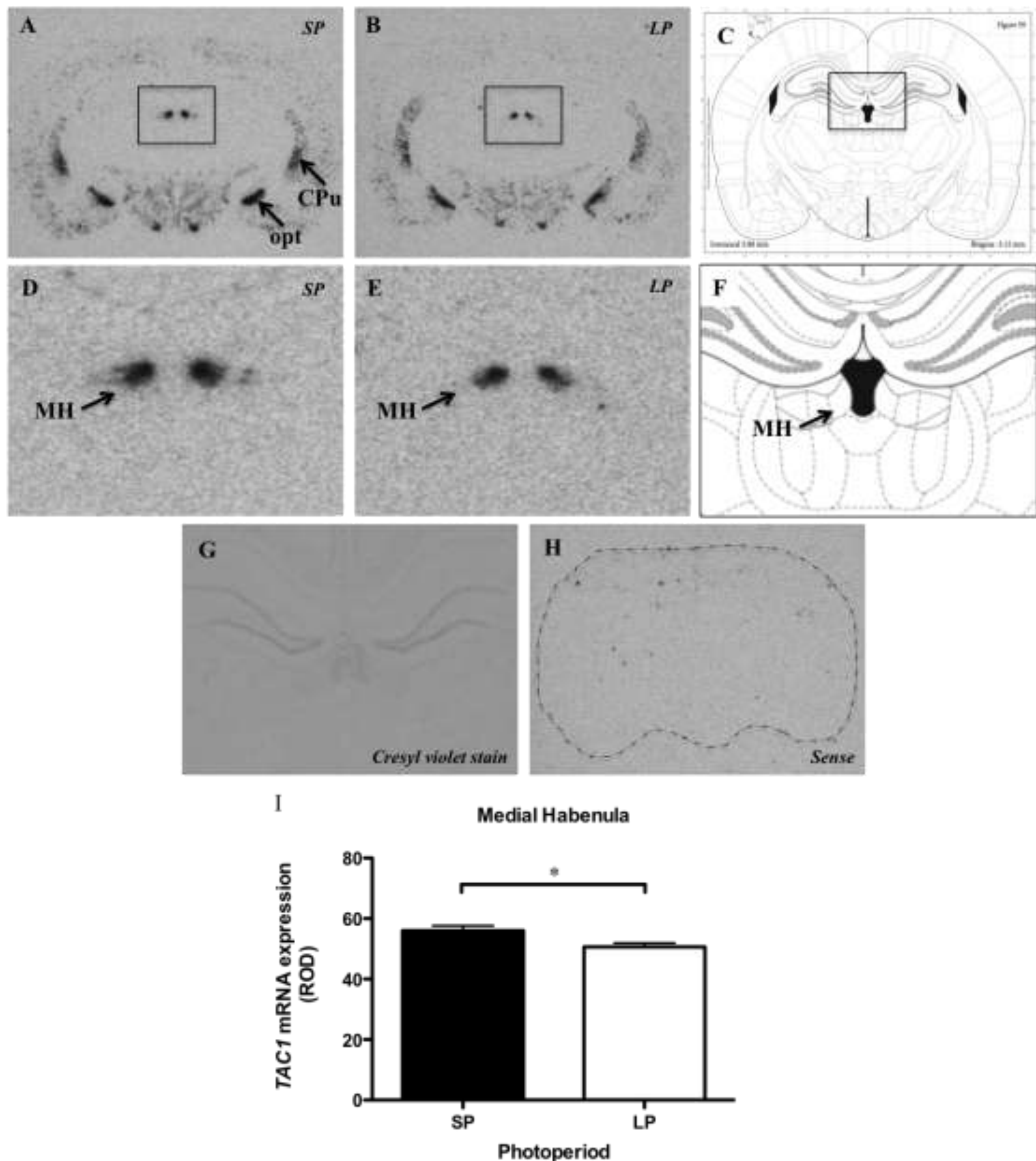


Figure 4.3: Expression of TAC1 mRNA in the medial habenula (MH) of the F344/NHsd rat following chronic exposure to SP and LP.

In situ hybridization autoradiographs of coronal sections from **(A)** SP and **(B)** LP F344/NHsd rats showing hybridization of antisense TAC1 riboprobes in the MH region. Arrows indicate the location of the MH, CPu and opt. **(C)** Coronal image of the rat brain taken from 'The Rat Brain' atlas, stereotaxic coordinates Bregma -3.12mm (Paxinos and Watson, 2005). **(D)**, **(E)** and **(F)** are magnifications of the selected areas in (A), (B) and (C) respectively. **(G)** Section stained with cresyl violet post *in situ* hybridization, confirming the intact structure of the tissue. **(H)** Section hybridized with sense riboprobe gave no specific signal. **(I)** Quantification of the TAC1 signal showed that expression was significantly reduced in LP compared to SP (n=6 animals/group). **CPu:** caudate putamen, **LP:** long photoperiod, **MH:** medial habenula, **opt:** optic tract, **ROD:** relative optic density, **SP:** short photoperiod. Data are expressed as means ± SE (*p<0.05; Student's t-test). Film exposed for 1 week.

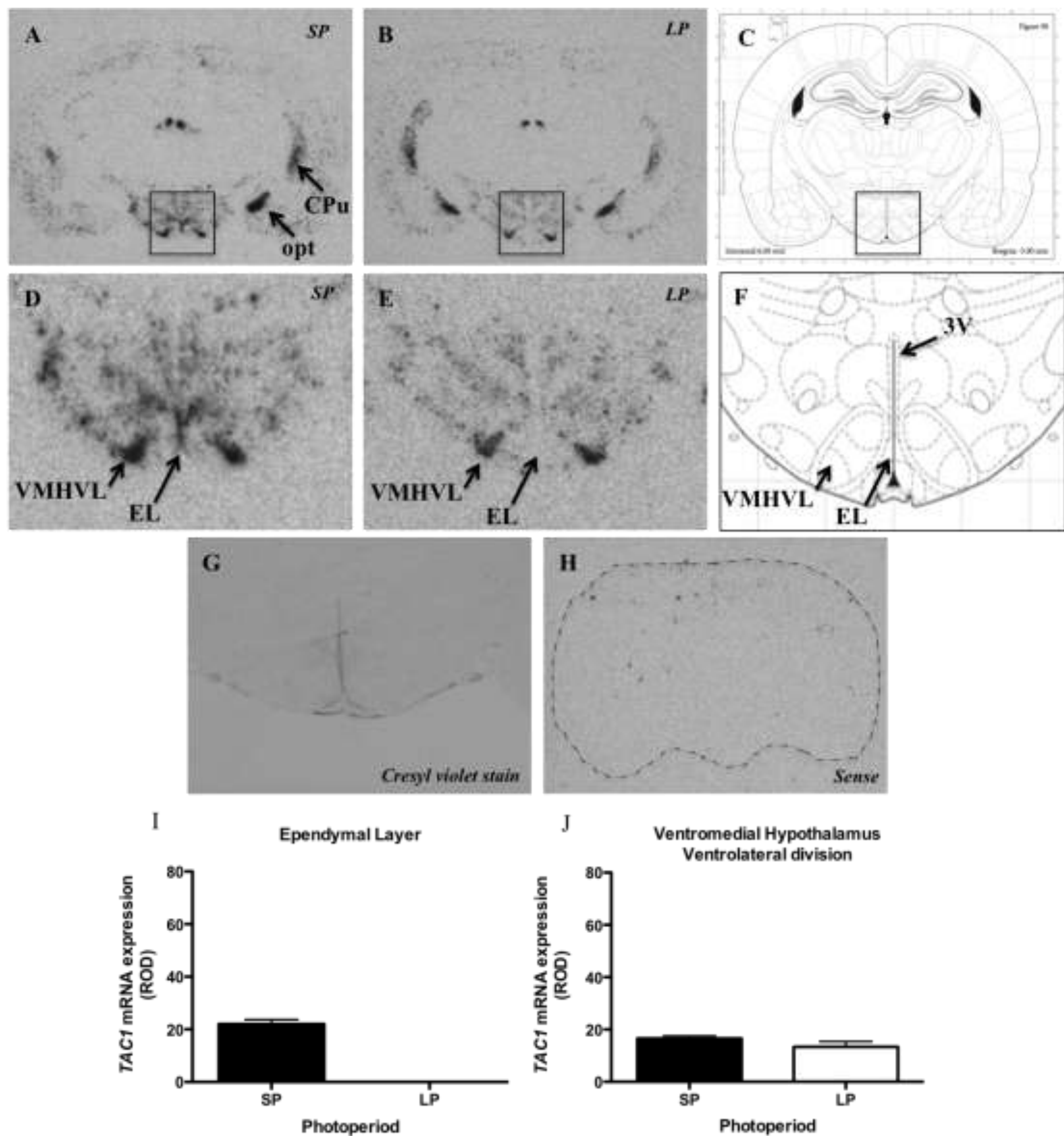


Figure 4.4: Expression of TAC1 mRNA in the ependymal layer (EL) and ventromedial hypothalamus ventrolateral division (VMHVL) of the F344/NHsd rat following chronic exposure to SP and LP.

In situ hybridization autoradiographs of coronal sections from **(A)** SP and **(B)** LP F344/NHsd rats showing hybridization of antisense TAC1 riboprobes in the EL region. Arrows indicate the locations of the EL, VMHVL, CPu and opt. **(C)** Coronal image of the rat brain taken from 'The Rat Brain' atlas, stereotaxic coordinates Bregma -3.00mm (Paxinos and Watson, 2005). **(D)**, **(E)** and **(F)** are magnifications of the selected areas in (A), (B) and (C) respectively. **(G)** Section stained with cresyl violet post *in situ* hybridization, confirming the intact structure of the tissue. **(H)** Section hybridized with sense riboprobe gave no specific signal. **(I)** Quantification of the TAC1 signal in the EL showed expression in SP but not in LP and in **(J)** the VMHVL showed no difference in expression between SP and LP. **CPu:** caudate putamen, **EL:** ependymal layer, **LP:** long photoperiod, **opt:** optic tract, **ROD:** relative optic density, **SP:** short photoperiod, **VMHVL:** ventromedial hypothalamus ventrolateral division. Data are expressed as means ± SE (n=6 animals/group). Film exposed for 1 week.

4.3.2 Effect of acute exposure to long day photoperiod on gene expression profiles in the F344/NHsd rat

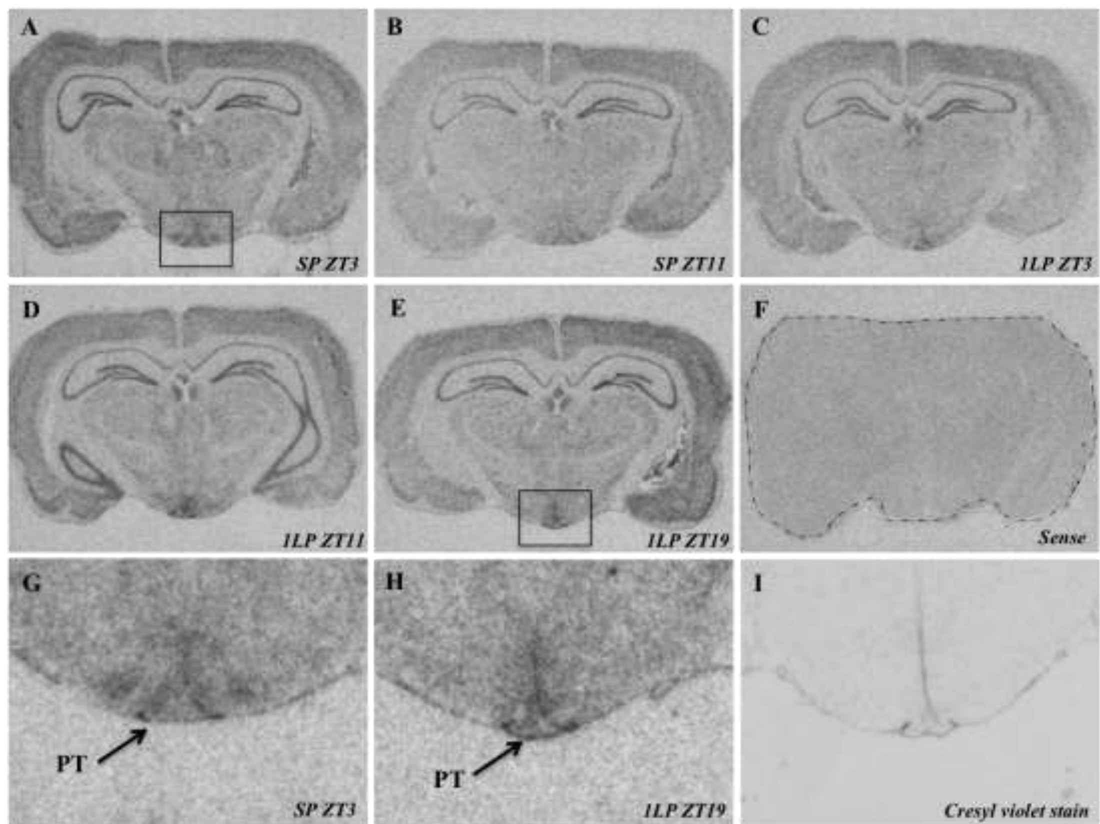
Eya3 and *TAC1* mRNA expression was detected in areas of the brain and PT of the F344/NHsd rat using the same riboprobes mentioned above, in section 4.3.1. Acute exposure of chronically-exposed SP animals, to one day of LP had a significant effect on gene expression profiles for *Eya3* and *TAC1*.

4.3.2.a Acute Eya3 mRNA expression in the F344/NHsd rat PT and brain

Eya3 mRNA was expressed in the PT and hippocampus of SP animals, however no difference was observed between the animals culled at ZT3 (Figure 4.5A) and ZT11 (Figure 4.5B). Upon transferring the animals to LP for one day, a significant increase in *Eya3* mRNA was observed at ZT19 (Figure 4.5E). The expression was therefore significantly affected by acute exposure to one day of LP and was higher at 1LP ZT19 compared to SP ZT11 and 1LP ZT3 (*$p<0.05$, Figure 4.5J). *Eya3* was also expressed at ZT3 and ZT11 following 1LP day with a gradual increasing trend, however the expression was not significantly different compared to that of the SP animals culled at ZT3 and ZT11. *Eya3* signal intensity in the PT was quantified using 8 PT sections from each animal (n=5/time-point).

4.3.2.b Acute TAC1 mRNA expression in the F344/NHsd rat brain

ISH revealed expression of *TAC1* mRNA in the F344/NHsd rat brain however no expression was detected in the PT of either SP (Figure 4.6A,B) or LP (Figure 4.6C-E) animals. *TAC1* mRNA was expressed in the medial habenula, ependymal layer, caudate putamen, optic tract and VMHVL of SP animals. Following acute exposure to one day of LP, a significant increase in *TAC1* mRNA was observed in the ependymal layer at ZT3 (*$p<0.05$, Figure 4.6J), with a gradual decrease of expression at ZT11 and ZT19. Furthermore, *TAC1* expression was significantly higher in the VMHVL at 1LP ZT3 (**$p<0.01$) and 1LP ZT19 (*$p<0.05$) compared to SP ZT3 (Figure 4.6K). In the medial habenula, *TAC1* was significantly lower at 1LP ZT11 compared to the SP ZT11 group, following one day of LP exposure (*$p<0.05$, Figure 4.6L). *TAC1* signal intensity in each region was quantified using 8 sections from each animal (n=5/timepoint).



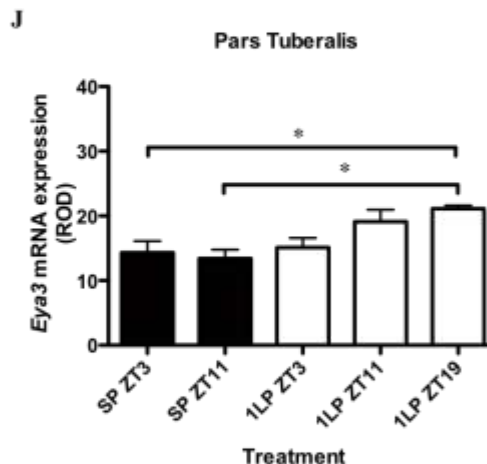


Figure 4.5: Expression of Eya3 mRNA in the pars tuberalis (PT) of the F344/NHsd rat following chronic exposure to SP and acute exposure to LP.

In situ hybridization autoradiographs of coronal sections showing hybridization of antisense Eya3 riboprobes in the PT region of F344/NHsd rats exposed to: **(A)** chronic SP and culled at ZT3, **(B)** chronic SP and culled at ZT11; **(C)** acute (1 day) LP and culled at ZT3; **(D)** acute LP and culled at ZT11; and **(E)** acute LP and culled at ZT19. Arrows indicate the location of the PT. **(F)** Section hybridized with sense riboprobe gave no specific signal. **(G)** and **(H)** are magnifications of the selected areas in (A) and (E) respectively. **(I)** Section stained with cresyl violet post *in situ* hybridization, confirming the intact structure of the tissue. **(J)** Quantification of the Eya3 signal showed that expression was significantly increased at 1LP time-point ZT19 compared to SP time-point ZT3 and SP time-point ZT11 (n=5 animals/group). **LP:** long photoperiod, **PT:** pars tuberalis, **ROD:** relative optic density, **SP:** short photoperiod, **ZT:** zeitgeber time. Data are expressed as means ± SE (*p<0.05; One-way ANOVA followed by Bonferroni's multiple comparison post-hoc analysis). Film exposed for 3 weeks.

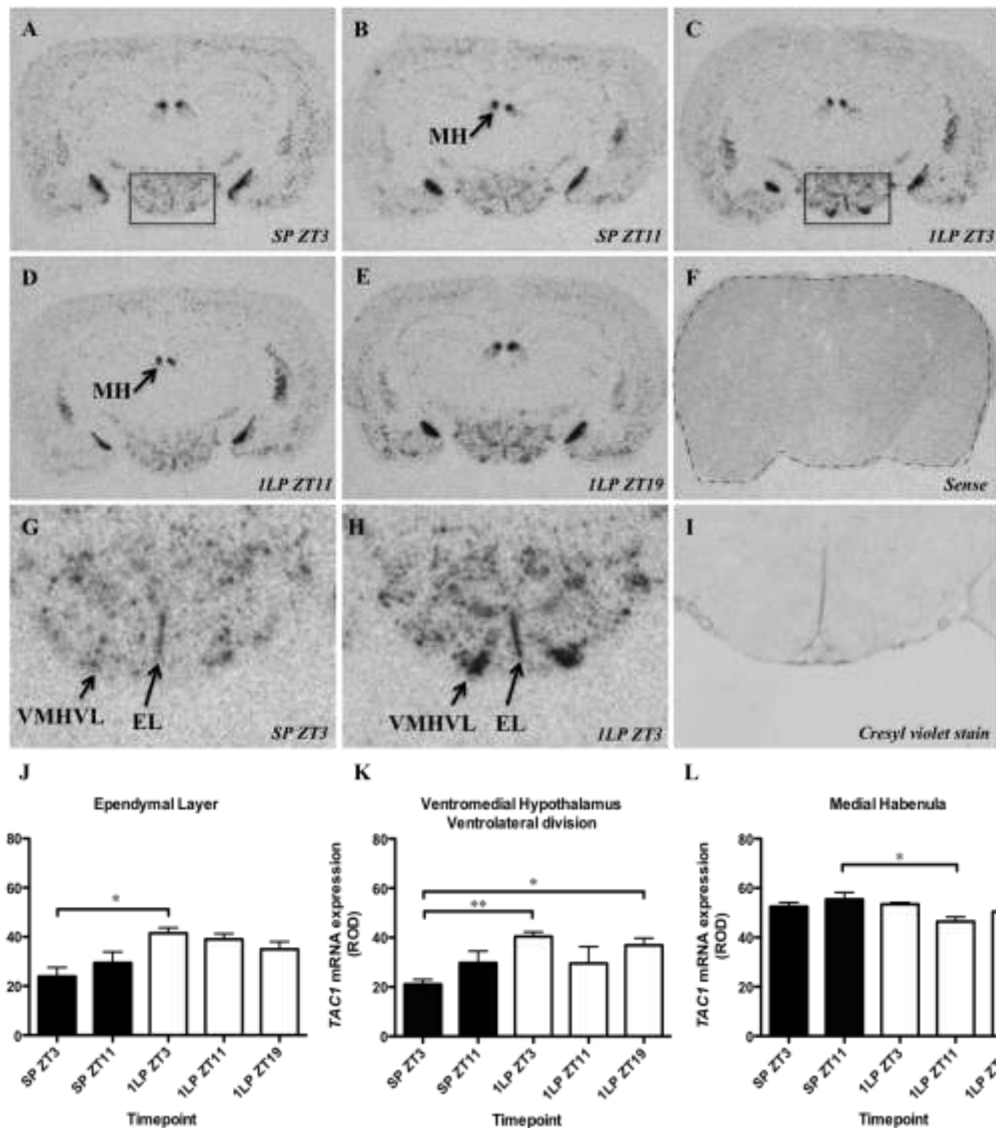


Figure 4.6: Expression of TAC1 mRNA in the EL, VMHVL and MH of the F344/NHsd rat following chronic exposure to SP and acute exposure to LP.

In situ hybridization autoradiographs of coronal sections showing hybridization of antisense TAC1 riboprobes in the EL, VMHVL and MH regions of F344/NHsd rats exposed to: **(A)** chronic SP and culled at ZT3, **(B)** chronic SP and culled at ZT11; **(C)** acute (1 day) LP and culled at ZT3; **(D)** acute LP and culled at ZT11; and **(E)** acute LP and culled at ZT19. Arrows indicate the locations of the EL, VMHVL and the MH. **(F)** Section hybridized with sense riboprobe gave no specific signal. **(G)** and **(H)** are magnifications of the selected areas in (A) and (C) respectively. **(I)** Section stained with cresyl violet post *in situ* hybridization, confirming the intact structure of the PT tissue. **(J)** Quantification of the TAC1 signal showed that expression in the EL was significantly increased at 1LP ZT3 compared to SP time-point ZT3; **(K)** in the VMHVL was significantly increased at 1LP ZT3 and 1LP ZT19 compared to SP ZT3; and **(L)** in the MH was significantly decreased at 1LP ZT11 compared to SP ZT11 (n=5 animals/group). **EL:** ependymal layer, **LP:** long photoperiod, **MH:** medial habenula, **ROD:** relative optic density, **SP:** short photoperiod, **VMHVL:** ventromedial hypothalamus ventrolateral division, **ZT:** zeitgeber time. Data are expressed as means ± SE (*$p<0.05$; One-way ANOVA followed by Bonferroni's multiple comparison post-hoc analysis). Film exposed for 1 week.

4.3.3 Effect of chronic exposure to long and short photoperiods on gene expression profiles in the Syrian hamster

Eya3 and *TAC1* mRNA expression was detected in areas of the brain and PT of Syrian hamsters following ISH with antisense riboprobes Ma_Eya3 and Ma_TAC1 respectively.

4.3.3.a Chronic Eya3 mRNA expression in the Syrian hamster PT and brain

ISH revealed expression of *Eya3* mRNA in the hippocampus and PT of Syrian hamsters following chronic exposure to SP and LP. Hybridization signal intensity was quantified in the PT of both SP (Figure 4.7A,D) and LP (Figure 4.7B,E) groups, using 12 PT sections from each animal. Subsequent analysis for the effect of photoperiod treatment revealed a significant difference between SP and LP groups with *Eya3* mRNA being significantly higher in LP animals (* $p<0.05$, Figure 4.7I).

4.3.3.b Chronic TAC1 mRNA expression in the Syrian hamster PT and brain

Widespread expression of *TAC1* mRNA was detected throughout the brain of the Syrian hamster following both SP and LP exposure, however no expression was detected in the PT. A hybridization signal was observed in the hypothalamus, medial habenula, caudate putamen, optic tract, and components of the hippocampus including the fasciola cinereum (FC), the indusium griseum (IG) and the *cornu Ammonis* CA_2 field (Figure 4.8A,B,D,E). A strong signal was observed in the fasciola cinereum and was quantified, revealing a significantly higher expression in the LP (Figure 4.8B,E) group compared to the SP (Figure 4.8A,D) group (* $p<0.05$, Figure 4.8I). *TAC1* intensity was also quantified in the MH however no significant difference was observed between the two groups (Figure 4.8J).

4.3.4 Effect of acute exposure to long day photoperiod on gene expression profiles in the Syrian hamster

ISH using the same antisense riboprobes as mentioned above (in section 4.3.3) revealed a weak *Eya3* hybridization signal in the hippocampus and PT, and *TAC1* expression in the hypothalamus, medial habenula and components of the hippocampus including the fasciola cinereum, the indusium griseum and the CA_2 field. The *Eya3* mRNA signal observed in the PT was very weak in both SP and LP groups across all time points (ZT4, ZT12 and ZT20) and was therefore not quantified. *TAC1* expression was quantified in the fasciola cinereum and medial habenula of SP animals culled at ZT4 and ZT12 as well as 1LP animals culled at ZT4, ZT12 and ZT20. Quantification of the *TAC1* signal in these areas of the brain revealed no difference between any of the aforementioned groups. Acute exposure to one day of LP therefore had no effect on the expression of *TAC1* mRNA in the Syrian hamster at the time points measured (data not shown).

A summary of the gene changes discussed in this chapter can be seen in table 4.1.

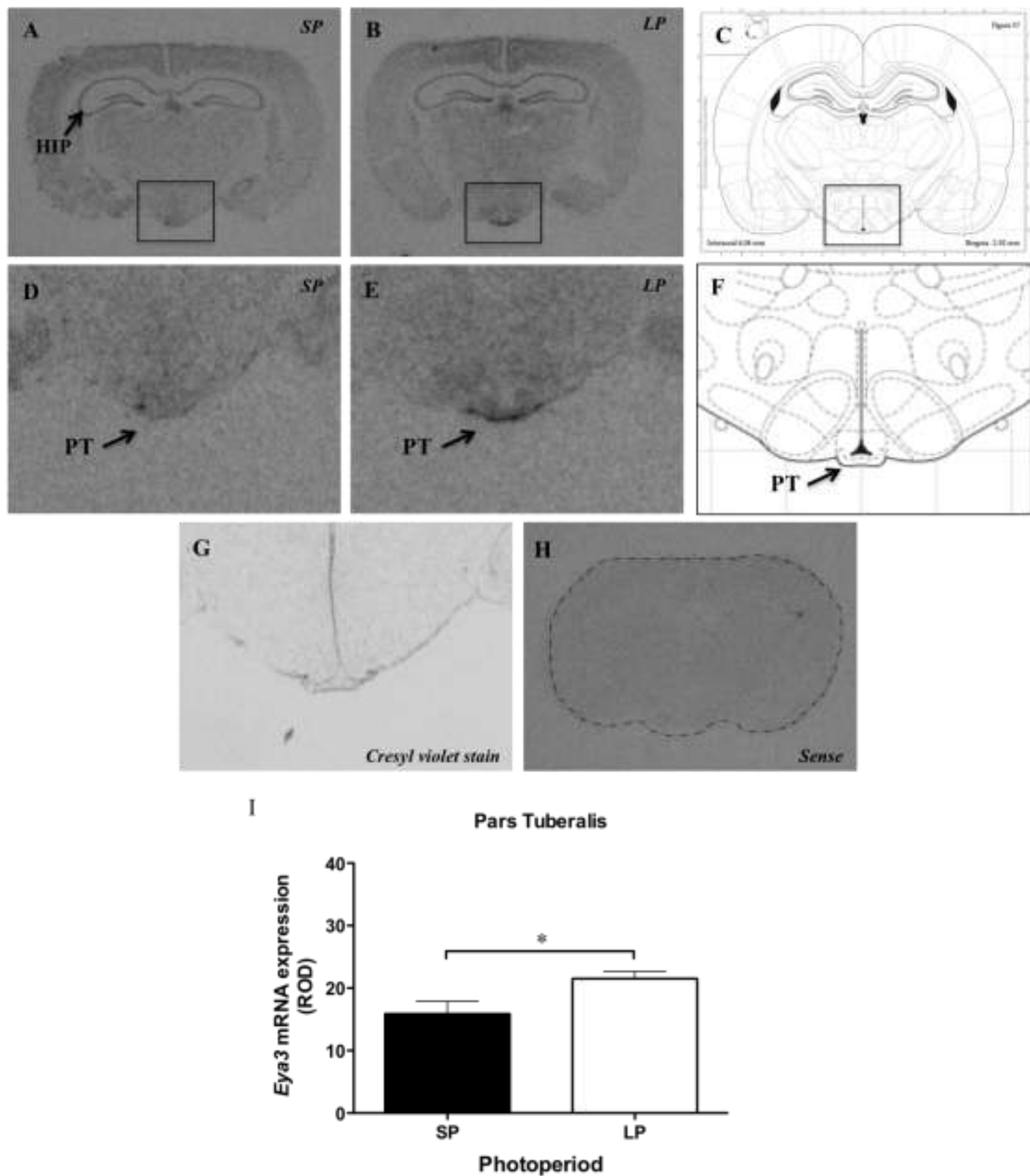


Figure 4.7: Expression of Eya3 mRNA in the pars tuberalis (PT) of the Syrian hamster following chronic exposure to SP and LP.

In situ hybridization autoradiographs of coronal sections from **(A)** SP and **(B)** LP Syrian hamsters showing hybridization of antisense Eya3 riboprobes in the PT region. Arrows indicate the location of the PT and HIP. **(C)** Coronal image of the rat brain taken from 'The Rat Brain' atlas, stereotaxic coordinates Bregma -2.92mm (Paxinos and Watson, 2005). **(D)**, **(E)** and **(F)** are magnifications of the selected areas in (A), (B) and (C) respectively. **(G)** Section stained with cresyl violet post *in situ* hybridization, confirming the intact structure of the tissue with PT attached. **(H)** Section hybridized with sense riboprobe gave no specific signal. **(I)** Quantification of the Eya3 signal showed that expression was significantly reduced in SP compared to LP (n=6 animals/group). **HIP:** hippocampus, **LP:** long photoperiod, **PT:** pars tuberalis, **ROD:** relative optic density, **SP:** short photoperiod. Data are expressed as means ± SE. (*p<0.05; Student's t-test). Film exposed for 3 weeks.

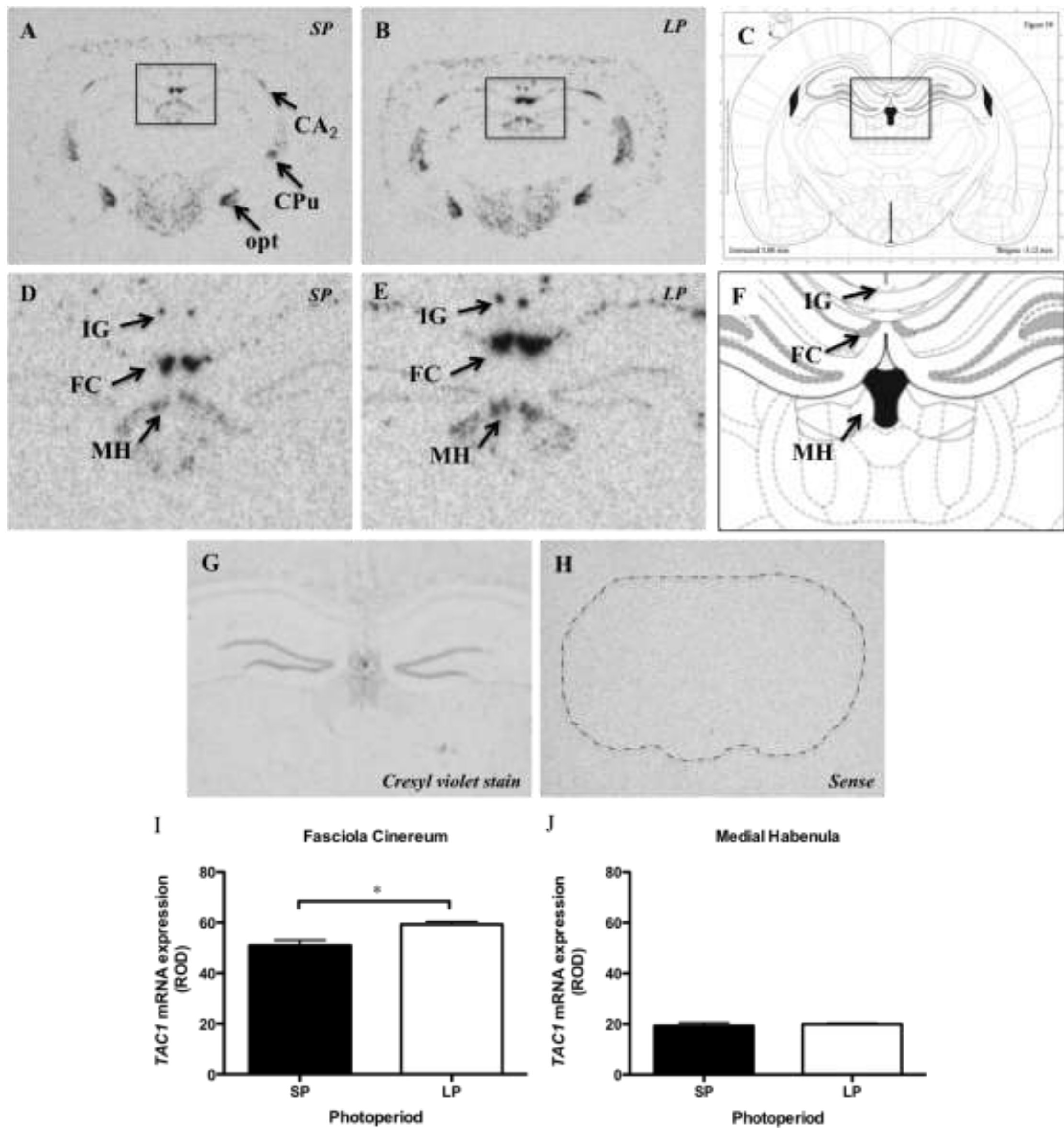


Figure 4.8: Expression of TAC1 mRNA in the FC and MH of the Syrian hamster following chronic exposure to SP and LP.

In situ hybridization autoradiographs of coronal sections from **(A)** SP and **(B)** LP Syrian hamsters showing hybridization of antisense TAC1 riboprobes in the FC region. Arrows indicate the location of the FC, IG, MH, CPu, opt and CA_2. **(C)** Coronal image of the rat brain taken from 'The Rat Brain' atlas, stereotaxic coordinates Bregma -3.12mm (Paxinos and Watson, 2005). **(D)**, **(E)** and **(F)** are magnifications of the selected areas in (A), (B) and (C) respectively. **(G)** Section stained with cresyl violet post *in situ* hybridization, confirming the intact structure of the tissue. **(H)** Section hybridized with sense riboprobe gave no specific signal. **(I)** Quantification of the TAC1 signal in the FC showed that expression was significantly reduced in SP compared to LP (n=6 animals/group), whilst in **(J)** the medial habenula, the signal was not significantly different between photoperiods. **CA_2:** *cornu Ammonis*, **CPu:** caudate putamen, **FC:** fasciola cinereum, **IG:** indusium griseum, **LP:** long photoperiod, **MH:** medial habenula, **opt:** optic tract, **ROD:** relative optic density, **SP:** short photoperiod. Data are expressed as means ± SE (*$p<0.05$; Student's t-test). Film exposed for 1 week.

A

	TAC1 Chronic SP	TAC1 Acute 1LP	TAC1 Chronic LP
F344/NHsd Rat PT	NOT EXPRESSED	NOT EXPRESSED	NOT EXPRESSED
F344/NHsd Rat Medial Habenula	↑	↓	EXPRESSED
F344/NHsd Rat Ependymal Layer	↑	↑	NOT EXPRESSED
F344/NHsd Rat Fasciola Cinereum	NOT EXPRESSED	NOT EXPRESSED	NOT EXPRESSED
F344/NHsd Rat VMHVL	EXPRESSED	↑	EXPRESSED
Syrian Hamster PT	NOT EXPRESSED	NOT EXPRESSED	NOT EXPRESSED
Syrian Hamster Medial Habenula	EXPRESSED	EXPRESSED	EXPRESSED
Syrian Hamster Ependymal Layer	NOT EXPRESSED	NOT EXPRESSED	NOT EXPRESSED
Syrian Hamster Fasciola Cinereum	EXPRESSED	EXPRESSED	↑
Syrian Hamster VMHVL	NOT EXPRESSED	NOT EXPRESSED	NOT EXPRESSED

B

	EYA3 Chronic SP	EYA3 Acute 1LP	EYA3 Chronic LP
F344/NHsd Rat PT	EXPRESSED	↑	↑
Syrian Hamster PT	EXPRESSED	WEAK NOT QUANTIFIED	↑

Table 4.1: Summary of (A) TAC1 and (B) Eya3 chronic and acute gene changes in the brain and PT of the F344/NHsd rat and Syrian hamster.

The arrows indicate either up-regulated (↑) or down-regulated (↓) expression. In chronic SP (column 1), changes are indicated with respect to chronic LP. In acute 1LP (column 2) and chronic LP (column 3), changes are indicated with respect to chronic SP.

4.4 Discussion

The ISH studies described in this thesis reveal a strong up-regulation of *Eya3* gene expression in the F344/NHsd rat PT following a 5-week exposure to LP compared to SP exposure. The collection of tissue at ZT4 (early light phase) was based on previous studies that revealed a marked increase in *Eya3* expression in the sheep PT at this time point (Dupré *et al*., 2010).

To identify immediate changes in PT gene expression upon LP stimulation, we also collected tissue at ZT3 (early light phase), ZT11 (late light phase) and ZT19 (early dark phase) following one day of LP exposure (preceded by chronic SP exposure) and compared mRNA expression with that of PT tissue collected from chronic SP-housed rats culled at two of those time points (ZT3, ZT11). Acute exposure to LP resulted in a marked increase in PT *Eya3* expression at ZT19 (early dark phase) compared to the SP animals (ZT3 and ZT11), but not during the early hours of the light phase (ZT4) as observed in the chronic studies. Furthermore, the magnitude of the response to acute LP at ZT3, following chronic SP exposure, is not similar to that observed in sheep which was greater than a two–fold increase following one day in LP (Dupré *et al*., 2010). In sheep, this early phase peak in *Eya3* after one LP day is then followed by a second peak at ZT15 (Dupré *et al*., 2010). *Eya3* was weakly present at ZT3 and ZT11 in the SP rats, with no significant variation between those two time points. The differences highlighted between our studies and those in sheep would therefore suggest that in the latter, *Eya3* expression is part of an immediate response to photoperiod change from as early as day 1 of LP exposure, whereas in rats this early light phase response of *Eya3* is only evident after several weeks of LP-housing.

Analysis of *Eya3* mRNA expression in the Syrian hamster also revealed an up-regulated expression in the PT of LP-exposed animals compared to SP-exposed, following 6 weeks of photoperiod treatment. The tissue was collected at ZT4 similarly to the F344/NHsd rat studies in order to compare the response in the two species. This comparison reveals that *Eya3* up-regulation in response to chronic LP treatment is of greater magnitude in the F344/NHsd rat PT (11.3-fold) than in the Syrian hamster PT (1.4-fold) at ZT4; an intriguing finding given the stronger seasonal nature of the Syrian hamster.

Overall these data on *Eya3* expression in the rat and hamster PT, parallel earlier studies in quail, sheep and mouse in which *Eya3* presents as a strong LP-induced gene that appears as one of the first molecular responses to photoperiod change (Nakao *et al*., 2008; Dupré *et al*., 2010; Dardente *et al*., 2010; Masumoto *et al*., 2010).

Contrary to expectation, *TAC1* gene expression was not observed in the PT following any photoperiod treatment in either the F344/NHsd rat or the Syrian hamster. However, a detectable hybridization signal of *TAC1* mRNA was observed in other areas of the brain, in both species. The work of Skinner *et al* (Skinner *et al*., 2009) demonstrated an abundant presence of SubP in the ovine PT that was substantially higher than in the anterior pituitary. Preprotachykinin A, the precursor of SubP and of other neurokinins, was also revealed in the ovine PT at the mRNA level (Skinner *et al*., 2009). These findings initially encouraged the hypothesis that neurokinins fulfil the role of the elusive 'tuberalin', the factor that is secreted by the PT and acts in the PD to stimulate prolactin secretion. Nonetheless, given the results of the present study, it is difficult to assign a similar role for neurokinins in the F344/NHsd rat and Syrian hamster PT as potential 'tuberalins', given the lack of *TAC1* gene expression in this region.

In the F344/NHsd rat, *TAC1* expression was measurable in distinct areas of the brain including the ependymal layer, medial habenula, caudate putamen, optic tract and the VMHVL. The profile of expression was similar between the two photoperiods, however there were some noticeable differences. Expression of *TAC1* mRNA was observed in the ependymal layer at ZT4 following chronic exposure to SP, however no expression was detected at the same time point following chronic exposure to LP. This clear difference in expression may signify an important switch with regards to photoperiod change, drawing attention to this region of the brain. Its potential significance is further highlighted by the acute induction of *TAC1* in this region at ZT3 following one day in LP. Our studies therefore demonstrate that *TAC1* is expressed in the ependymal layer at a considerable level following chronic exposure to SP and while its expression is initially induced by acute LP exposure, it later subsides and is ultimately completely abolished following several weeks in LP.

The ependymal layer has already been associated with seasonal physiological changes since a functional relationship was demonstrated between the ependymal cells lining the third ventricle and the PT. These bipolar ependymal cells are known as tanycytes and

possess a long process connecting them to the capillaries of the hypothalamo-hypophyseal portal system (Löfgren, 1958; Löfgren, 1961). This structural arrangement is sufficient to allow transport of compounds from the ventricular cerebrospinal fluid (CSF) to the portal blood and subsequently the intercellular channels of the PT (Peruzzo *et al*., 2004; Rodríguez *et al*., 2005). Four types of tanycyte cells have been desbribed, $\alpha_1, \alpha_2, \beta_1$ and β_2 (Rodríguez *et al*., 2005). Guerra *et al* suggest that a subpopulation of the β_1 tanycytes projects through the basal lamina and establishes direct contact with the PT-specific cells (Guerra *et al*., 2010). Thyroid hormone synthesis, which leads to important seasonal changes in physiology, is now considered to be regulated in tanycytes by PT-derived thyrotrophin (TSH) (Hanon *et al*., 2008; Nakao *et al*., 2008). It is therefore clear that cells of the ependymal layer are involved in mediating seasonal responses and given the photoperiod-regulated expression of *TAC1* in the ependymal region it would not be unreasonable to assume a role for neurokinins in the seasonal response pathway, despite the absence of *TAC1* in the PT. What is more, circadian oscillations have also been demonstrated in the ependymal cell layer in the form of rhythmic clock gene expression (Guilding *et al*., 2010).

Additionally, microscopy studies revealed a strong immunocytochemical expression of the ligand binding domain of the PRL-R in the ependymal lining of the third ventricle and in the tanycyte processes projecting to the median eminence (Lerant and Freeman, 1998). These data suggest that the PRL-R in this region may be involved in a short prolactin feedback loop, with the possibility of tanycytes mediating bi-directional trafficking (Mezey and Palkovits, 1982) of PRL between the pericapillary spaces of the median eminence and the CSF. It is therefore highly interesting that *TAC1* expression is also present in this region and is regulated in a seasonal manner, and may therefore be involved in a seasonal feedback mechanism of PRL regulation.

Neurokinins have already been associated with the hypothalamo-pituitary-gonadal axis as potential regulators of reproductive functions, with a number of studies having identified high concentrations of neurokinin-containing cell bodies in hypothalamic structures and other areas of the brain related to neuroendocrine function (Debeljuk and Lasaga, 1999). *TAC1* mRNA expression has previously been reported in the hypothalamus of the female rat (Gautreau *et al*., 1997) and specific regions of the human and monkey brain including the hypothalamus and ventromedial nuclei (Hurd *et al*., 1999). The arcuate nucleus has also been identified as a major neurokinin-

containing area of the rat hypothalamus (Jessop *et al*., 1991; Villanúa *et al*., 1992). Intriguingly, immunohistochemical studies have demonstrated that certain SP/NKA neurons in the arcuate nucleus may project fibres onto GnRH neurons, the density of which varies during the estrous cycle and lactation, thus suggesting that neurokinins may have a role in the regulation of gonadotrophin secretion and that hypothalamic neurokinin concentrations may be affected by circulating sex steroids through a feedback mechanism (Tsuruo *et al*., 1987). It is therefore not unreasonable to suggest that neurokinins in the brain may also be associated with prolactin regulation through such a feedback system.

Chronic SP exposure had an up-regulating effect on *TAC1* expression in the medial habenula, and acute exposure to LP significantly reduced *TAC1*. A number of previous studies have identified a high concentration of SubP and NKA neurons in the habenula (Ljungdahl *et al*., 1978; Shults *et al*., 1984; Harlan *et al*., 1989). Furthermore, a recent study characterising the sub-nuclear organization of the rat habenula revealed the expression of *TAC1* mRNA in the medial habenula (Aizawa *et al*., 2012). The habenula is an epithalamic complex, which consists of the medial and lateral regions, and has been implicated in learning, memory, sleep/wake cycles and anxiety (Lecourtier and Kelly, 2007; Geisler and Trimble, 2008; Hikosaka *et al*., 2008). Circadian rhythms in spontaneous electrical activity were demonstrated in the lateral habenula neurons when isolated from the SCN (Zhao and Rusak, 2005), whilst rhythmic clock gene expression was later discovered in the lateral habenula cells (Guilding *et al*., 2010). These findings clearly highlight the involvement of this structure in biological time-keeping mechanisms and the results of the present study support a further potential involvement in seasonal pathways.

In the Syrian hamster, an area of the brain demonstrating strong *TAC1* mRNA expression was the fasciola cinereum. Chronic LP exposure resulted in up-regulation of *TAC1* in this area, compared to SP. The fasciola cinereum (FC), also known as gyrus fasciolaris, is a small continuation of the most anterior part of the hippocampal formation, which is a functional unit composed of the entorhinal area, the gyrus dentatus, the cornu Ammonis and the subiculum (Powell and Hines, 1975; Rakic and Nowakoswki, 1981; Teyler and DiScenna, 1984; Amaral and Campbell, 1986; Squire, 1986). Very little is known about the FC, however it does appear to be composed of granular and pyramidal cells, which are clustered in distinct parts. Measurements of

TAC1 in the FC following acute exposure to LP did not reveal a significant difference between any of the time points, suggesting that expression in this region is not part of an acute response to LP (data not shown), but a result of chronic exposure.

TAC1 expression was also found in other components of the hippocampus including the CA_2 field, which is a histological division of the *cornu Ammonis* structure, otherwise known as Ammon's horn (Lewis, 1923). SubP-immunoreactive cells have previously been demonstrated in the CA_1 and CA_3 fields in association with acute prolonged epileptic seizures (Liu *et al*., 1999). In addition, nerve fibre terminals of neuromediators such as SubP, vasopressin, somatostatin, neuropeptide Y and α–melanocyte stimulating hormone (α-MSH) have also been found in the hippocampus (Nieuwenhuys, 1985), however their role in this tissue structure is not yet clear. Furthermore, through its projections to the paraventricular hypothalamic nucleus, the hippocampus may inhibit hypophyseal ACTH secretion (Duvernoy, 2005). This finding is in favour of a hippocampal regulation of the hypothalamo-hypophyseal axis, which could prove useful in determining an association between the seasonal changes in hippocampal gene expression and the regulation of hypophyseal hormone secretion.

The hippocampus has long been associated with learning and memory (DeJong, 1973), however more recently, studies have revealed the importance of this structure in motivational processes and the control of behaviour related to food and appetite (Tracy *et al*., 2001). Considering the photoperiod-regulated effects on body weight and food intake in seasonal mammals, there could potentially be a link associating the seasonal differences in *TAC1* expression in this region of the brain to the substantial physiological changes induced by this same structure.

Intriguingly, these three distinct regions of the hippocampal formation, FC, IG and CA_2, that express *TAC1* in the Syrian hamster, have previously been linked with a strong expression of basic fibroblast growth factor (bFGF) mRNA, using ISH in the adult rat brain (Emoto *et al*., 1989). The cells expressing bFGF were determined to be neurons based on their morphology and location within the hippocampus. Furthermore, bFGF is found in the ovine PT and its regulation in this region is thought to be sensitive to photoperiod, with a higher expression of mRNA detected in LP animals compared to SP (Graham *et al*., 1999). However, bFGF is not thought to be directly involved in the seasonal regulation of lactotrophs as it does not increase prolactin secretion from ovine

PD cells (Hazlerigg *et al*., 1996; Graham *et al*., 1999). On the other hand, there is evidence suggesting that oestrogen-induced bFGF can increase prolactin secretion in rat pituitary tumours (Heaney *et al*., 1999). It would therefore be of interest to see whether bFGF co-localizes with the seasonally-regulated expression of *TAC1* in the hippocampal FC cells and whether these two factors are indirectly involved in a seasonal mechanism.

The likelihood of a photoperiod-regulated event occurring in the hippocampus is further strengthened by the presence of melatonin binding sites and transcripts of melatonin receptors in this region of the brain (Laudon *et al*., 1988; Mazzuchelli *et al*., 1996; Nonno *et al*., 1995; Musshoff *et al*., 2002). In addition, in the rat hippocampus the melatonin binding sites appear to display diurnal variations in the density of expression, with high expression during the night and low expression during the day (Laudon *et al*., 1988), which most probably explains the nocturnal increase in neuronal excitability of the hippocampal cells in response to melatonin (Musshoff *et al*., 2002). Further work is needed in order to decipher the exact role of the seasonal *TAC1* changes observed in the hippocampus of the Syrian hamster and how these may be melatonin-related.

To summarize, in this chapter I observed the expression of *Eya3* in the PT following LP exposure in both F344/NHsd rats and Syrian hamsters, a finding which is consistent with previous studies carried out in birds, sheep and mice, and which supports the proposed role of *Eya3* as a common first response LP-induced gene amongst different species. The precise functional interactions downstream of *Eya3* up-regulation remain to be determined. However, given the differences in timing of *Eya3* induction observed in the acute rat studies compared to those in sheep it would not be unreasonable to suggest that perhaps the immediate downstream seasonal mechanisms regulated by Eya3 differ depending on the extent of the photoperiodic nature of the animal. Furthermore, given the lack of *TAC1* expression in the rat PT it is not yet possible to assign a direct involvement of this gene in an Eya3-driven pathway, or indeed in the PT-mediated regulation of prolactin. Although the present study did not reveal an expression of *TAC1* in the PT, it did demonstrate the photoperiod-regulated expression of this gene in other areas of the brain, such as the ependymal layer and hypothalamus, that have previously been associated with seasonal mechanisms controlling thyroid hormone regulation and reproduction. It would therefore be of interest in future studies,

to determine the role of neurokinins in the brain with regards to seasonality, as it is currently unclear.

CHAPTER 5: Developing an *in vitro* model for studying prolactin regulation in the rat pituitary

5.1 Introduction

In temperate zones, the primary cue for species to adapt their physiology according to the time of year is photoperiod. This information is received by the eyes and translated into a melatonin signal via the pineal gland. The PT, with its high density of MT1 receptors, receives the signal and is thought to communicate it to the anterior pituitary and ultimately regulate seasonal prolactin secretion. Plentiful evidence is in support of the hypothesis that the PT acts to convey the seasonal signal by releasing messenger molecules, in an anterograde manner, to control functions of the PD (Lincoln and Clarke, 1994; Lincoln and Clarke, 1995; Hazlerigg *et al*., 1996; Morgan *et al*., 1996; Stirland *et al*., 2001; Graham *et al*., 2002). These messengers, known as 'tuberalins', although not yet identified, display certain characteristics. They are secreted from the PT and are found in PT-conditioned medium; they act on the PD to stimulate prolactin secretion and their secretion is dependent on photoperiod, enhanced by forskolin and this effect reversed by melatonin (Morgan *et al*., 1992; Morgan *et al*., 1994b; Morgan *et al*., 1996; Hazlerigg *et al*., 1996; Morgan, 2000).

The strongest evidence implicating the PT as a mediator of the photoperiodic effects of melatonin in the regulation of prolactin secretion comes from the sheep hypothalamo-pituitary disconnection studies of Lincoln and Clarke (Lincoln and Clarke, 1994). By surgically removing the nerve fibres connecting the median eminence to the pituitary whilst sparing the blood supply, they created a model whereby the hypothalamic input to the pituitary was lost. Despite the absence of this connection, the sheep continued to display seasonal rhythmicity in prolactin regulation when exposed to alternating photoperiods, thus demonstrating the importance of the PT in relaying seasonal information to the PD region of the pituitary.

Several studies have been conducted in pursuit of PT-specific secretory factors that could fulfil the role of a messenger molecule. The polypeptides secreted by PT-specific cells were first identified by exposing ovine PT primary cultures to [^{35}S]methionine and then analysing the biosynthetically labelled products of the conditioned medium using fluorography (Morgan *et al*., 1992). This method resulted in the identification of various compounds between 14-100kDa, the most intensely labelled of these being a 72-kDa protein, designated p72 (Morgan *et al*., 1992; Morgan *et al*., 1994b). Furthermore, the forskolin-induced synthesis and secretion of some of these compounds appeared to be

reversed by melatonin (Morgan *et al*., 1992). A similar study using bovine PT explants also identified a protein in the conditioned medium that was 72-kDa, as well as another one of 21-kDa (Guerra at Rodriguez, 2001). Antibodies were then raised against these two compounds and immunostaining of the bovine PT revealed binding to secretory granules of PT cells, suggesting that both these compounds are stored and then released from PT endocrine cell populations. Although much progress has been made in the field, the elusive PT-secreted factor, which was termed 'tuberalin', currently remains unidentified.

One of the most recent breakthroughs in the search for tuberalin came from the studies by Skinner and colleagues (Skinner *et al*., 2009a; Skinner, 2009) who demonstrated an abundant presence of SubP-immunoreactive cells in the PT of sheep, which was considerably higher than in the anterior pituitary gland. Further characterisation of the PT revealed the expression of mRNA encoding preprotachykinin A, the precursor of the neurokinins Substance P (SubP) and Neurokinin A (NKA), that have long been associated with prolactin regulation (Kato *et al*., 1976; Pisera *et al*., 1994). These results provided strong evidence to support the hypothesis that neurokinins have an important role in seasonal hormone regulation. Later studies further encouraged the proposed role of these compounds as potential tuberalins, by revealing that *TAC1,* the gene encoding neurokinins, is photoperiodically-regulated in the sheep PT, being strongly activated by LP exposure, whilst the products of this gene, Substance P(1-7), a known bioactive cleavage product of SubP (Hall *et al*., 1989), and NKA, were found to induce PRL secretion when applied to primary pituitary cells (Dupré *et al*., 2010).

In this study, neurokinins, which are currently the most likely candidates believed to have a role in seasonal prolactin release, were tested with the help of a transgenic Fischer-344 (F344) rat, the generation of which has been previously described (Semprini *et al*., 2009). In this transgenic model, firefly luciferase gene expression is directed by a large fragment of the human prolactin locus, providing a useful molecular tool for measuring prolactin promoter activity, through the expression of the reporter gene luciferase. The present study utilises the line 49 transgenic animals (F344-Luc49), which had the transgene inserted on an autosome, allowing it to be integrated into the genome in high copy number (Semprini *et al*., 2009). The F344 transgenic model has been successfully utilised in studies characterizing prolactin gene expression in living

pituitary tissue slices and dispersed primary cell cultures (Harper *et al*., 2010; Featherstone *et al*., 2011).

The objectives of the work described in this chapter were firstly to establish a functional luciferase reporter system using either pituitary tissue slices or dispersed pituitary cells from the transgenic F344 rat and secondly to test the action of neurokinin molecules on prolactin promoter activity. In order to further investigate the involvement of these molecules within the rat pituitary, measurements of prolactin and luteinizing hormone (LH) release in the cell culture medium were also made, following incubation with the candidate molecules. Finally, backcrossing of the transgenic F344 rat onto a photoperiodic F344/NHsd genetic background was carried out and third generation offspring carrying the luciferase transgene within a photoperiodic genotype, were also used to test the action of neurokinins.

5.2 Methods

In order to study the effects of neurokinins on prolactin promoter activity, a luciferase reporter system was set up using tissue from adult transgenic F344-Luc49 rats. Initially, pituitary glands of either male or female F344-Luc49 transgenic rats were cultured *in vitro* either as tissue slices or enzymatically-dispersed cells, allowing monitoring of prolactin regulation through the use of real-time recording of luciferase expression. Using luminometer luciferase assays, an end-point measurement of total luciferase activity was made following each treatment incubation period. The luminometer luciferase assay was validated with the use of forskolin as a positive control, and then the substances Neurokinin A, Substance P and Substance P(1-7), were tested for their effects on prolactin promoter activity, measured in relative luminescence units (RLU). Subsequently, PRL and LH release in the culture medium, were measured using ELISA (section 2.7).

5.3 Results

5.3.1 Establishing a luciferase reporter system for measuring prolactin promoter activity using transgenic F344-Luc49 rat pituitary tissue

5.3.1.a Use of F344-Luc49 pituitary tissue slices in the Lumicycle system

A luciferase reporter system was initially set up using pituitary tissue slices taken from transgenic F344-Luc49 rats. The use of this system was successful in confirming the

action of the adenylyl-cyclase activator forskolin, of inducing prolactin promoter activity (Figure 5.1). The main advantage of the Lumicycle system is that it offers a real-time monitoring of luciferase expression and thus of transcriptional activity of the prolactin gene. Luciferase expression is measured by adding 1mM of luciferin substrate to the tissue culture medium, which is converted into oxyluciferin in the presence of luciferase, resulting in the emission of light, in the form of detectable luminescent counts. Prior to any treatment, the tissue was left un-stimulated for 24h, in the presence of luciferin, during which luminescence imaging was performed. The effect of treatment with 5μM forskolin was observed as a sharp increase in luminescent counts, which peaked at approximately 11.5-12h following the addition of the compound (Figure 5.1B). Furthermore, forskolin appeared to have an effect in the presence of both culture media supplemented with regular FBS and media supplemented with dextran (dxt) charcoal-stripped FBS. Charcoal-stripping is an effective way of removing many steroid, peptide and thyroid hormones from fetal bovine serum (Cao *et al*., 2009). The response seen in the presence of regular FBS was significantly greater compared to the dxt-FBS, suggesting that perhaps the endogenous molecules in the regular serum, which are not present in the charcoal-stripped serum, were having an inducing effect on the response to forskolin treatment (Figure 5.1B). For that reason, all subsequent treatments were carried out in media containing charcoal-stripped FBS. Tissue slices were able to survive in culture for approximately 30 days and repeatedly responded to forskolin treatment, which was demonstrated by an increase in luminescent signal. This confirmed viability of the tissue in the culture conditions used, as only viable cells can generate adenosine triphosphate (ATP), which is required by luciferase to catalyze the production of light (DeLuca and McElroy, 1984). Slices that did not show any response in luminescent signal following treatment with forskolin were presumed to have died in culture. Treatment of tissue slices with forskolin revealed that the magnitude of the response varied across different slices. This inter-slice variability was a limitation, as it prevents the accurate assessment of other treatments with perhaps less robust responses than forskolin. For this reason, the tissue slice method was not considered appropriate to investigate further the action of neurokinins.

5.3.1.b Use of F344-Luc49 dispersed pituitary cells in the PMT-Lumicycle system

In an attempt to overcome the limitation of slice to slice variability and thus eliminate the inconsistency observed when using the tissue slice method described above, the

reporter system was set up using dispersed pituitary cells instead of tissue slices, allowing a homogeneous starting point of 1 x 10^6 cells per culture dish. Forskolin (5μM) induced a more than 5-fold increase in luminescent counts compared to the control group (Figure 5.2A). Nonetheless, the responses often varied in amplitude and when other treatments were applied, such as 1μM thyrotrophin-releasing hormone (TRH), a known regulator of PRL expression (Semprini *et al.*, 2009), no response was observed (data not shown). The sensitivity of this system was considered limited as it only provided a distinct acute response in the presence of forskolin and therefore would not be very efficient in measuring responses to less active secretagogues. In addition, the culturing of cells using this method was very technically demanding and if chosen to set up the *in vitro* system would have required the use of large numbers of animals, to ensure proper experimental design and statistical analysis.

5.3.1.c Use of F344-Luc49 dispersed pituitary cells in luminometer luciferase assay measurements

The issues encountered with the two systems described above were addressed by considering the use of a luminometer luciferase assay for measuring prolactin promoter activity. This assay involved culturing dispersed transgenic F344-Luc49 pituitary cells in 96-well plates for 3 days, before treating with the candidate substances for the required time, and then calculating total luminescent output at the end of treatment by lysing the cells and measuring luciferase content. Treatment with 1μM forskolin resulted in a >4-fold increase in luminescent counts compared to the control group (Figure 5.2B) and this was reproducible. The uniformity achieved with this method, made it the most functional and efficient system for measuring luciferase and thus prolactin promoter activity in response to different treatments. Furthermore, it was the most ethical in terms of animal welfare, as it allowed the use of a minimum number of animals.

5.3.2 Backcrossing F344-Luc49 rats onto an NHsd genetic background

Transgenic F344-Luc49 male rats were backcrossed with non-transgenic F344/NHsd females over 3 generations (Figure 5.3). The resulting offspring that carried the Luc-49 transgene were termed F344-Luc49/NHsd and were used to test the effects of neurokinins NKA, SubP and SubP(1-7), on PRL promoter activity and hormone release. This allowed a comparison to be made between the responses of F344-Luc49 and F344-

Luc49/NHsd rats, the latter carrying 87.5% of the genetic material of the photoperiodic parental F344/NHsd strain.

5.3.3 Effect of neurokinins on PRL promoter activity in transgenic F344-Luc49 and F344-Luc49/NHsd rat cell cultures using luminometer assays

5.3.3.a Effect of NKA, SubP and SubP (1-7) on PRL promoter activity in F344-Luc49 male- and female-derived cell cultures

F344-Luc49 male- and female-derived cell cultures treated with NKA, SubP and SubP (1-7) each at two different concentrations, 10^{-7} M and 10^{-6} M, for 11.5h of treatment did not reveal any significant effects on PRL promoter activity. Forskolin (1μM) was used as a positive control and induced a 4.1-fold and 2.2-fold increase in PRL promoter activity in males and females respectively (Figure 5.4A-B). DMSO (0.1%) was used as a control treatment. The mean raw luciferase data for the control groups revealed a 15-fold higher baseline of expression in the female-derived cultures (646618.4 ± 138908.6 RLU), compared to the male-derived (42131.3 ± 6409.4 RLU).

5.3.3.b Effect of NKA, SubP and SubP (1-7) on PRL promoter activity in F344-Luc49/NHsd male- and female-derived cell cultures

Treatment with NKA, SubP and SubP (1-7) did not have a significant effect on PRL promoter activity in F344-Luc49/NHsd male- or female-derived cell cultures following either 6h or 11.5h of incubation (Figure 5.5A-D). Forskolin (1μM) induced a 3.7-fold increase in PRL promoter activity following 11.5h of treatment, in the male cultures and a 1.5-fold increase in the female cultures following the same duration of treatment. The induction caused by forskolin after 6h of treatment was only 1.5-fold in the males, whilst no induction was seen in the corresponding cultures using cells derived from female rats. DMSO (0.1%) was used as a control treatment. The mean raw luciferase data for the control groups revealed a 7-fold and 14-fold higher baseline of expression in the female-derived cultures compared to the male-derived, at 6h and 11.5h of treatment respectively.

5.3.4 Effect of neurokinins on hormone release in the medium of dispersed cell cultures of transgenic F344-Luc49 and F344-Luc49/NHsd rats

5.3.4.a Effect of NKA, SubP and SubP (1-7) on PRL and LH release in F344-Luc49 male- and female-derived cell cultures

PRL release was not induced following 11.5h of treatment with NKA, SubP or SubP(1-7) in the F344-Luc49 male- or female-derived cultures (Figure 5.6A-B). Forskolin did have a significant inducing effect on prolactin release, which was 1.4-fold in the male and 1.3-fold in the female cultures. The baseline concentration of prolactin release for the control groups was 8-fold higher in the female-derived cultures (5668.7 ± 646.9 ng/ml) compared to the male-derived (495.7 ± 85.5 ng/ml).

Measurements of LH release in the medium did not reveal a significant effect of the neurokinin treatments, however forskolin did increase LH release by 1.3-fold in the male- and by 4-fold in the female-derived cultures, compared to the corresponding controls (Figure 5.7A-B). The baseline concentration of LH release for the control groups was 3.9-fold higher in the female-derived cultures (10.9 ± 1.2 ng/ml) compared to the male-derived (2.8 ± 0.3 ng/ml).

5.3.4.b Effect of NKA, SubP and SubP (1-7) on PRL and LH release in F344-Luc49/NHsd male- and female-derived cell cultures

Forskolin was used as a positive control in these experiments and only induced a significant 1.6-fold increase in PRL release in the F344-Luc49/NHsd male-derived cultures following 11.5h of treatment (Figure 5.8B), and a 1.4-fold increase in LH release in the F344-Luc49/NHsd male-derived cultures (Figure 5.9B), whilst no effect was observed at 6h of treatment or in any of the female-derived cultures. The neurokinin treatments did not have an effect in either of these experiments. For both PRL and LH release, the baseline concentration for the control groups was approximately 10-fold higher in the female-derived cultures compared to the male-derived, at both 6h and 11.5h of treatment.

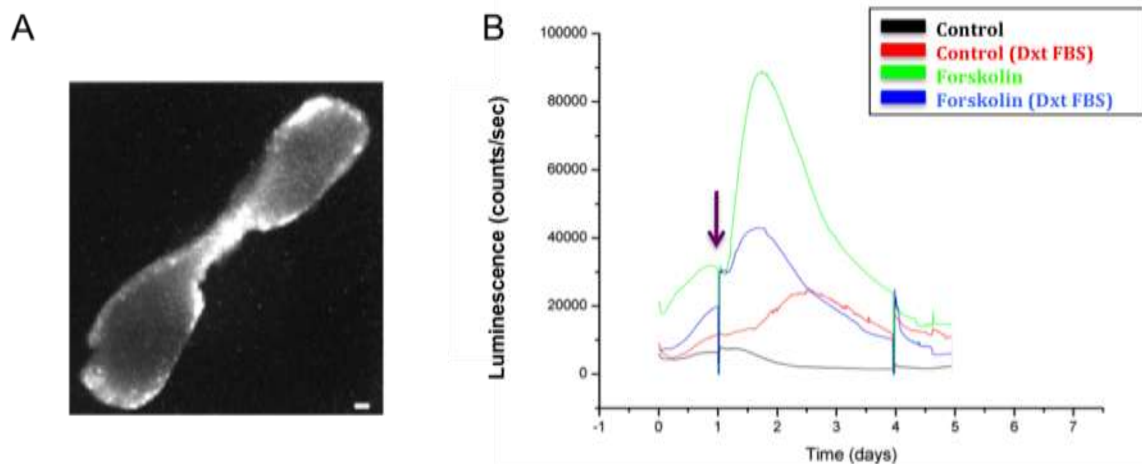


Figure 5.1: Real-time bioluminescent imaging of PRL promoter activity in transgenic pituitary tissue slices.

(A) Luminescence image of a 400μm-thick coronal-orientated tissue slice from an adult F344-Luc49 male pituitary. The luminescent signal indicates transcriptionally active lactotrophs cells. The image was collected using a photon-counting charge-coupled device camera (Hamamatsu Photonics) attached to a microscope (Zeiss) and using a Fluar 2.5X magnification 0.12NA (numerical aperture) objective. Generated by K Featherstone and CV Harper and reproduced from Featherstone *et al* (Featherstone *et al*., 2011). **(B)** Luminescence activity in four pituitary tissue slices, each treated with either 0.1% DMSO (control) or 5μM FSK in two different types of culture media, one supplemented with dextran charcoal stripped FBS (Dxt FBS) and the other with regular FBS. The arrow indicates time of treatment (Day 1). Scale bar in A: 200μm.

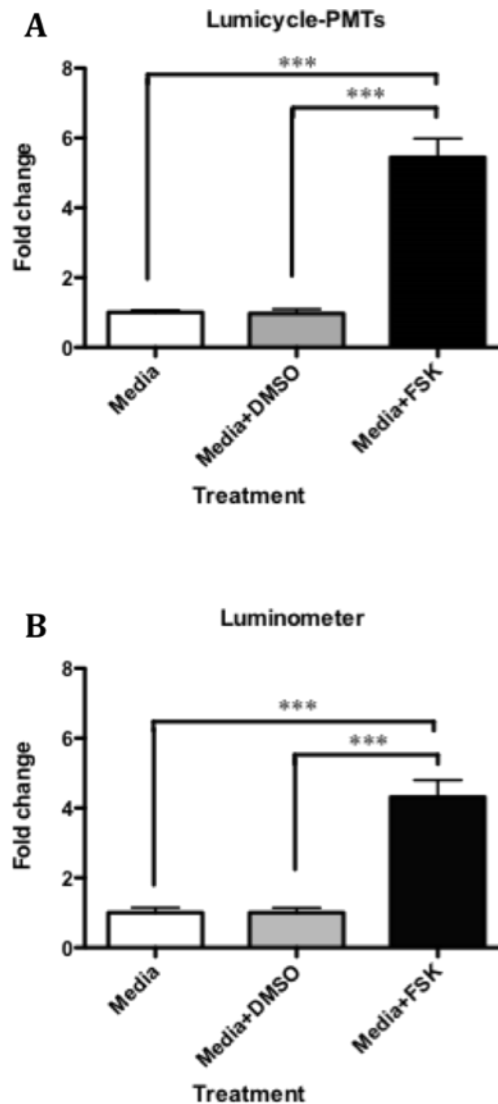


Figure 5.2: Establishing a functional luciferase reporter system for measuring prolactin promoter activity.

Measuring the effects on prolactin promoter activity (luminescent signal), of treatment with culture media, DMSO and FSK (11.5h treatment), using **(A)** the Lumicycle-PMT reporter system (DMSO-0.1%; FSK-5μM) and **(B)** the luminometer assay system (DMSO-0.1%; FSK-1μM). In both systems, the increase in luminescent signal following treatment with media + FSK was significantly higher compared to the media alone (***$p<0.001$) and media + DMSO controls (***$p<0.001$; One-way ANOVA with Dunnett's Multiple Comparison post hoc analysis). Data shown are means ± SE and represent fold changes in luminescent signal compared to control values (n=3 independent experiments).

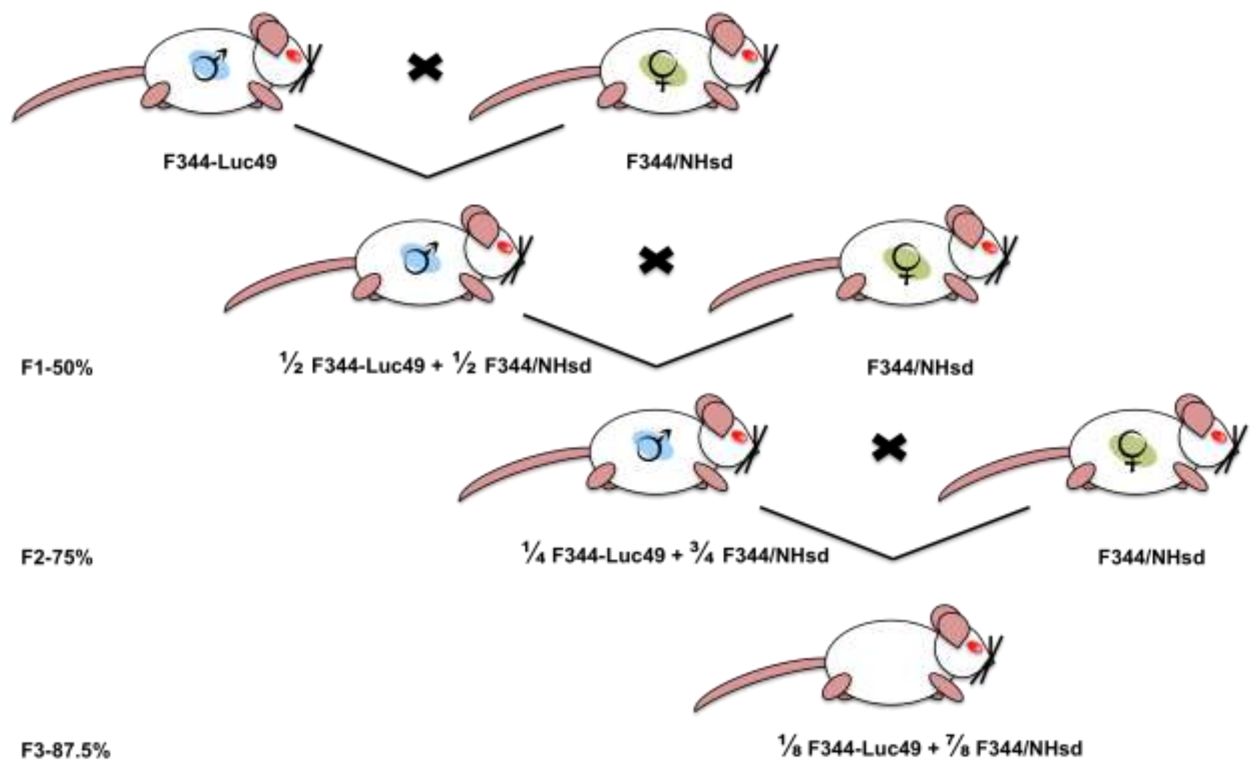


Figure 5.3: Backcrossing of transgenic F344-Luc49 rats onto a photoperiodic F344/NHsd background.

Male transgenic F344-Luc49 rats were crossed with wild-type F344/NHsd females over 3 generations (F1-F3) to produce F344-Luc49/NHsd rats carrying 87.5% of the genetic material of the parental F344/NHsd strain whilst also maintaining the Luc49 transgene.

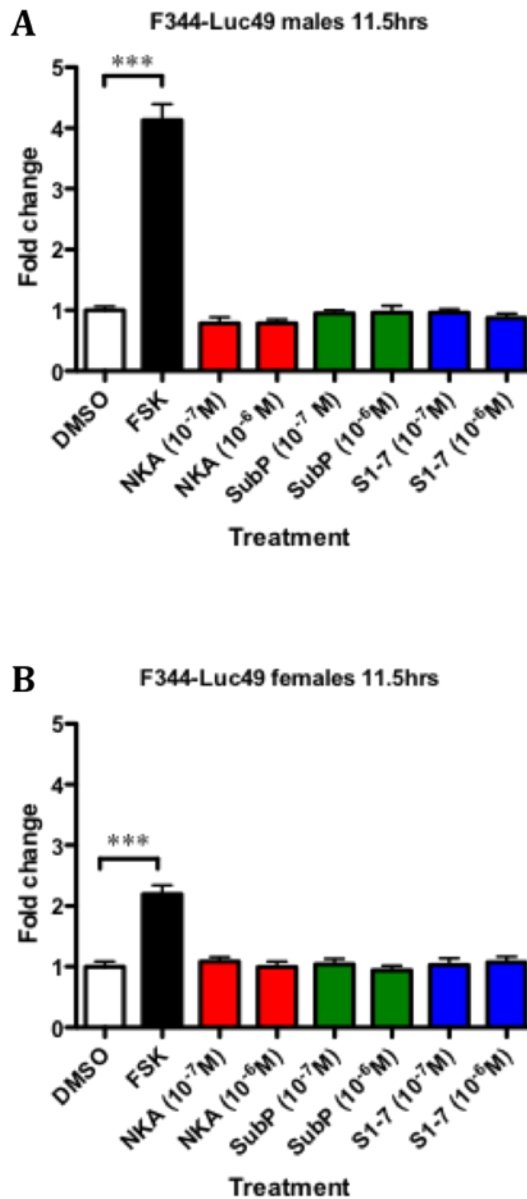


Figure 5.4: Neurokinin A, Substance P and Substance P (1-7) have no effect on the prolactin promoter activity of dispersed F344-Luc49 rat pituitary cells.

Fold changes in PRL promoter activity (luminescence), relative to DMSO (control), in **(A)** F344-Luc49 male- and **(B)** F344-Luc49 female-derived cultures, following 11.5h treatment with Forskolin (FSK) 1µM, Neurokinin A (NKA) at 10^{-7} M and 10^{-6} M, Substance P (SubP) at 10^{-7} M and 10^{-6} M; and a short fragment of Substance P (S1-7) at 10^{-7} M and 10^{-6} M. FSK induced a significant fold change in PRL promoter activity, compared to control, in both males and females (***$p<0.001$; One-way ANOVA with Dunnett's Multiple Comparison post hoc analysis). Data shown are means ± SE and represent fold changes compared to control values (n=3 independent experiments). The mean raw luciferase data for the control group in (A) was 42131.3 ± 6409.4 RLU, and in (B) was 646618.4 ± 138908.6 RLU.

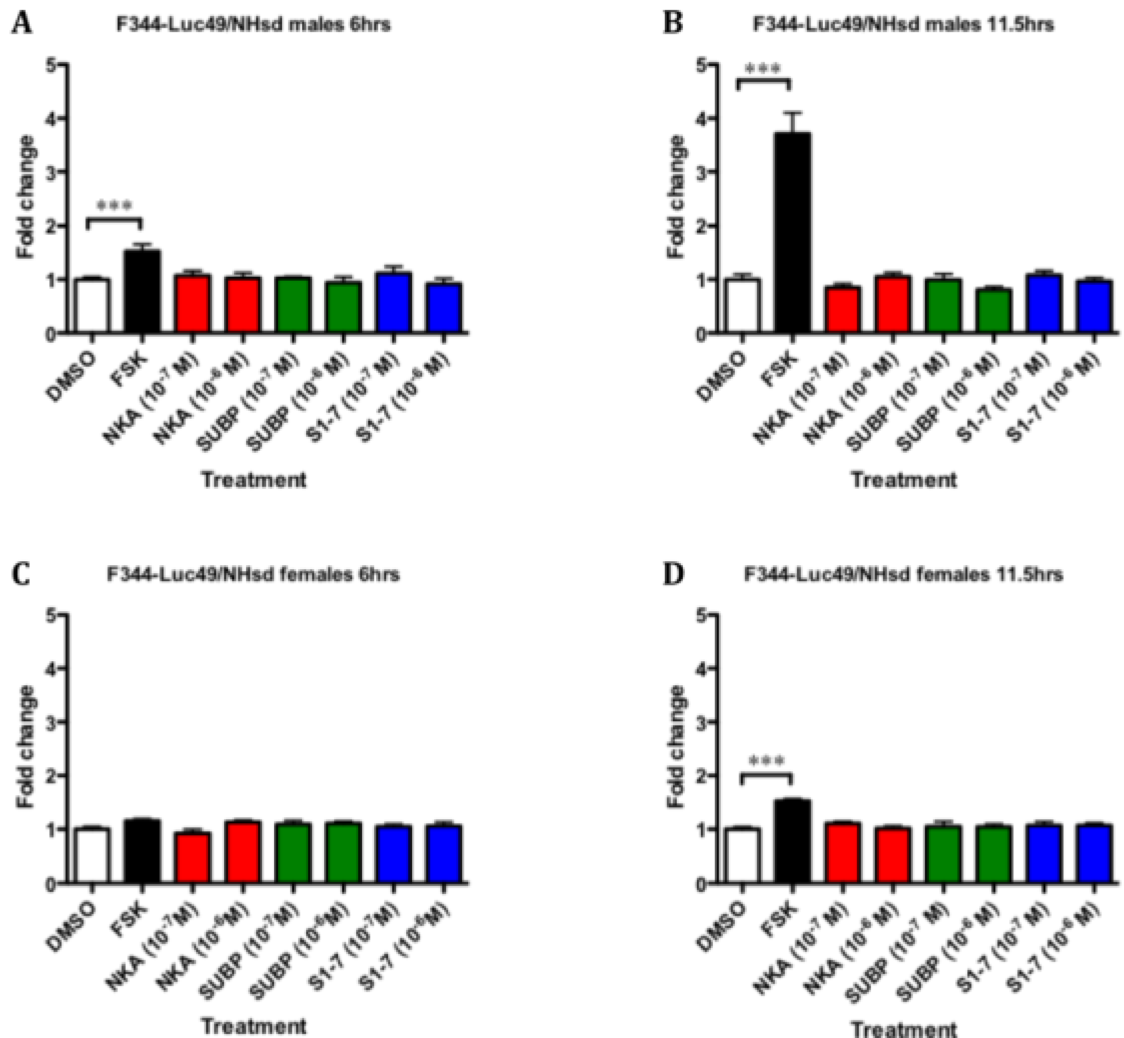


Figure 5.5: Neurokinin A, Substance P and Substance P (1-7) have no effect on the prolactin promoter activity of dispersed F344-Luc49/NHsd rat pituitary cells.

Fold changes in PRL promoter activity (luminescence), relative to DMSO (control), in F344-Luc49/NHsd male-derived cultures following **(A)** 6h treatment and **(B)** 11.5h treatment, and in F344-Luc49/NHsd female-derived cultures following **(C)** 6hr treatment and **(D)** 11.5h treatment with Forskolin (FSK) 1μM, Neurokinin A (NKA) at 10^{-7} M and 10^{-6} M, Substance P (SubP) at 10^{-7} M and 10^{-6} M; and a short fragment of Substance P (S1-7) at 10^{-7} M and 10^{-6} M. FSK induced a significant fold change in PRL promoter activity, compared to control, in males following 6h and 11.5h treatment, and in females following 11.5h treatment (***$p<0.001$; One-way ANOVA with Dunnett's Multiple Comparison post hoc analysis). Data shown are means ± SE and represent fold changes compared to control values (n=3 independent experiments). The mean raw luciferase data for the control group in (A) was 80948.3 ± 25432 RLU, in (B) was 46350 ± 16808.3, in (C) was 582367.7 ± 70355.4 and in (D) was 670805.5 ± 149956.1.

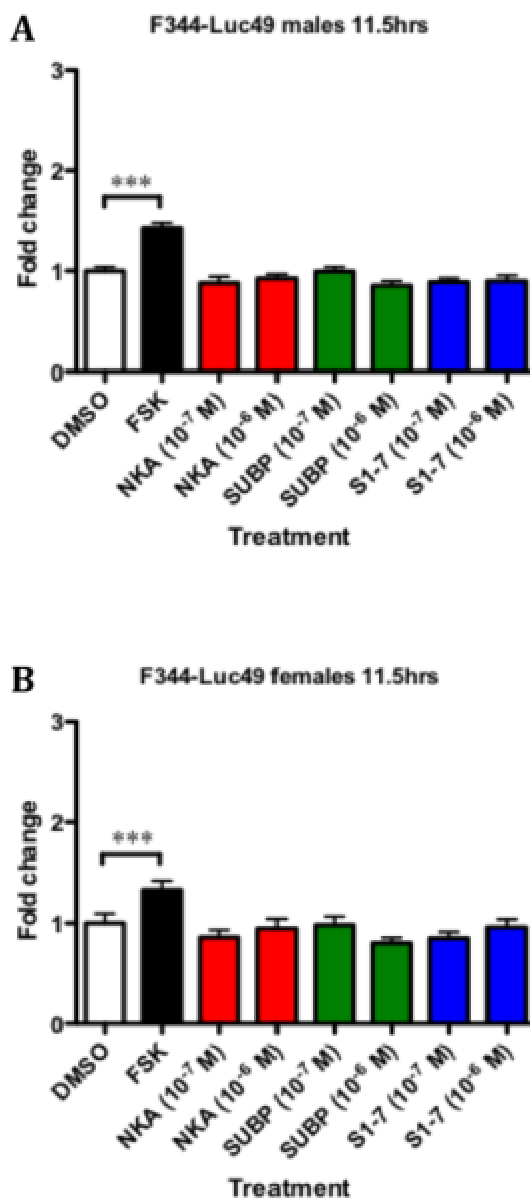


Figure 5.6: Neurokinin A, Substance P and Substance P (1-7) have no effect on prolactin release in the medium of dispersed F344-Luc49 rat pituitary cells.

Fold changes in PRL release (concentration) in the medium, relative to DMSO (control), in **(A)** F344-Luc49 male- and **(B)** F344-Luc49 female-derived cultures, following an 11.5h treatment with Forskolin (FSK) 1μM, Neurokinin A (NKA) at 10^{-7} M and 10^{-6} M, Substance P (SubP) at 10^{-7} M and 10^{-6} M; and a short fragment of Substance P (S1-7) at 10^{-7} M and 10^{-6} M. FSK induced a significant fold change in PRL release in the medium, compared to control, in both males and females (***$p<0.001$; One-way ANOVA with Dunnett's Multiple Comparison post hoc analysis). Data shown are means ± SE and represent fold changes compared to control values (n=3 independent experiments). The mean raw prolactin concentration data for the control group in (A) was 495.7 ± 85.5 ng/ml and in (B) was 5668.7 ± 646.9 ng/ml.

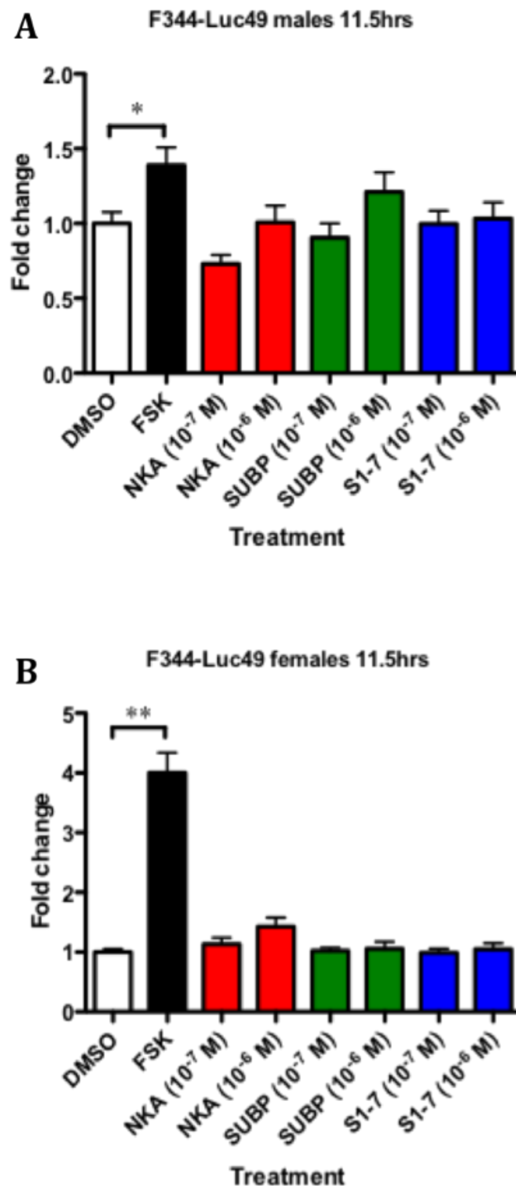


Figure 5.7: Neurokinin A, Substance P and Substance P (1-7) have no effect on LH release in the medium of dispersed F344-Luc49 rat pituitary cells.

Fold changes in LH release (concentration) in the medium, relative to DMSO (control), in **(A)** F344/NCrHsd male- and **(B)** F344/NCrHsd female-derived cultures, following an 11.5h treatment with Forskolin (FSK) 1μM, Neurokinin A (NKA) at 10^{-7} M and 10^{-6} M, Substance P (SubP) at 10^{-7} M and 10^{-6} M; and a short fragment of Substance P (S1-7) at 10^{-7} M and 10^{-6} M. FSK induced a significant fold change in LH release in the medium, compared to control, in males (*$p<0.05$) and a highly significant fold change in females (***$p<0.001$; One-way ANOVA with Dunnett's Multiple Comparison post hoc analysis). Data shown are means ± SE and represent fold changes compared to control values (n=3 independent experiments). The mean raw LH data for the control group in (A) was 2.8 ± 0.3 ng/ml and in (B) was 10.9 ± 1.2 ng/ml.

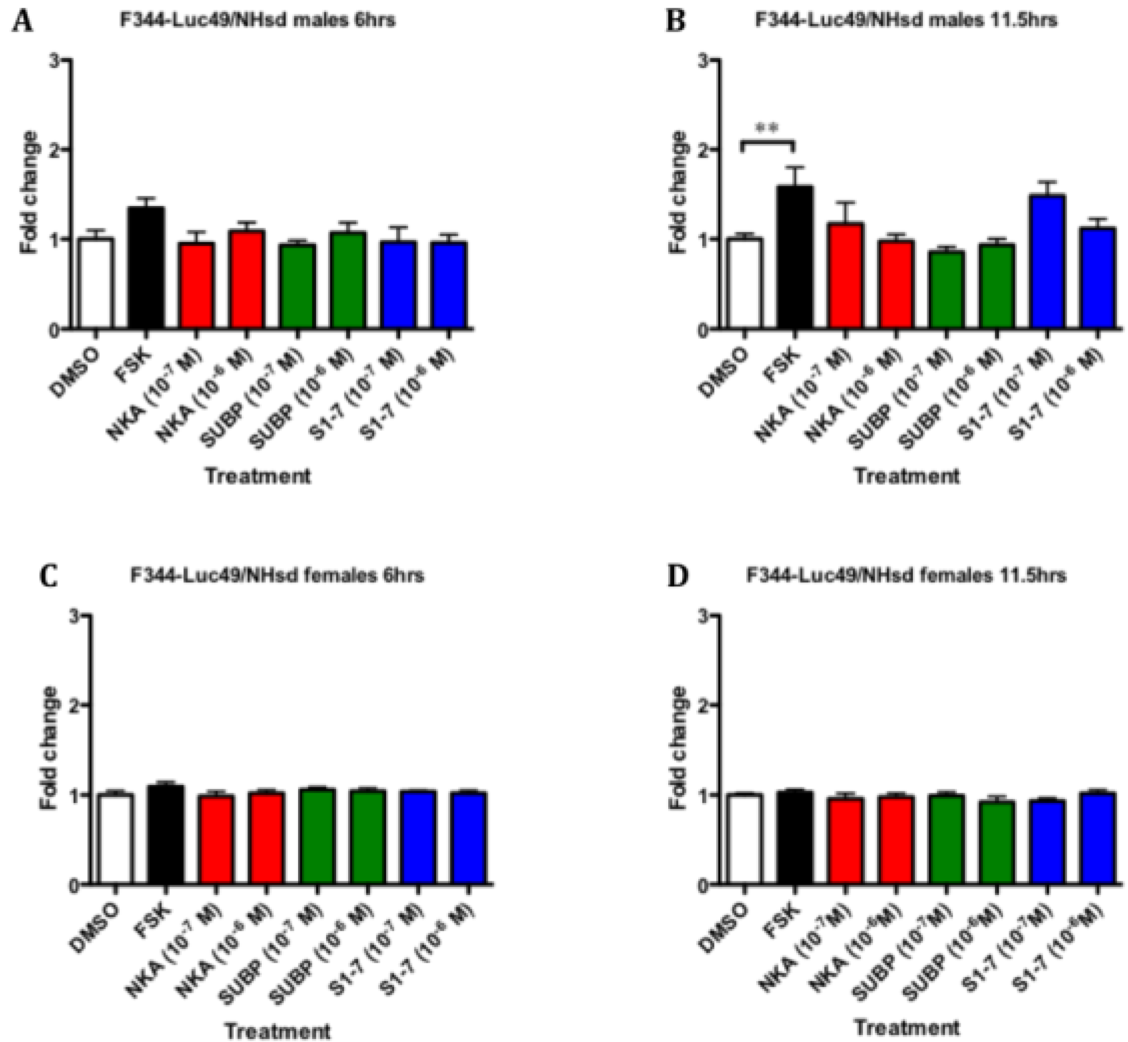


Figure 5.8: Neurokinin A, Substance P and Substance P (1-7) have no effect on prolactin release in the medium of dispersed F344-Luc49/NHsd rat pituitary cells.

Fold changes in PRL release (concentration) in the medium, relative to DMSO (control), in F344-Luc49/NHsd male-derived cultures following **(A)** 6h treatment and **(B)** 11.5h treatment, and in F344-Luc49/NHsd female-derived cultures following **(C)** 6hr treatment and **(D)** 11.5h treatment with Forskolin (FSK) 1µM, Neurokinin A (NKA) at 10^{-7} M and 10^{-6} M, Substance P (SubP) at 10^{-7} M and 10^{-6} M; and a short fragment of Substance P (S1-7) at 10^{-7} M and 10^{-6} M. FSK induced a significant fold change in PRL release in the medium, compared to control, in males following 11.5h treatment, (**$p<0.01$; One-way ANOVA with Dunnett's Multiple Comparison post hoc analysis). Data shown are means ± SE and represent fold changes compared to control values (n=3 independent experiments). The mean raw prolactin data for the control group in (A) was 510.1 ± 147.6 ng/ml, in (B) was 590.2 ± 175.5 ng/ml, in (C) was 5095 ± 744.1 ng/ml and in (D) was 6253.5 ± 892.8 ng/ml.

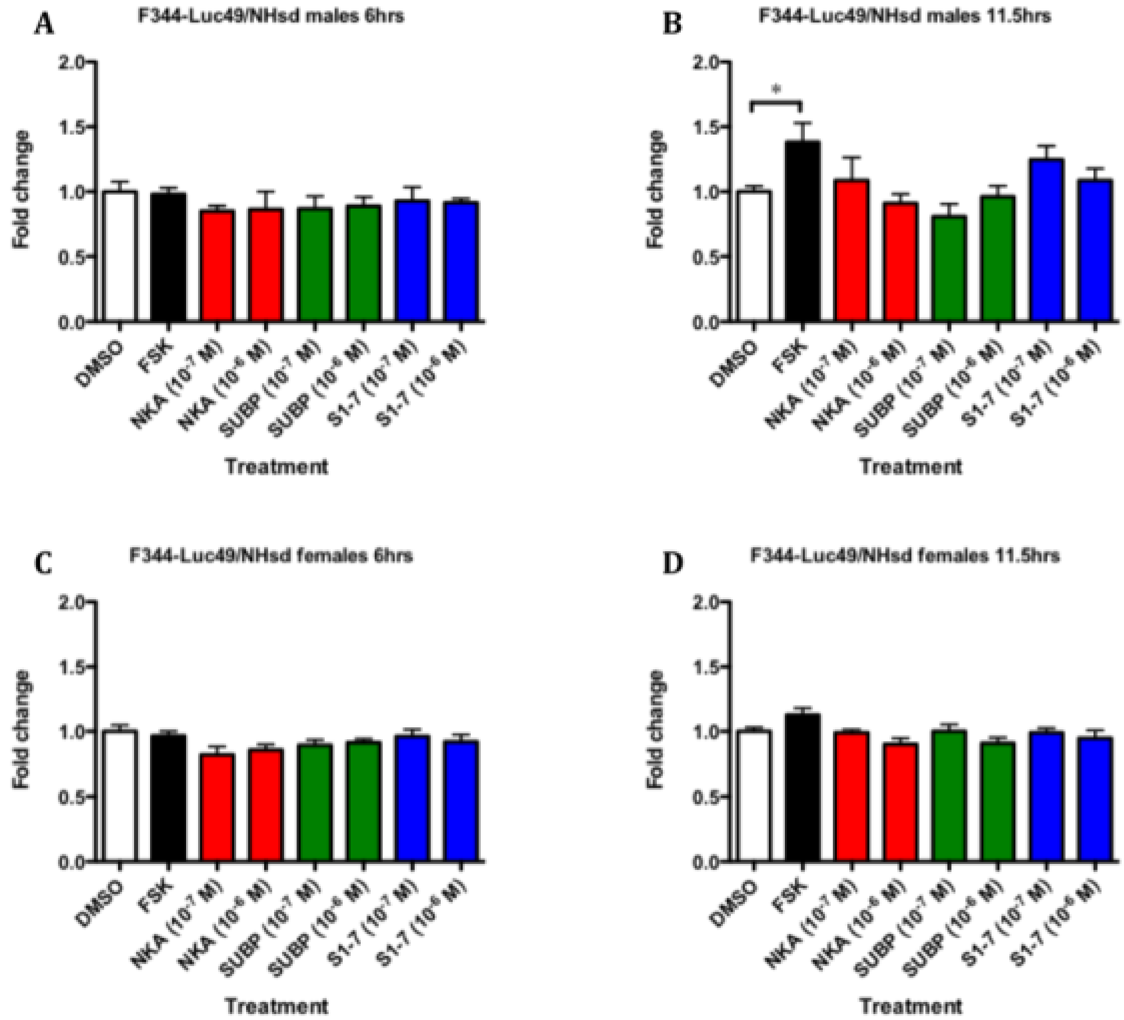


Figure 5.9: Neurokinin A, Substance P and Substance P (1-7) have no effect on LH release in the medium of dispersed F344-Luc49/NHsd rat pituitary cells.

Fold changes in LH release (concentration) in the medium, relative to DMSO (control), in F344-Luc49/NHsd male-derived cultures following **(A)** 6h treatment and **(B)** 11.5h treatment, and in F344-Luc49/NHsd female-derived cultures following **(C)** 6h treatment and **(D)** 11.5h treatment with Forskolin (FSK) 1μM, Neurokinin A (NKA) at 10^{-7} M and 10^{-6} M, Substance P (SubP) at 10^{-7} M and 10^{-6} M; and a short fragment of Substance P (S1-7) at 10^{-7} M and 10^{-6} M. FSK induced a significant fold change in LH release in the medium, compared to control, in males following 11.5h treatment, (*$p<0.05$; One-way ANOVA with Dunnett's Multiple Comparison post hoc analysis). Data shown are means ± SE and represent fold changes compared to control values (n=3 independent experiments). The mean raw LH data for the control group in (A) was 9.2 ± 1.1 ng/ml, in (B) was 7.9 ± 0.8 ng/ml, in (C) was 87.2 ± 12.7 ng/ml and in (D) was 85.5 ± 13.9 ng/ml.

	F344-Luc49	F344-Luc49	F344-Luc49/NHd	F344-Luc49/NHsd
	Male-derived	**Female-derived**	**Male-derived**	**Female-derived**
(A) PRL promoter activity fold-change:				
At 6h			**1.5** (96,407.1± 28,667.5 RLU)	**-** (662,691.5 ± 77,878.1 RLU)
At 11.5h	**4.1** (168,372.7± 23,193.1 RLU)	**2.2** *** (1,395,514.1± 291,809.8 RLU)	**3.7** (130,094.2± 36,994.2 RLU)	**1.5** *** (1,144,146.8 ± 232,740.3 RLU)
(B) PRL release fold-change:				
At 6h			**-** (658.1 ± 193.7 ng/ml)	**-** (5775.2 ± 769.7 ng/ml)
At 11.5h	**1.4** (722.6 ± 129.7 ng/ml)	**1.3** (7531.6 ± 729.1 ng/ml)	**1.6** (712.8 ± 164.3 ng/ml)	**-** (6656.2 ± 873.2 ng/ml)
(C) LH release fold-change:				
At 6h			**-** (8.6 ± 0.9 ng/ml)	**-** (77.8 ± 8.9 ng/ml)
At 11.5h	**1.3** (3.7 ± 0.6 ng/ml)	**4** (42 ± 5 ng/ml)	**1.4** (12.6 ± 2.4 ng/ml)	**-** (85.8 ± 10.1 ng/ml)

Table 5.1: Summary of effects of forskolin on (A) PRL promoter activity, (B) PRL release and (C) LH release, of F344-Luc49 and F344-Luc49/NHsd male- and female- derived primary cell cultures following the incubation times indicated.

The response to forskolin is presented as a fold-change (in bold) compared to the appropriate control, the symbol (-) indicates no effect compared to control. The corresponding raw data for each mean forskolin response is also indicated in brackets, shown as means ± SE, in RLU or ng/ml. The asterisks in row 5 indicate a significant difference between the two groups (***$p<0.001$; Unpaired t-test).

5.4 Discussion

This chapter presents the establishment of a functional *in vitro* luciferase reporter system, using dispersed pituitary cell cultures derived from transgenic F344 rats, which was subsequently used to investigate the action of neurokinins on the regulation of prolactin promoter activity.

In order to establish a functional reporter system, different methods were tested and the first involved real-time imaging of transgenic pituitary tissue slices. However, the preliminary results following the use of pituitary slices to examine the effect of forskolin, did not give a clear outcome and there are several factors that may have contributed to this. Slice to slice variation and thickness of slices which may result in necrosis of the inner tissue layer due to unavailability of culture medium to these cells, along with the regional distribution of lactotrophs and other cell-types throughout the pituitary, are determining factors which may have given variable results between different slice samples, making it hard to assess the true effect of treatment. Although the tissue slice system was not the method of choice to investigate the actions of neurokinins, it did prove useful in establishing the time-point of peak forskolin induction following treatment, through real-time visualisation of the luminescence recordings.

Previous imaging of adult pituitary tissue slices has revealed that the luminescent signal is greater at the periphery of the tissue, suggesting that lactotrophs in this region have the highest transcriptional activity (Harper *et al*., 2010; Featherstone *et al*., 2011). This may be reflective of a spatial organisation that coincides with functional differences between lactotrophs located peripherally and centrally, something that has previously been demonstrated (Boockfor and Frawley, 1987). It is therefore evident that by imaging intact tissue slices there is a clear benefit of preserved tissue architecture and paracrine signalling, which is inevitably lost during enzymatic dispersion of the tissue, and may significantly affect transcriptional activity of the cells (Harper *et al*., 2010; Featherstone *et al*., 2011). Furthermore, studies have demonstrated that the PRL promoter activity of dispersed primary cell cultures, generated from rat pituitaries, appears pulsatile and heterogeneous between individual cells, displaying no signs of synchronisation (Shorte *et al*., 2002; Semprini *et al*., 2009; Harper *et al*., 2010; Featherstone *et al*., 2011), unlike the co-ordinated response observed in pituitary tissue

slices (Harper *et al*., 2010; Featherstone *et al*., 2011). Seemingly therefore, the tissue slice method would be the most favourable approach, however due to the limitations discussed above, this system was not considered appropriate for the purposes of the present study.

Subsequently, real-time imaging of dispersed cell cultures was assessed as a potential reporter system. Although this method was successful in demonstrating an inducing-effect of forskolin on transcriptional activity within the cell cultures, the responses varied in amplitude and when other activators were applied to the system such as TRH, no effect was observed (data not shown). Furthermore the culturing of primary cells using this system was very technically demanding and several experimental repeats were required before statistical conclusions could be made. This approach was therefore not considered to be sensitive or efficient enough to use as an *in vitro* system for studying the effect of neurokinin treatments.

Dispersed cell cultures were subsequently used in a different reporter system, whereby luciferase activity was measured using a microplate luminometer assay. This involved culturing the cells in 96-well plates, applying treatment for the required time and then lysing the cells and measuring total luciferase activity. Use of the luminometer luciferase system demonstrated that forskolin treatment (1μM) triggered a strong fold-induction of PRL promoter activity, compared to control, that was of approximately double the magnitude in male cell cultures compared to female cell cultures in both the F344-Luc49 and the F344-Luc49/NHsd strains. However, the mean baselines of raw luminescence data, as well as hormone concentration in the medium, were consistently higher in the female cultures. Early molecular characterisation studies of these luciferase transgenic rats indeed demonstrated a higher expression of luciferase in the female transgenics compared to the male transgenics (Semprini *et al*., 2009). Furthermore immunohistochemistry revealed a significantly denser expression of lactotrophs in the female pituitary compared to the male, whilst quantitative PCR (qPCR) revealed greater expression of endogenous rat prolactin in the females (Semprini *et al*., 2009).

Once established, the *in vitro* reporter system was used to investigate the action of neurokinins on PRL promoter activity. Although several previous studies report an effect of neurokinins on PRL secretion, there is no evidence of neurokinins being tested

for their direct effect on transcriptional activity of the PRL promoter. This study therefore provides the first investigation into the action of neurokinins on transcriptional mechanisms. The results demonstrate that treatment with NKA, SubP and SubP(1-7) did not induce an effect on transcriptional activity of the prolactin promoter, in either the F344-Luc49 or F344-Luc49/NHsd rats.

Furthermore, this study was unable to demonstrate an effect of NKA, SubP or SubP(1-7) on prolactin release in the pituitary cultures of F344-Luc49 or F344-Luc49/NHsd rats. Measurements of prolactin promoter activity and hormone release were made following incubation of the cells with each treatment for 11.5h in the F344-Luc49-derived cell cultures and following either 6h or 11.5h in the F344-Luc49/NHsd-derived cultures. In addition, neurokinins did not provide a measurable response in LH release at the time-points considered. Female cell cultures appeared to respond to forskolin treatment more dramatically, with a 4-fold increase in LH release compared to only a 1.3-fold induction in the male cultures. This would suggest that the female rats had larger amounts of LH stored in their cells compared to the males. This is likely to be reflective of the fact that the female rats were collected on the day of proestrous when LH reserves would have been at their peak, in anticipation of the preovulatory LH surge, which is known to occur on the afternoon of proestrous (Kalra *et al*., 1983).

Although the results described here do not support a prolactin-releasing role for neurokinins in the rat pituitary, there is evidence in the literature to suggest otherwise. SubP has long been associated with the *in vivo* secretion of prolactin in rats following intravenous injection (Kato *et el*., 1976), whilst its involvement with LH secretion, both positive and negative, was only later discovered *in vitro* (Shamgochian and Leeman, 1992; Duval *et al*., 1998). A study by Henriksen *et al* (Henriksen, 1995) showed that SubP had a dose-dependent effect on prolactin secretion from cultured peripubertal female rat anterior pituitary cells which were grown on collagen-coated micro beads and placed in a perfusion system. However, the authors were unable to explain why the release of prolactin persisted in this system even after the neurokinins were removed from the medium to which the cells were exposed. The possibility of NKA having an effect on prolactin secretion was first suggested following *in vivo* studies in rats and hamsters whereby anti-NKA serum injection resulted in a significant decrease of serum prolactin (Pisera *et al*., 1991). The doses chosen in the present study in order to investigate the effects of neurokinins in the rat pituitary *in vitro,* were based on the

analysis of previous rodent studies that suggest a dose of at least 100nM in order to induce a hormonal response (Shamgochian and Leeman, 1992; Henriksen *et al*., 1995; Mau *et al*., 1997; Pisera *et al*., 1998; Duvilanski *et al*., 2000). However it is not known whether this is a physiological dose.

Early reports on the effects of neurokinins on hormone secretion in the rat pituitary provided conflicting results. The discrepancies observed may have been due to the age and sex of the animals used, as well as other methodological differences between experiments. For these reasons, all the animals used in this study were of adult age (6-8 months), and both males and females were used separately in order to eliminate potential gender inconsistencies.

Interestingly, it has been shown that the number of anterior pituitary SubP binding sites varies over the estrous cycle of female rats and is highest during the day of proestrous (Kerdelhué *et al*., 1985). This would imply that the effects of neurokinins on the anterior pituitary also depend on the hormonal environment, a suggestion that has been made by a few studies (Kalra *et al*., 1992; Sahu and Kalra, 1992; Shamgochian and Leeman, 1992). This proposal is further supported by evidence showing that oestradiol can influence the effect of other known prolactin-releasing agents such as TRH (Giguére *et al*., 1982) and VIP (Pizzi *et al*., 1992), on the responsiveness of lactotrophs.

An investigation into the effect of hormonal status on the action of NKA on PRL secretion in female rats (Pisera *et al*., 1998), showed that NKA had a significant dose-dependent inducing effect on prolactin secretion from hemipituitaries and anterior pituitary cell cultures of rats culled on the days of proestrous and estrous, when the endogenous concentrations of estradiol are high, but not of diestrous (Pisera *et al*., 1998). For this reason, in the present study we only collected pituitaries from rats during proestrous, to investigate the actions of neurokinins. The protocol used for enzymatic dispersion and culturing of the cells was similar between the present study and that of the Pisera group, and included a 3-day incubation period of the cell cultures, prior to treatment with the neurokinins. However, some differences do exist in the methodology between the two studies. For example, in the study by Pisera *et al* (Pisera *et al*., 1998) treatment with NKA was limited to 60min, whereas in this study treatment was applied for 11.5h in the F344-Luc49 rats, and for both 6h and 11.5h in the F344-Luc49/NHsd rats. Furthermore, the culture serum used in the Pisera studies (Pisera *et al*., 1998) was

not dextran charcoal-stripped and would therefore have included endogenous molecules, which may have influenced the action of the neurokinins on prolactin release, possibly giving a false positive result. A previous study by the same authors also demonstrated an effect of NKA on cultured anterior pituitary cells of male rats (Pisera *et al*., 1994), thus suggesting that the endogenous sex steroids in the male are sufficient to induce NKA action. However, the results of the present study are not in accordance with any of the above observations by the Pisera group. NKA did not have an *in vitro* effect on prolactin secretion in either the male- or female-derived cell cultures.

More recently, NKA and SubP(1-7) have also been shown to stimulate prolactin secretion from dispersed ovine pituitary cells after 1hr of treatment (Dupré *et al*., 2010). These measurements were made by radioimmunoassay, unlike the measurements made in this study, which were carried out using ELISA. It remains possible that the longer duration of treatment with neurokinins in the present study, may not have been optimal for detecting a response, however, a preliminary experiment which was carried out and involved treating the cells for 1h, failed to demonstrate a significant effect of either forskolin or the neurokinins, and so this time-point was not used for further investigations (data not shown).

When comparing the responses to forskolin of the two different strains of rats, the F344-Luc49 and the F344-Luc49/NHsd, there does not appear to be a significant difference in the male-derived cultures in terms of PRL promoter activity, PRL release or LH release. In the female-derived cultures however, there is an obvious difference in response to forskolin between the two strains, across PRL promoter activity, PRL release and LH release. In the female F344-Luc49/NHsd cultures, forskolin did not have an inducing effect on either PRL or LH release, however a 1.3-fold and 4-fold induction in PRL and LH respectively, is observed in the female F344-Luc49 cultures. Furthermore there was a significant difference between the responses in PRL promoter activity between the two female strains at 11.5h of treatment with forskolin, with a 2.2-fold induction in the F344-Luc49 versus a 1.5-fold induction in the F344-Luc49/NHsd-derived cultures (***$p<0.001$; Unpaired t-test; Table 5.1). This indicates that the F344-Luc49/NHsd-derived cultures were for some reason less responsive to forskolin compared to the F344-Luc49-derived cultures. It could be suggested that the 12L:12D light schedule under which all the rats were housed in the animal facility, may have been inhibitory for

the backcrossed photo-responsive F344-Luc49/NHsd rats, and this may have had a dampening effect on the later responses of the cells to treatment, in culture. It has previously been shown that newly weaned F344 rats kept under intermediate photoperiods (12L:12D) can demonstrate inhibited responses such as reduced reproductive maturation, lower food intake and lower body weight, relative to rats kept under long photoperiods, (16L:8D) in the first few weeks of treatment and also following longer-term treatment (Shoemaker and Heideman, 2002). Evidence of tissue sensitivity to photoperiod history comes from previous studies using cultured Syrian and Siberian hamster PD cells that demonstrated increased sensitivity to dopamine when derived from SP-housed animals (Steger *et al.*, 1983; Badura, 1996). In addition, the stimulatory effect of VIP on prolactin secretion from Siberian hamster anterior pituitaries in culture was also dependent on the prior photoperiod-housing of the animals (Badura and Goldman, 1997). It is therefore possible that pre-exposure of the F344-Luc49/NHsd rats to an intermediate photoperiod may have influenced the responsiveness of the cells in culture, and this would only have occurred in the photo-responsive strain.

In summary, the present findings suggest that the neurokinins tested [NKA, SubP and SubP(1-7)] do not directly influence PRL promoter activity, PRL release or LH release in rat pituitary cultures. These observations therefore do not support the hypothesis that neurokinins fulfil the role of 'tuberalin' in the rat pituitary. However, certain considerations must be taken into account, one being the duration of treatment with the neurokinins, which may not have been optimal for the particular system used. Furthermore, disruption of the normal architecture of the pituitary through dispersion of the tissue may have been detrimental on the normal responsiveness of the lactotrophs, as it renders the pituitary cells dissociated from their physiological environment and natural communicative partners, whilst it is also possible that prolonged exposure of the cells to the proteolytic activity of trypsin during enzymatic dispersion may have resulted in damage to the transmembrane neurokinin receptors, leading to dysregulation of cellular functions. Consequently, treatment with neurokinins would not have evoked a prolactin response. Intriguingly, in the study by Pisera and colleagues (Pisera *et al.*, 1998) trypsin was also used during the enzymatic dispersion of the anterior pituitary cells, however a trypsin inhibitor was included in the dispersion buffer, which may have had an instrumental role in limiting the disruptive activity of trypsin. As an alternative, collagenase could be considered for use in future studies, as it is known to be less

damaging to membrane proteins and receptors, compared to trypsin. Whilst the present study was unable to investigate the action of neurokinins using pituitary tissue slices, future use of such a system, or perhaps even of *in vivo* studies, could be helpful in resolving the issue of whether or not the three-dimensional structure of the pituitary gland and paracrine communication between the cells, is important in regulating the prolactin response to neurokinin treatment. As previously mentioned, dopamine is the primary neuroendocrine inhibitor of prolactin secretion from the anterior pituitary gland *in vivo*. In an *in vitro* setting and in the absence of dopaminergic control, lactotroph cells continuously produce prolactin, and therefore it is possible that in the cell culture experiments prolactin secretion was already maximal, thus rendering the cells unresponsive to any further stimulation by the neurokinins. This reasoning could also potentially explain the unresponsiveness of the F344-Luc49/NHsd-derived pituitary cells to forskolin, which is known to be a strong inducer of adenylyl-cyclase activity. Future studies could include the use of alternative cAMP-inducers as positive controls, such as adenosine and PACAP. Additionally, it would be useful if prior to any further testing of neurokinin action on prolactin release, an assay was performed, to confirm that the neurokinins are indeed intact and reaching their targets. The former could be achieved through western blot analysis, a technique used to detect specific proteins in a sample, by size, through gel electrophoresis and then staining with antibodies specific to the target protein. In order to determine whether or not the peptides are reaching their targets, a fluorescent protein tag could be added to the peptides of interest and then fluorescent microscopy used to visualise the peptides and determine whether or not they are binding to the cell membrane.

In conclusion, whilst the data presented in this chapter do not currently support a role for neurokinins in the regulation of prolactin, future studies would be needed to fully confirm the nature of neurokinin action within the rat anterior pituitary, with regards to prolactin, and to unequivocally discount the possibility of a tuberalin role for these molecules. Nonetheless, these results provide an extension of the literature and support the hypothesis that a wide array of determining factors synergize to influence the responsiveness of anterior pituitary lactotrophs to secretagogues and other stimuli, thus demonstrating the complexity of the neuroendocrine system.

CHAPTER 6: Neurokinin receptor expression in the rat pituitary

6.1 Introduction

In mammals, the biological actions of neurokinins are exerted through binding to the neurokinin receptors (NKRs), members of the superfamily of G-protein coupled transmembrane receptors. There are three main types of NKRs, namely neurokinin receptor 1 (NK1R), neurokinin receptor 2 (NK2R) and neurokinin receptor 3 (NK3R), to which all neurokinins can bind, albeit with varying degrees of affinity (Pennefather *et al*., 2004). So whilst SubP preferentially binds NK1R, NKA has a preferential affinity for NK2R and NKB has a preferential affinity for NK3R. The mammalian NKRs were identified and characterised through electrophysiological measurements (Harada *et al*., 1987) and molecular cloning studies (Masu *et al*., 1987; Yokota *et al*., 1989; Sasai and Nakanishi, 1989; Shigemoto *et al*., 1990). The signal transduction mechanism of activation of the NKR, following the binding of its ligand, involves phosphatidylinositol hydrolysis and mobilization of intracellular calcium (Harada *et al*., 1987; Nakanishi, 1990).

Despite the extensive literature surrounding NKRs and the studies reporting the presence of neurokinin binding sites in the rat anterior pituitary (Kerdelhué *et al*., 1985; Larsen *et al*., 1989; Mikkelsen *et al*., 1989; Winkler *et al*., 1995), there is still conflicting evidence regarding their distribution in the different cell-types of the rat PD. Given the potential involvement of neurokinins in the photoperiodic transmission of information in the PD, during the seasonal pathway of prolactin regulation (Skinner *et al*., 2009; Skinner, 2009; Dupré *et al*., 2010), it was of major interest to try and identify their receptors in the rat PD tissue.

The objectives of the work described in this chapter were firstly to detect the expression of neurokinin receptors within the F344/NHsd rat pituitary PD region, secondly to identify potential co-localized expression of these receptors to the different secretory cell-types and folliculo-stellate cells in this region and finally to test the hypothesis that expression of the different cell-types or the neurokinin receptors is regulated by photoperiod.

6.2 Methods

In order to investigate NKR expression in the pituitary and the effect of photoperiod on this expression, tissue was collected from male F344/NHsd rats, which were housed

under SP (8L:16D) or LP (16L:8D) conditions for 5 weeks (n=6 animals per photoperiod), during which time body weight and food intake were measured weekly to ensure the animals were responding appropriately to each photoperiod (data not shown). At the end of photoperiod treatment, the animals were killed at ZT4, and hypothalamic tissue blocks with the pituitary attached and the PT intact were collected, fixed and processed in order to generate tissue slices to be used for dual immunohistochemistry. Double immunostaining was carried out to investigate co-localization of PRL, LH, ACTH, S100, TSH and GH antigens with the neurokinin receptors, NK1R and NK2R in both SP and LP tissue (described in section 2.6). All double immunostaining images are shown at magnification x60. Cell counting (analysis of tissue) was carried out by choosing 6 areas per section of PD tissue analysed, using 4 sections per animal (3 animals per photoperiod) and then counting the number of hormone-expressing cells and the number of neurokinin receptor-expressing cells. The number of cells counted within each experiment was normalized and therefore different experiments could be pooled thus reducing inter-experimental variability. RT-PCR was also used to investigate NKR expression in rat pituitary tissue, which had not been pre-exposed to either SP or LP (details in section 2.8). Data in this chapter were analysed using unpaired t-test.

6.3 Results

6.3.1 Neurokinin receptor expression in the PD region of the F344/NHsd rat pituitary

Immunohistochemistry was used to detect the expression of neurokinin receptors in the PD region of the F344/NHsd rat pituitary. This revealed that neurokinin receptor 1 (NK1R) and neurokinin receptor 2 (NK2R) were expressed in the rat PD. Neurokinin receptor 3 (NK3R) could not be detected using this method. However, all three neurokinin receptors were detected using RT-PCR of total RNA from isolated rat anterior pituitaries. This technique resulted in the observation of clear signals of the expected size, allowing identification of NK1R, NK2R and NK3R. The three band signals were sized 393 base pairs (bp), 447bp and 505bp respectively (Figure 6.1).

6.3.2 Characterizing the cell types expressing neurokinin receptors in the F344/NHsd rat PD region of the pituitary

The cell-types identified in the pars distalis using immunohistochemistry were: lactotrophs (PRL-expressing), corticotrophs (ACTH-expressing), gonadotrophs (LH-expressing), folliculo-stellate cells (S100-expressing), thyrotrophs (TSH-expressing) and somatotrophs (GH-expressing). PRL expression was further detected in the rat anterior pituitary using diaminobenzidine staining, which revealed widespread expression of lactotrophs throughout the tissue (Figure 6.2). Dual immunohistochemistry was carried out to reveal potential co-localization patterns of PRL (Figure 6.3), ACTH (Figure 6.4), LH (Figure 6.5), S100 (Figure 6.6), TSH (Figure 6.7) and GH (Figure 6.8) antigens with the neurokinin receptors NK1R and NK2R in the rat pituitary PD region. NK1R, the main receptor for the neurokinin SubP, was localized in the corticotrophs (Figure 6.4C), whilst NK2R, the main receptor for NKA, was localized to gonadotrophs (Figure 6.5F) and folliculo-stellate cells (Figure 6.6F). A summary of all the merged expression profiles can be seen in Figure 6.9.

6.3.3 The effect of photoperiod on the expression of neurokinin receptors and of the different cell types in the pars distalis of the pituitary

Immunohistochemical analysis of rat anterior pituitary tissue sections taken from animals that had been exposed to either SP or LP for 5 weeks did not reveal a significant effect of photoperiod on the number of NK1R-expressing (Figure 6.10A) or NK2R-expressing cells (Figure 6.10B). However, analysis of the different cell types in the PD region of SP and LP animals revealed a significant effect of photoperiod on the number of PRL-expressing cells. The mean number of PRL-expressing cells per $225\mu m^2$ of pituitary tissue was significantly increased in LP compared to SP ($*p<0.05$; Figure 6.11A). No difference was observed in the number of the other hormone-expressing cells or the folliculo-stellate cells (Figure 6.11B-F). The number of cells expressing ACTH and NK1R, LH and NK2R, or S100 and NK2R were also counted in SP and LP, however no differences were found between the groups (data not shown). Thus, photoperiod did not significantly affect the co-localization patterns observed in the pituitary, but did alter the expression of PRL.

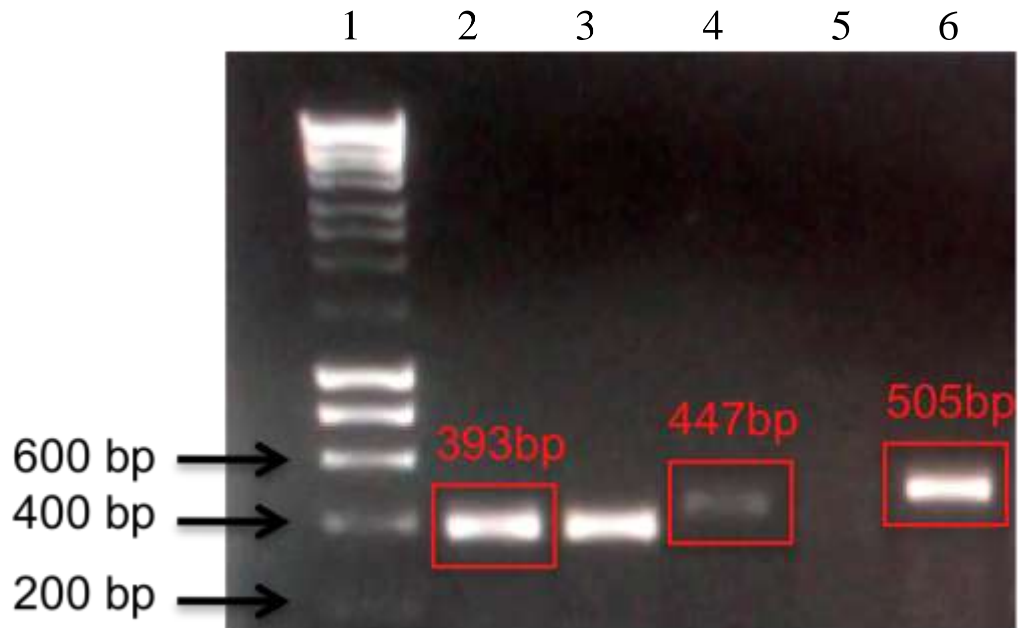


Figure 6.1: Gel electrophoresis of PCR products from rat pituitary mRNA demonstrating NKR expression.

SafeView staining of PCR products run on an agarose gel showed bands of the expected sizes: 393bp for NK1R (lane 2), 447bp for NK2R (lane 4) and 505bp for NK3R (lane 6). Specific primers were used to amplify the regions of NK1R, NK2R and NK3R, using RT-PCR of rat pituitary mRNA. Lane 1 contains DNA HyperLadder I. Lane 5 contains reaction negative control.

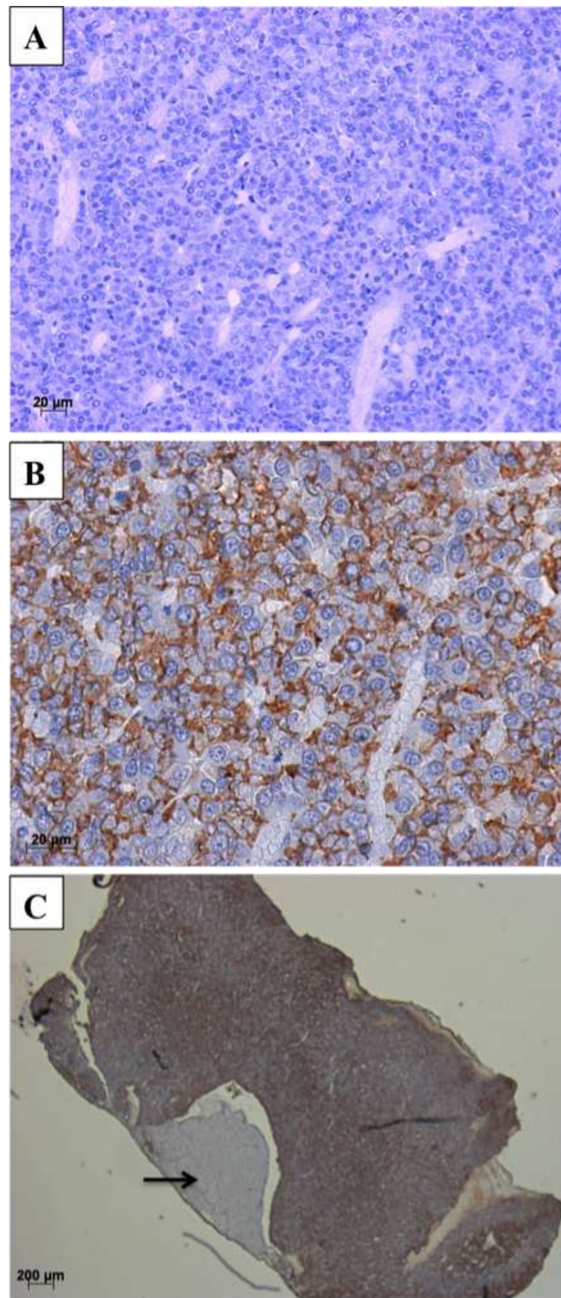


Figure 6.2: Diaminobenzidine staining showing the expression of PRL in the rat anterior pituitary.

Microscopic view of **(A)** Negative control, no PRL expression (no secondary antibody), **(B)** PRL expression (brown staining) shown in the pars distalis **(C)** Cross-section of the whole pituitary showing the anterior (indicated by brown PRL staining) and posterior (indicated by arrow) regions. Hematoxylin (blue) in A-C is an indicator of nuclear staining. Magnification of (A) is x20; (B) is x40 and (C) is x2. Scale bar: A,B =20µm; C = 200µm.

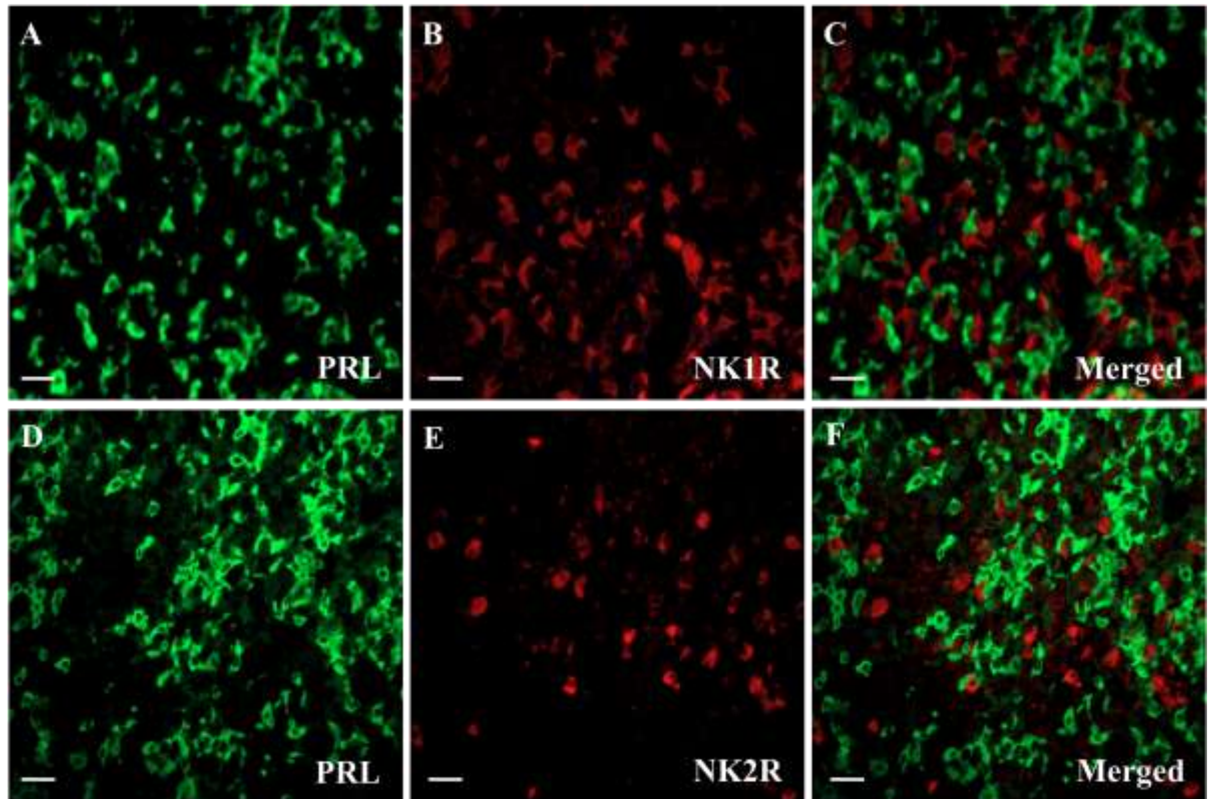


Figure 6.3: Localization of PRL, NK1R and NK2R protein immunofluorescence in the F344/NHsd rat PD region of the pituitary.

Microscopic view of double immunofluorescent staining showing the expression of (**A**,**D**) PRL (green), (**B**) NK1R (red), (**E**) NK2R (red) and the respective merged images (**C**) PRL+NK1R and (**F**) PRL+NK2R. No co-localizations were observed. Scale bar: 20µm

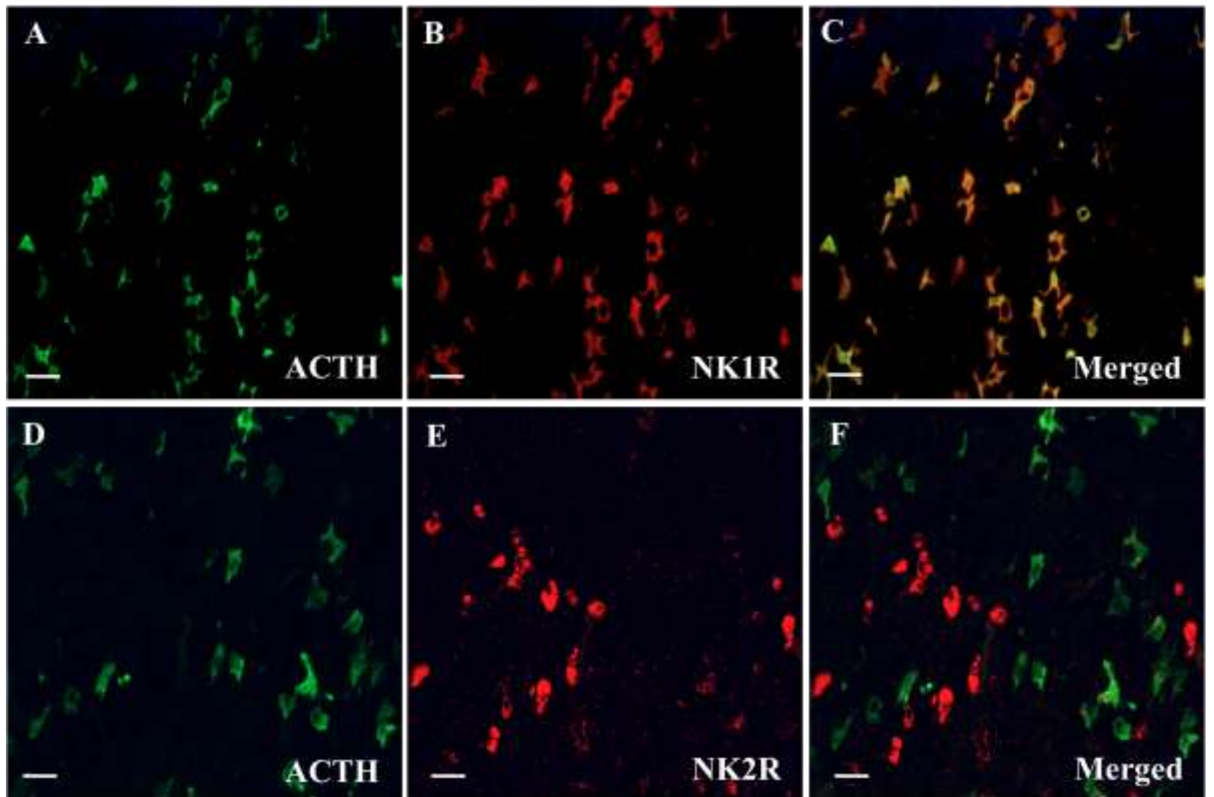


Figure 6.4: Localization of ACTH, NK1R and NK2R protein immunofluorescence in the F344/NHsd rat PD region of the pituitary.

Microscopic view of double immunofluorescent staining showing the expression of (**A**,**D**) ACTH (green), (**B**) NK1R (red), (**E**) NK2R (red) and the respective merged images (**C**) ACTH+NK1R (yellow indicates co-localization) and (**F**) ACTH+NK2R. Scale bar: 20µm

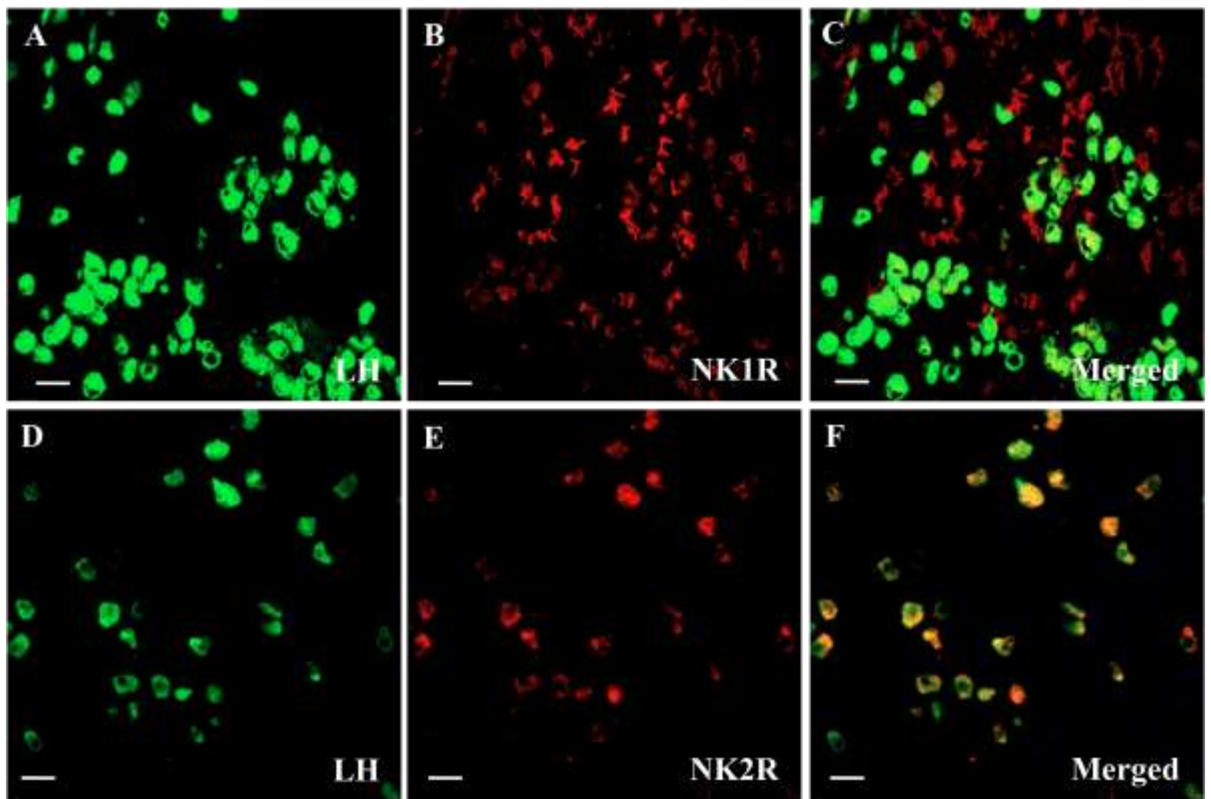


Figure 6.5: Localization of LH, NK1R and NK2R protein immunofluorescence in the F344/NHsd rat PD region of the pituitary.

Microscopic view of double immunofluorescent staining showing the expression of (**A**,**D**) LH (green), (**B**) NK1R (red), (**E**) NK2R (red) and the respective merged images (**C**) LH+NK1R and (**F**) LH+NK2R (yellow indicates co-localization). Scale bar: 20µm

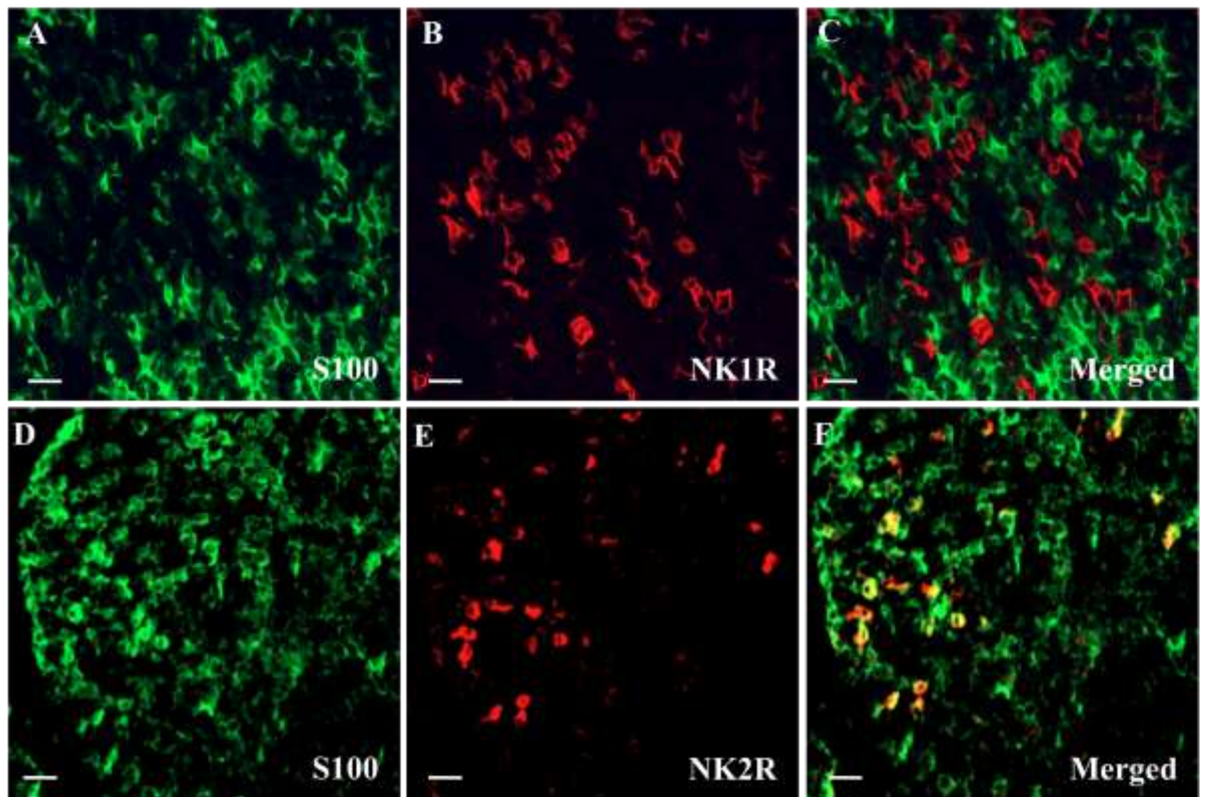


Figure 6.6: Localization of S100, NK1R and NK2R protein immunofluorescence in the F344/NHsd rat PD region of the pituitary.

Microscopic view of double immunofluorescent staining showing the expression of (**A**,**D**) S100 (green), (**B**) NK1R (red), (**E**) NK2R (red) and the respective merged images (**C**) S100+NK1R and (**F**) S100+NK2R (yellow indicates co-localization). Scale bar: 20µm

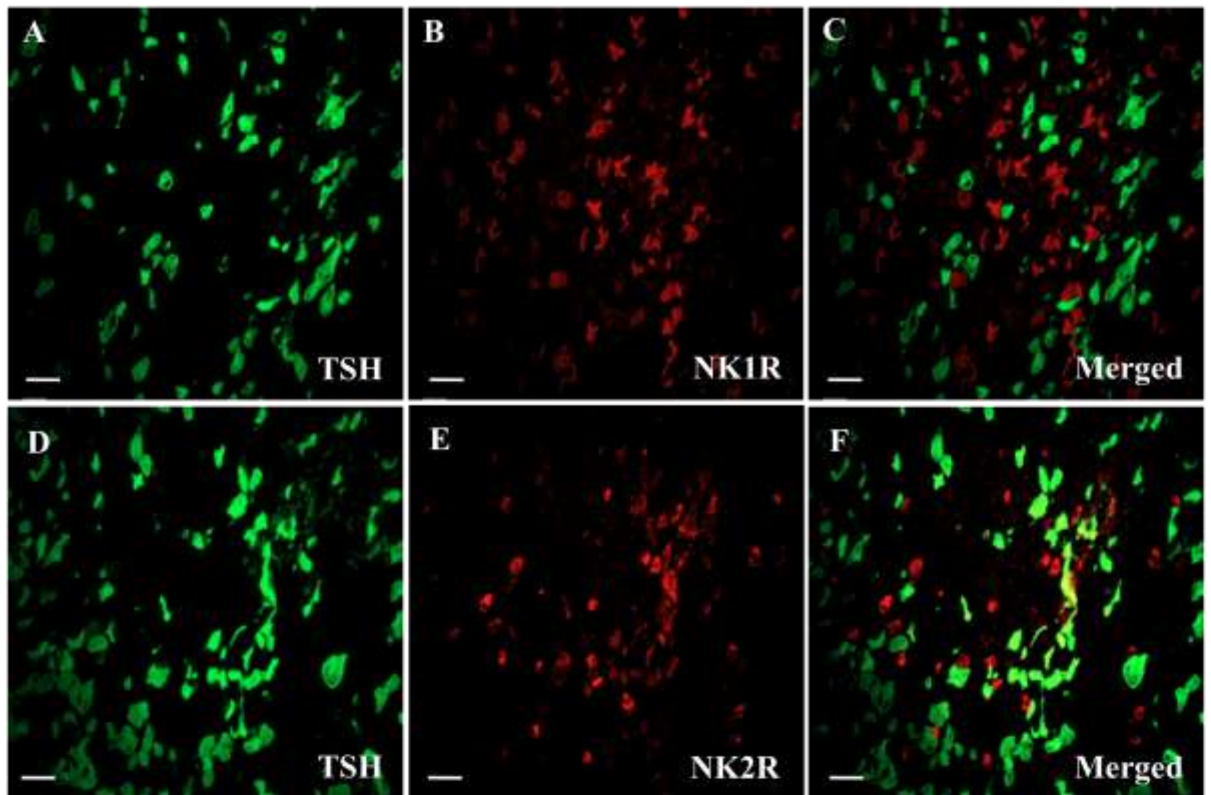


Figure 6.7: Localization of TSH, NK1R and NK2R protein immunofluorescence in the F344/NHsd rat PD region of the pituitary.

Microscopic view of double immunofluorescent staining showing the expression of (**A**,**D**) TSH (green), (**B**) NK1R (red), (**E**) NK2R (red) and the respective merged images (**C**) TSH+NK1R and (**F**) TSH+NK2R. No co-localizations were observed. Scale bar: 20µm

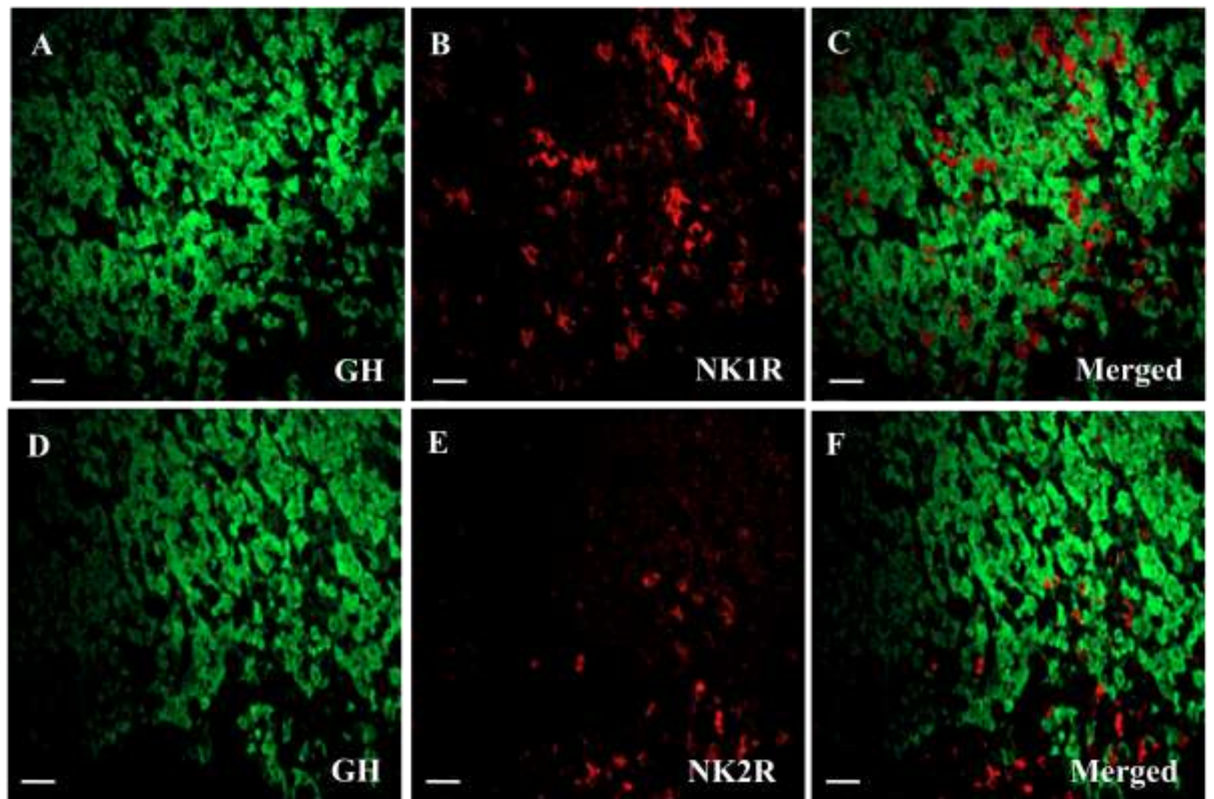


Figure 6.8: Localization of GH, NK1R and NK2R protein immunofluorescence in the F344/NHsd rat PD region of the pituitary.

Microscopic view of double immunofluorescent staining showing the expression of (**A**,**D**) GH (green), (**B**) NK1R (red), (**E**) NK2R (red) and the respective merged images (**C**) GH+NK1R and (**F**) GH+NK2R. No co-localizations were observed. Scale bar: 20µm

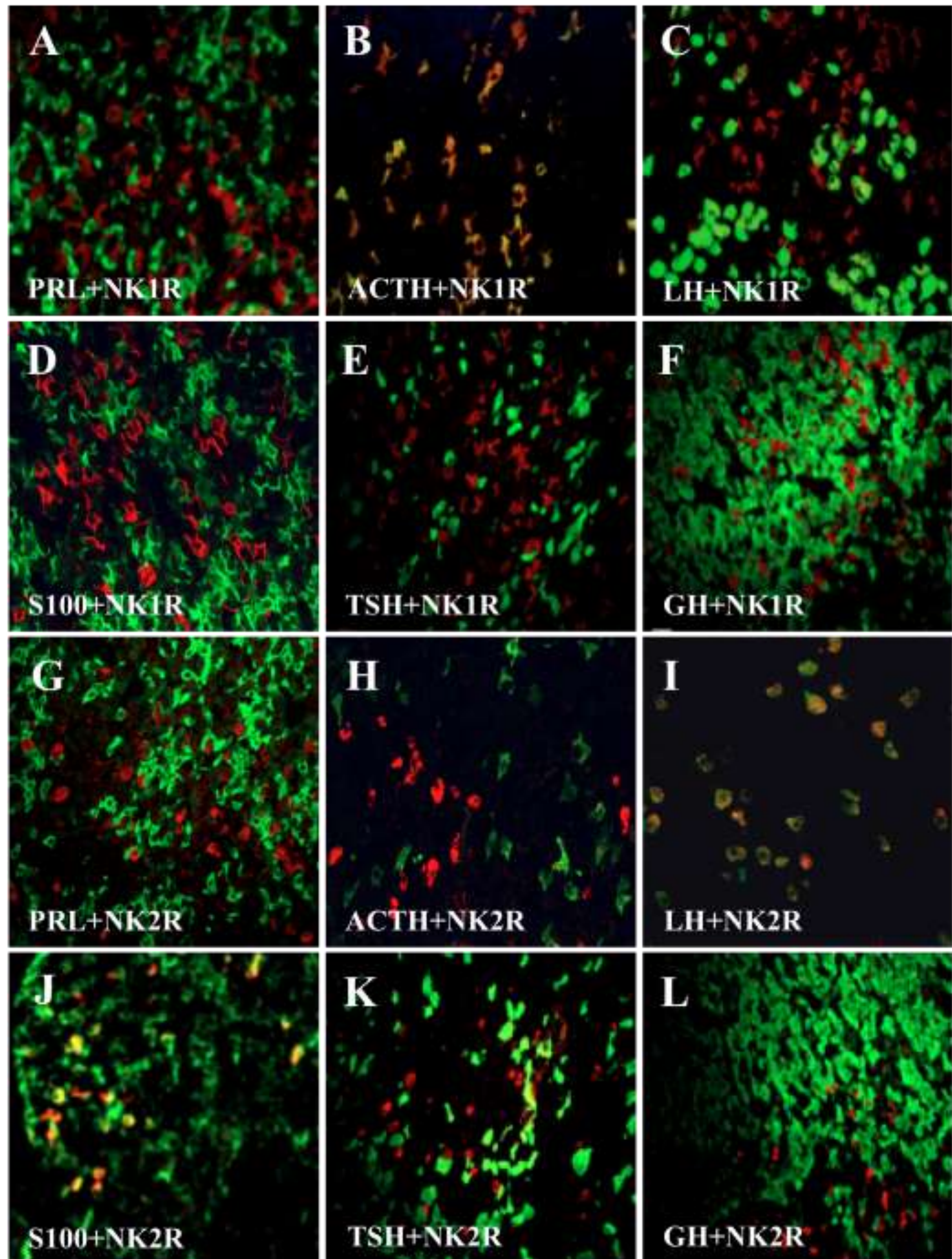


Figure 6.9: Summary of merged expression of NKRs and markers of the different pituitary cell-types in the PD.

Double immunofluorescent images showing the merged expression of either (**A-F**) NK1R (red) or (**G-L**) NK2R (red), with the main cell-types (green) in the rat pituitary: (A,G) lactotrophs (PRL), (B, H) cotricotrophs (ACTH), (C, I) gonadotrophs (LH), (D, J) folliculo-stellate cells (S100), (E, K) thyrotrophs (TSH) and (F, L) somatotrophs (GH). ACTH is co-localized with NK1R (B), LH with NK2R (I), and S100 with NK2R (J), as indicated by the merged expression (red+green=yellow).

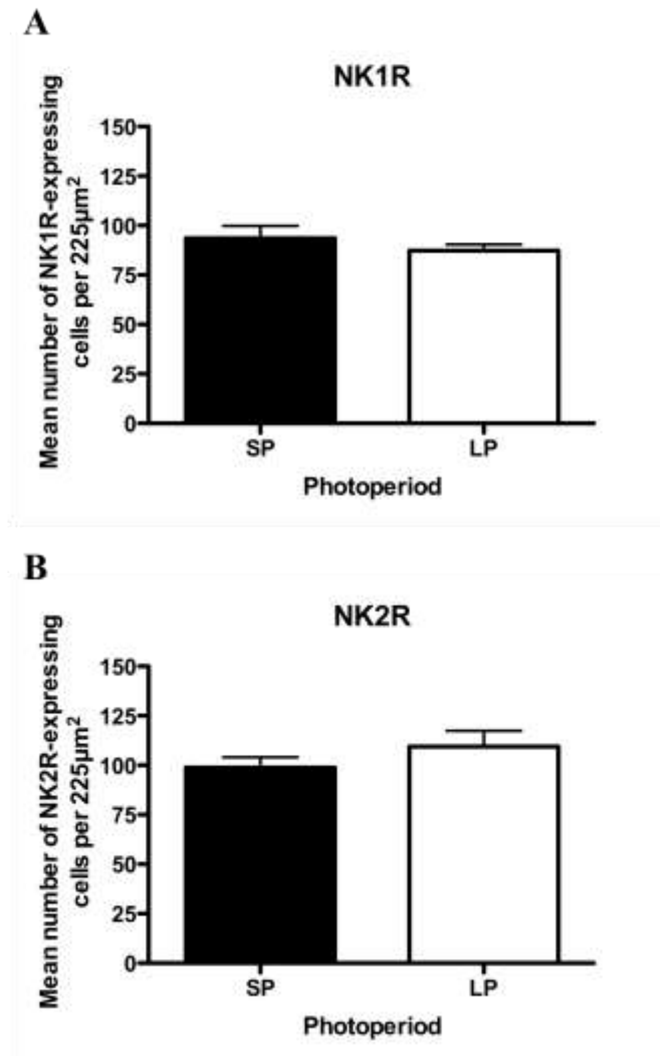


Figure 6.10: Quantification of NK1R- and NK2R- expressing cells in the rat anterior pituitary following exposure to short and long photoperiods.

The mean number of (**A**) NK1R-expressing and (**B**) NK2R-expressing cells per 225µm^2 of rat anterior pituitary tissue following SP and LP exposure. Statistical analysis using unpaired t-test did not reveal significant differences between groups. Data are expressed as means ± SE and represent normalized changes compared to control values. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

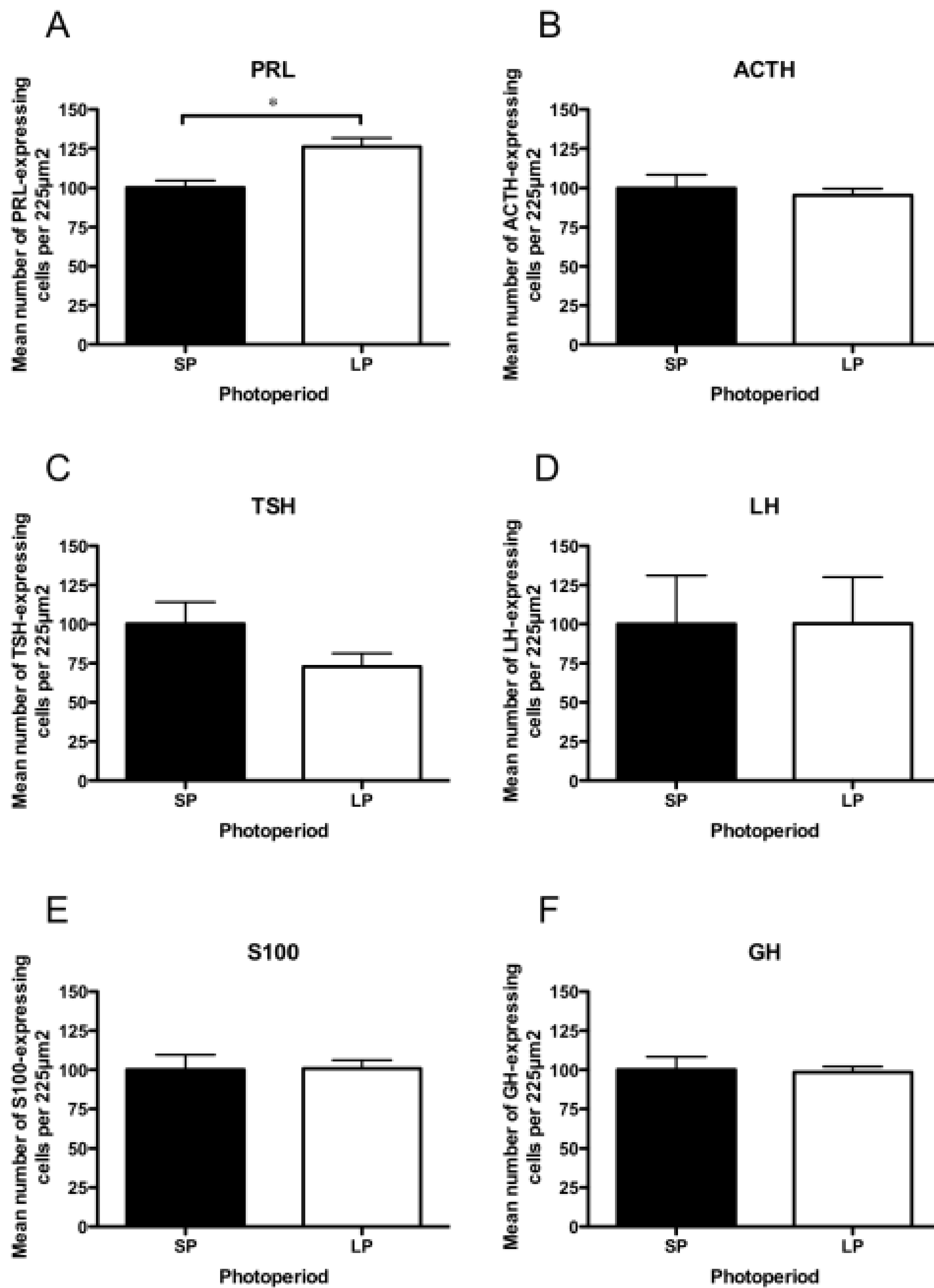


Figure 6.11: Quantification of the different endocrine cell types and folliculo-stellate cells in the rat anterior pituitary PD region, following exposure to short and long photoperiods.

The effect of photoperiod on the mean number of **(A)** PRL, **(B)** ACTH, **(C)** TSH, **(D)** LH, **(E)** S100 and **(F)** GH – expressing cells, per 225μm^2 of rat anterior pituitary tissue. The number of PRL-expressing cells was significantly increased in LP compared to SP (*p<0.05; unpaired t-test). Data are expressed as means ± SE and represent normalized changes compared to control values. **LP:** Long Photoperiod; **SP:** Short Photoperiod.

6.4 Discussion

Neurokinins exert their effects by interacting with any one of the three receptor subtypes, NK1R, NK2R and NK3R. In order to fully investigate the potential involvement of neurokinins within the intra-pituitary signalling pathway of seasonal hormone regulation in the rat pituitary, this study examined the neurokinin receptor expression profiles within the pituitary PD region. RT-PCR experiments demonstrated the presence of mRNA encoding all three neurokinin receptors, NK1R, NK2R and NK3R. Furthermore, through the use of immunohistochemistry, NK1R and NK2R were visualised and co-localized with markers of the different cell-types in the PD region of the pituitary.

There are five types of hormone-producing cells and a non-hormone-producing cell in the rat anterior pituitary. Evidence that each pituitary hormone originates from a specific cell-type came from early immunocytochemical studies (Nakane, 1970), which demonstrated that each pituitary hormone (PRL, ACTH, GH, TSH) was stored in a separate cell-type, with the exception of LH and FSH, the expression of which appeared to coincide in many gonadotrophs. The study further revealed that the endocrine cells were not homogeneously distributed throughout the pituitary and that particular associations existed between somatotrophs and corticotrophs, and between gonadotrophs and lactotrophs, which led the author to suggest a functional significance (Nakane, 1970). These topographic affinities were later confirmed through the use of double immunostaining and microscopic examinations, revealing high attachment rates between these cell types (Noda *et al*., 2001). High affinities were also observed between somatotrophs and thyrotrophs (Noda *et al*., 2001).

In this study I was able to localize the main receptor for SubP (NK1R) to corticotroph cells, and the main receptor for NKA (NK2R) to gonadotrophs and folliculo-stellate cells, via double immunostaining. The expectation was that at least one of the receptors would be expressed in lactotroph cells. However the results are not in support of such a co-localization, and the location of these receptors in other cell types raises the question as to whether these cells are involved in a more complex paracrine communication system, in which lactotrophs receive a signal following neurokinin binding to the receptors of the neighbouring cells. Such a suggestion was made in a recent study in

sheep, where it is also the case that lactotrophs lack NK1R and NK2R expression (Dupré *et al*., 2010).

The immunohistochemical investigation of NKR expression in the highly seasonal sheep pituitary revealed localization of NK1R in corticotrophs and folliculo-stellate cells, and of NK2R in corticotrophs, gonadotrophs and a few thyrotrophs, whilst NK3R was localized to gonadotrophs (Dupré *et al*., 2010). These findings are partly consistent with the present study, which also co-localized NK1R to corticotrophs and NK2R to gonadotrophs. It would therefore appear likely that expression of these receptors on these particular cell types is physiologically significant, and therefore may be conserved more generally in mammals.

Previous immunocytochemistry and radio-ligand binding assays localized the NK1R to PRL- and LH-secreting cells in the rat pituitary, suggesting a direct effect of SubP on these cell types (Larsen *et al*., 1992). NK1R was later detected at the mRNA level in rat pituitary tissue (Winkler *et al*., 1995). The effects of blockade of NK1R in the rat pituitary, in the presence and absence of SubP, are consistent with SubP binding to NK1R and influencing GnRH-induced LH secretion (Duval *et al*., 1998). Furthermore, the action of SubP can be either stimulatory or inhibitory, depending on the hormonal environment (Duval *et al*., 1998). The evidence was therefore in support of a physiological role for SubP in the pituitary, directly regulating the secretion of the gonadotrophin hormones. In the present study however, NK1R was localized to corticotrophs and not to gonadotrophs, suggesting that the actions of SubP on GnRH-induced LH secretion may be indirect.

Given the role of SubP as a neurotransmitter involved in stress responses (Unger *et al*., 1988; Culman and Unger, 1995; Ebner and Singewald, 2006) it is perhaps not surprising that its preferred receptor, NK1R, has been co-localized to corticotrophs, which are responsible for transducing the stress response from the brain to peripheral organs through the release of stress hormones, such as ACTH (Antoni, 1986; Dallman *et al*., 1992; Aguilera, 1994). Corticotrophs are involved in autocrine and paracrine communications within the corticotroph population, through the release of factors such as arginine-vasopressin (Chateau *et al*., 1979; Loh *et al*., 1988; Lolait *et al*., 1986; Terrier *et al*., 1991), corticotrophin-releasing hormone (CRH) (Pecori Giraldi and Cavagnini, 1998) and acetylcholine (Carmeliet and Denef, 1989). Anterior pituitary

acetylcholine has also been associated with paracrine effects on PRL and GH release (Carmeliet and Denef, 1988; Carmeliet *et al*., 1989a; Carmeliet *et al*., 1989b), suggesting a possible involvement of corticotrophs in modulating hormone release during stress responses. The exact influence of SubP on corticotrophs is not yet established. However, it should be noted that a study investigating the action of SubP on corticotrophin-releasing hormone- (CRH) and arginine-vasopressin- (AVP) stimulated corticotrophin release from rat quartered-pituitaries, was able to demonstrate an inhibiting effect of the neurokinin when used at high concentrations, whilst at lower doses no effect was observed (Nicholson *et al*., 1984). A lack of corticotrophin response to SubP was also observed when using dispersed pituitary cell cultures (Chowdrey *et al*., 1990). Intriguingly though, *in vivo* treatment with SubP resulted in decreased corticotrophin and increased AVP release (Chowdrey *et al*., 1990). Whilst these contradictory results demonstrate the complexity of the hypothalamic-pituitary-adrenal (HPA) response to neurokinins, they also show that SubP can indeed influence the activity of corticotrophs and therefore potentially that of other neighbouring cells. Subsequent studies using a mouse pituitary tumour cell line also showed an inhibitory effect of SubP on CRH-induced ACTH secretion and on proopiomelanocortin (POMC) gene expression (Fickel *et al*., 1994).

The presence of NK2R has previously been indirectly demonstrated in the pituitary of male rats following the *in vitro* release of LH in response to NKA treatment (Kalra *et al*., 1992). Furthermore, blockade of NK2R in the Siberian hamster anterior pituitary resulted in the altered secretion of the gonadotrophins LH and FSH, whilst immunoneutralisation of NKA decreased gonadotrophin secretion, thus highlighting the involvement of NKA in intrapituitary mechanisms and the regulation of gonadotrophs (Debeljuk and Bartke, 1994); a modulatory effect that has also been shown in the rat (Debeljuk *et al*., 1997). However, blockade of NK2R in the rat pituitary was also shown to decrease prolactin secretion (Debeljuk *et al*., 1997) whilst immunocytochemical staining has localized NK2R to pituitary lactotrophs (Pisera *et al*., 2003). This would suggest that neurokinins have a direct effect on prolactin release through binding to NK2R and that paracrine communication between lactotrophs and gonadotrophs may be responsible for the NK2R-mediated modulation of gonadotrophin release. However, the theory of a direct effect on lactotrophs is not supported by the present study, which localizes NK2R in gonadotrophs and folliculo-stellate cells.

Folliculo-stellate cells are agranular stellate-shaped cells first described in the anterior pituitary by Rinehart and Farquhar (Rinehart and Farquhar, 1953). Their potential role in modulating hormone production through paracrine action on neighbouring endocrine cells was later demonstrated in the rat pituitary (Baes *et al*., 1987; Allaerts *et al*., 1994; Soji *et al*., 1997). Several studies investigating the effect of FS cell-derived factors (e.g. S100, bFGF and IL-6) on anterior pituitary hormone release have revealed a stimulatory effect on the secretion of prolactin in cultured pituitary cells or cell lines (Ishikawa *et al*., 1983; Baird *et al*., 1985; Spangelo *et al*., 1989; Koike *et al*., 1997).

The close interactive relationship between folliculo-stellate cells and lactotrophs is highlighted in a study by Oomizu and colleagues (Oomizu *et al*., 2004), which demonstrates that folliculo-stellate cells derived from high-oestrogen responsive female F344 rats, are able to regulate oestrogen-induced proliferation of lactotrophs. Their data further suggests that production and secretion of basic fibroblast growth factor (bFGF) by folliculo-stellate cells affected the growth response of lactotrophs. These findings clearly signify the presence of a functional communication between these two cell types, with a resulting increase in the lactotrophic population. Furthermore, it has been demonstrated that the secretory activity of folliculo-stellate cells (S100-production) is influenced by photoperiod (Acosta and Mohamed, 2011), thus reinforcing the potential of this cell-type being involved in seasonal paracrine communications within the pituitary to influence the secretory activity of other cells.

The intercellular communication between gonadotrophs and lactotrophs has been well documented over the years. The first evidence of paracrine interactions between these two cell-types came from studies using postnatal rat pituitary cell cultures whereby the presence of gonadotrophs was proven to be instrumental in the secretory activity of lactotrophs in response to GnRH stimulation of the gonadotroph-lactotroph culture (Denef and Andries, 1983). Whilst there is plentiful data to support a PRL-inducing action of GnRH (Denef and Andries, 1983; Andries *et al*., 1995; Andries and Denef, 1995; Chabot *et al*., 2001) and an effect on lactotroph development (Bégeot *et al*., 1984; Aubert *et al*., 1985; Jennes, 1990; Van Bael *et al*., 1998; Seuntjens *et al*., 1999), it appears that in adult pituitaries, the PRL response to GnRH stimulation is not as consistent and may differ depending on various factors such as hormonal status (Amsterdam *et al*., 1983; Mais *et al*., 1986; Serafini *et al*., 1987; De Marinis *et al*., 1990), gender (Barbarino *et al*., 1982; Gooren *et al*., 1985), circadian rhythms

(Rossmanith *et al*., 1993) and pathological conditions (Tamai *et al*., 1987; Mauras *et al*., 1991).

Paracrine factors such as glycoprotein hormone alpha-subunit (α-GSU) and N-POMC (the glycosylated N-terminal fragment of POMC) that are found in gonadotroph-conditioned medium, have been shown to be involved in lactotroph development. The synthesis of N-POMC by a subpopulation of gonadotrophs was demonstrated at the mRNA level (Roudbaraki *et al*., 1999; Pals *et al*., 2006) with some cells co-expressing N-POMC and α-GSU (Pals *et al*., 2006). The α-GSU subunit was shown to both induce lactotroph differentiation and stimulate PRL release (Bégeot *et al*., 1984; Oguchi *et al*., 1996; Chabot *et al*., 2000; Chabot *et al*., 2001). N-POMC was also shown to stimulate the development, differentiation and mitogenic action of lactotrophs (Tilemans *et al*., 1994; Van Bael *et al*., 1996; Tilemans *et al*., 1997) as well as PRL mRNA expression (Lu *et al*., 2002).

Associations between gonadotrophs and all other cell-types in the PD have also been investigated in relation to photoperiod, in adult male viscachas, a rodent with photoperiod-dependent reproduction. The double immunohistochemical examinations revealed an abundant association between LH- and PRL-cells during short compared to long photoperiods (Filippa *et al*., 2012). The opposite was observed in the percentage of FSH and PRL association, which decreased significantly during winter. This novel study by Filippa and colleagues (Filippa *et al*., 2012) of gonadotroph associations demonstrates the presence of specific seasonal cytological configurations, thus providing evidence that paracrine interactions between gonadotrophs and other cell-types occur according to the reproductive requirements.

Morphological associations between gonadotrophs and lactotrophs have also been studied in the rat and equine pituitary gland (Allaerts *et al*., 1991; Tortonese *et al*., 2001; Townsend *et al*., 2004). Townsend and colleagues (Townsend *et al*., 2004) demonstrate that these intercellular associations are influenced by gonadal status and season, in the equine pituitary. Both gonadotrophs and lactotrophs increase in number during the breeding season and this appears to be linked to a stimulatory gonadal effect. Furthermore these studies show that the number of lactotrophs is also influenced by photoperiod independently of the gonadal response, being increased in the breeding season compared to the non-breeding season.

In short-day seasonal breeders, such as sheep, the secretion patterns of LH and PRL are inversely correlated, with PRL being high in the summer (non-breeding season) and low in the winter (breeding season). This inverse relationship appears to be driven by the inhibitory effect that prolactin has on the gonadotrophic axis, as it has been shown to directly suppress LH release at the pituitary level (Smith, 1978; Winters and Loriaux, 1978; Cheung, 1983; Sortino and Wise, 1989; Hodson *et al*., 2010). Furthermore it has been shown that PRL and dopamine interact within the pituitary gland to exert a suppressive effect on GnRH-induced LH secretion (Gregory *et al*, 2004). This event appears to be photoperiod-regulated, with gonadotrophs from the non-breeding season being much more responsive to the PRL/dopamine inhibitory action, compared to gonadotrophs of the breeding season (Gregory *et al*., 2004; Hodson *et al*., 2012). It therefore appears evident that seasonal changes have quite an influential effect on intercellular communication within the anterior pituitary amongst different species.

Interestingly, a study investigating viscacha corticotroph cells in relation to season, has shown that the morphology of ACTH-expressing cells varies according to the environmental conditions and the authors further suggest that ACTH secretion may be inhibited during the winter months by melatonin, as the corticotrophs appeared to remain de-granulated (Filippa and Mohamed, 2006). This finding could therefore imply a seasonally-dependent communication between corticotrophs and other PD cell types, perhaps even lactotrophs. Nonetheless, the present study did not reveal a photoperiod-dependent change in the number of ACTH-expressing cells in the PD.

The results presented here demonstrate an increased number of PRL-expressing cells in the rat anterior pituitary following LP exposure compared to SP. This would suggest that seasonal changes occur in the number of lactotrophs, something that has previously been shown in deer (Schulte *et al*., 1980) and sheep (Stroud *et al*., 1992) pituitary glands. In addition, photoperiod has been shown to affect the ultrastructure of the lactotroph cells, which appear to display an increase in size and number of secretory granules as well as a highly developed rough endoplasmic reticulum under LP conditions (Schulte *et al*., 1980; Schulte *et al*., 1981). All these morphological changes occurring in the cell, are indicative of increased synthetic capacity.

The present study further sought to investigate whether or not photoperiod had an effect on the number of neurokinin receptors in the PD region of the pituitary. Analysis of

both NK1R and NK2R expression did not reveal a significant difference between long and short photoperiod-exposed tissue. It is therefore unlikely that the seasonal regulation of prolactin is modulated through regulation of neurokinin receptor expression. However, studies using the Siberian hamster as a model, have demonstrated an effect of photoperiod on the neurokinin content of the anterior pituitary during development, with higher concentrations being detected under short photoperiods (Rao *et al*., 1996). Previous investigations by the same group had also revealed that the concentrations of SubP and NKA in the anterior pituitary were increased in the Siberian hamster compared to both the Syrian hamster and the rat (Debeljuk and Bartke, 1994). Those results, in association with the dramatic gonadal responses of Siberian hamsters to photoperiod changes and the effects of NKR- blockade on gonadotrophin secretion, led to the proposal that neurokinins could be involved in modulating the gonadotrophin responses (Debeljuk and Bartke, 1994). It would therefore be interesting to investigate whether neurokinin content is also photoperiod-dependent in the rat pituitary.

A study carried out in female rats looking at the effect of age and exogenous melatonin on SubP and NKA concentrations in different tissues of the hypothalamo-pituitary axis, revealed that in 25-month-old (acyclic) rats, hypothalamic SubP and NKA were significantly increased compared to 5-month-old (cyclic) and 15-month-old (preacyclic) rats, and the rise in the acyclic rats was blunted by melatonin treatment (Fernandez *et al*., 2002). Furthermore, whilst no significant differences in neurokinin concentrations were found between the three age groups in the anterior pituitary gland, melatonin treatment of the rats resulted in significantly increased concentrations of NKA in preacyclic and acyclic groups compared to controls. These results suggest that melatonin has an inducing effect on neurokinin content in the pituitary of aging rats.

As a final point, the immunohistochemical studies carried out here, demonstrate a cytoplasmic expression of the NKRs; an intriguing finding considering that they are transmembrane receptors which would be expected to localize to the cell surface. This discrepancy is thought to be due to endocytosis of the receptor and subsequent trafficking through the cytoplasm following agonist binding, something which has previously been demonstrated in rat and guinea-pig neurons (Southwell *et al*., 1996; Southwell *et al*., 1998; Mann *et al*., 1999; Schultz *et al*., 2006). In order to confirm the cytoplasmic or membrane localization of the receptors, future studies could employ immunohistochemical techniques, using antibodies raised against the extracellular N-

terminus of the receptors or any other extracellular domain, followed by measurements of the intracellular and cell surface distributions of receptor immunoreactivity using microscopy. The specificity of the antibodies can be accurately tested, by showing specific reaction with the peptide antigen through the use of immunoblotting and/or immunoprecipitation. These techniques should demonstrate that the antibody binds a single protein of the appropriate molecular weight. Alternatively, antibody specificity could be tested using absorption or pre-absorption controls, which involve mixing the antibody of interest with the peptide or protein used to generate the antibody, with the aim of eliminating the binding of the antibody to the protein in the tissue. This is based on the theory that incubation of the peptide with the antibody results in occupancy of all the binding sites on the antibody for that specific antigen and so immunostaining of the tissue with the absorption controls should not give a positive signal. If positive staining does occur, this suggests that the antibody is not specific to the antigen in question.

In conclusion, the results presented in this chapter demonstrate a lack of neurokinin receptor expression on pituitary lactotrophs, suggesting that neurokinins are unlikely to have a direct role in modulating lactotroph activity in the rat anterior pituitary. However, there remains the possibility of an indirect role in modulating PRL regulation, through intercellular paracrine communication (Figure 6.12). The exact role of neurokinins in this region of the rat pituitary, with regards to the seasonal regulation of PRL, remains unclear, however their actions appear to be mediated through specific cell types. Whilst the expression of NKRs appears to be species-dependent, the co-localization of the receptors to corticotrophs and gonadotrophs, appears to be a common feature shared with sheep (Dupré *et al.*, 2010). The association of lactotrophs with gonadotrophs in particular, has been widely documented in the literature, rendering a functional PRL-modulating relationship between these cell types highly plausible. Furthermore, photoperiod has been shown to regulate the number of PRL-expressing cells in the anterior pituitary, consistent with the well-known LP-induced rise of the concentration of this hormone. Finally, it is becoming increasingly evident that neurokinins are involved in a plethora of mechanisms within the anterior pituitary and their actions are highly dependent on other factors, including hormonal status, age, gender, species, photoperiod and melatonin.

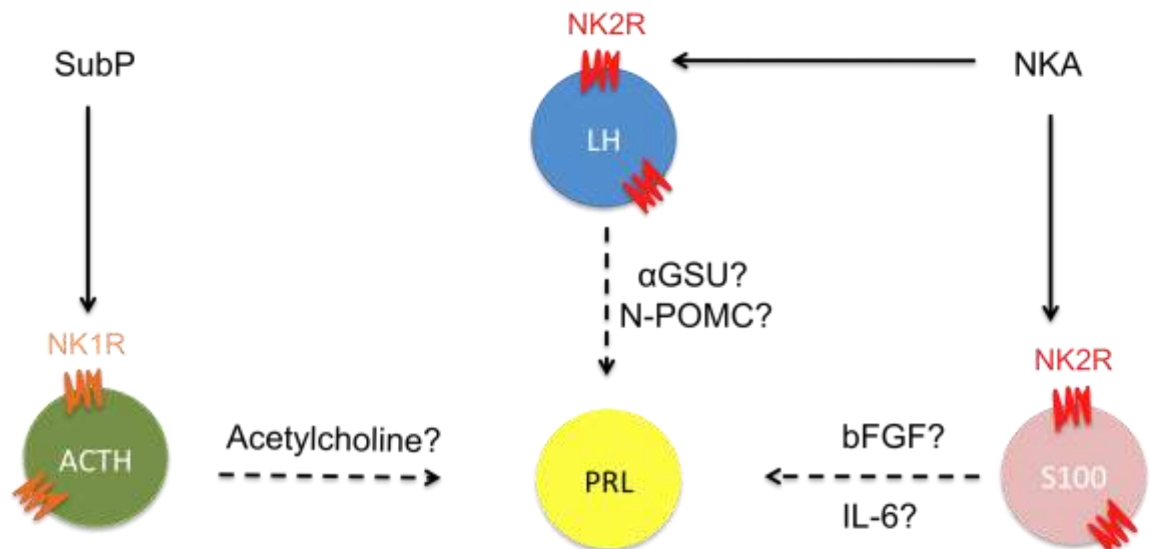


Figure 6.12: Putative intercellular communications in the rat anterior pituitary involving lactotrophs as indirect target cells of neurokinins.

Immunohistochemical studies reveal expression of NK1R, the main receptor for SubP in corticotrophs (ACTH-expressing cells) and expression of NK2R, the main receptor for NKA, in gonadotrophs (LH-expressing cells) and folliculo-stellate cells (S100-expressing cells). The dashed arrows indicate putative paracrine signals involved in regulating the activity of lactotrophs (PRL-expressing cells), following binding of SubP and NKA to NK1R and NK2R, respectively. **ACTH**: adrenocorticotropin; **αGSU**: alpha glycoprotein subunit; **bFGF**: basic fibroblast growth factor; **IL-6**: interleukin-6; **LH**: luteinizing hormone; **NK1R**: neurokinin receptor 1; **NK2R**: neurokinin receptor 2; **N-POMC:** N-terminal fragment of proopiomelanocortin; **PRL**: prolactin; Figure by the author.

CHAPTER 7: General Discussion

7.1 Summary of key observations

This study extends our knowledge of photoperiodic regulation in the F344/NHsd rat and Syrian hamster pars tuberalis (PT) and brain, revealing chronic and acute photoperiod-induced changes in the expression of *Eya3* and *TAC1*, two genes previously shown to be involved in photoperiodic response pathways (Dupré *et al*., 2010). A key finding presented within this thesis was the strong up-regulation of *Eya3* gene expression in the PT of the F344/NHsd rat and Syrian hamster, in response to long photoperiod (LP) exposure. This result is in agreement with the recent identification of Eya3 as a conserved early molecular response to LP activation, considered to be involved in a switch mechanism ultimately responsible for seasonal changes in physiology in both birds and mammals (Dardente *et al*., 2010; Masumoto *et al*., 2010). Unexpectedly, *TAC1* gene expression was not detected in the PT of either the rat or hamster, in either SP or LP. This result is not in line with previous findings in ovine studies, which demonstrate strong activation of this gene in LP and suggest that products of the *TAC1* gene (neurokinins) may fulfil the role of the elusive PT-derived 'tuberalin'. However, *TAC1* expression was strongly activated by SP exposure, in the ependymal layer lining the third ventricle of the hypothalamus. Interestingly, this region of the brain has previously been associated with the seasonal regulation of thyroid hormone availability, body weight and reproduction. An *in vitro* luciferase reporter system, which was set up using dispersed primary pituitary cell cultures derived from transgenic F344 rats, revealed no effect of neurokinins on the transcriptional activity of the prolactin promoter. Furthermore, these compounds did not reveal an effect on PRL or LH release either. Finally, an examination carried out on the anatomy of the rat anterior pituitary gland, revealed neurokinin receptor (NKR) expression in the pars distalis (PD), the region responsible for seasonal prolactin release. Whilst the NKRs did not co-localize with PRL-expressing cells, their co-localization with corticotrophs, gonadotrophs and folliculo-stellate cells, allows for the possibility of an indirect action of neurokinins on PRL regulation through paracrine communication.

7.2 The F344 rat as a model for studying seasonality and photoperiodism

In the study of mammalian photoperiodism, sheep and hamsters have been the two predominant model species considered, due to the robust seasonal responses displayed in terms of their physiology. However, more recent studies that have included the use of laboratory rodents, which are not generally considered to be photo-responsive, have

revealed that they can be extremely useful models in understanding the underlying mechanisms of seasonality. A strong example of such a species can be seen in the F344/NHsd rat, which displays a reduction in body weight, food intake, gonadal weight and circulating prolactin concentration, in response to short photoperiod exposure. Although the magnitude of these responses may not match those of a truly seasonal species, it does provide evidence that at least part of the underlying machinery controlling photoperiodic responses is present in the rat. Intriguingly however, not all laboratory strains or even sub-strains of rats have retained the ability to respond to photoperiod. In an attempt to identify the reasons accounting for these inter-strain differences, studies by Yasuo and colleagues (Yasuo *et al.*, 2007) were the first to demonstrate the photoperiod-dependent expression of the type II deiodinase gene (*Dio2*), an enzyme involved in the conversion of inactive thyroxine (T4) into the active tri-iodothyronine (T3), in hypothalamic areas of the F344 rat. Their study suggested that the differential response of *Dio2* to photoperiod, which was observed in the F344 but not the Wistar rat, was an indication of the photoperiod-sensitive nature of the former strain.

More recently, it has been shown that much of the thyroid hormone signalling machinery, which is thought to have a central role in the seasonal regulation of body weight and reproduction in seasonal species, is present in the F344/NHsd rat hypothalamus (Ross *et al.*, 2011). Photoperiodic signal transduction mechanisms regulating thyroid hormones in the brain are considered to be mediated through the photoperiod-regulated expression of thyroid-stimulating hormone (TSH) in the PT of both birds and mammals (Nakao *et al.*, 2008; Hanon *et al.*, 2008; Hazlerigg and Loudon, 2008). Key molecular events thought to be associated with the TSH signalling pathway, which have been demonstrated in the F344 rat, include the photoperiod-dependent expression of the genes regulating the two subunits that form a functional TSH heterodimer in the PT {thyroid-stimulating hormone beta subunit (TSHβ) and glycoprotein hormone alpha polypeptide (CGA)}, the photoperiod-regulated activity of the deiodinase enzymes Dio2 and Dio3, the expression of the TSH receptor in the ependymal region of the hypothalamus and the strongly photoperiodic expression of thyroid hormone transporter genes *MCT8* (monocarboxylate transporter) and *Oatp1c1* (organic anion transporter family member 1c1) (Ross *et al.*, 2011). These findings demonstrate how mechanisms responsible for photoperiod-regulated changes in physiology are in place in a rodent model, similarly to other species. On the other hand,

there are also findings indicative of significant inter-species differences, which are likely to reflect the divergence in the degree of photoperiodism in various species. In view of the fact that photoperiod-driven alteration of physiological traits is an adaptive approach to the seasonally changing environment, it is perhaps not surprising that domesticated and laboratory-bred animals that are raised under constant conditions and do not require environmental cues in order to reproduce or indeed survive, may present differences in the mechanisms regulating their physiological responses.

Laboratory mice are generally considered to be a non-seasonal species and therefore an inappropriate model for studying photoperiodism. However, studies have demonstrated that much of the photoperiodic machinery required to elicit a response to photoperiod-change is in place in melatonin-proficient mice and the same response can be mimicked in melatonin-deficient mice through the administration of exogenous melatonin (Ono *et al*., 2008). Despite not appearing to be photo-responsive in terms of their reproductive status, it has been shown that mice display robust photoperiodic responses in genes associated with the regulation of thyroid hormone signalling (Ono *et al*., 2008), which is thought to impact the seasonal regulation of reproduction in mammals (Barrett *et al*., 2007). Furthermore, the photoperiod-induced molecular changes observed in mice are consistent with those seen in the Japanese quail, a strongly seasonal bird species (Nakao *et al*., 2008).

Although many responses in the photoperiodic signal transduction pathway appear conserved in birds and mammals, it is becoming increasingly clear that marked differences also exist from one species to another. An example of this can be seen in terms of the entry route through which the light signal is received in photoperiodic species. In mammals the input pathway of the signal is through the eyes whilst in birds it is received through extra-retinal deep brain photoreceptors. Further differences lie in the role of melatonin, which in mammals acts as a signalling molecule directly implicated in the translation of the photoperiodic stimulus into a reproductive output, but in birds has very little to do with this process (Sharp, 2005). It would therefore be unreasonable to assume that the study of a single model is sufficient in unravelling the complexities of photoperiodism, a system that is far from universal. The data presented within this thesis provide further insight into the conserved nature of certain elements of the seasonal machinery in photo-responsive species, but also highlight some important differences.

7.3 Photoperiodic regulation of *Eya3* gene expression in the PT

In an attempt to unravel the photoperiodic transduction pathways downstream of melatonin action, studies have focused on the effects of this molecule on gene expression profiles in the PT, a highly melatonin-responsive tissue. *Eyes absent 3 (Eya3)* is a circadian clock-controlled gene encoding a transcriptional coactivator with enzymatic capabilities (Rebay *et al*., 2005), and appears to be a critical target of melatonin action in the PT region. Through its synergistic action with other clock-driven transcription factors, whose expression is also influenced by melatonin, Eya3 is believed to be involved in the LP-induction of *TSHβ* expression in the PT (Dardente *et al*., 2010). The action of melatonin on *Eya3* is thought to be two-fold; firstly, with the rhythmic expression of *Eya3* set by the evening onset of melatonin, *Eya3* expression peaks 12h after dark onset whilst secondly, it is directly suppressed by melatonin such that the peak expression of *Eya3* is reduced under SP conditions where it coincides with the dark phase, when melatonin is high. According to this proposed mechanism, also known as the external coincidence timing model (Goldman, 2001), the amplitude of peak *Eya3* expression and subsequently of *TSHβ* induction, would be significantly higher under LP compared to SP, and would thus signify the onset of summer conditions.

Eya3 and *TSHβ* are among the first genes whose expression is increased upon transfer to LP, and this appears to be conserved in quail (Nakao *et al*., 2008), sheep (Hanon *et al*., 2008; Dupré *et al*., 2010) and melatonin-proficient mice (Ono *et al*., 2008; Masumoto *et al*., 2010). Furthermore it has been demonstrated in sheep and mice that Eya3 synergistically acts with the transcription factors Six1 (Sine oculis homeobox homolog 1) and TEF (thyrotroph embryonic factor) to induce *TSHβ* expression in the PT (Dardente *et al*., 2010; Masumoto *et al*., 2010), however this has not been confirmed in the quail model. It is therefore proposed that Eya3 is a key regulator involved in the initiation of the photoperiodic responses observed in LP conditions (Dardente *et al*., 2010; Masumoto *et al*., 2010). The novel demonstration in the present study, of LP-induced *Eya3* expression in both the F344/NHsd and Syrian hamster PT is in clear agreement with this proposal and further accentuates the conserved nature of the Eya3 signal.

As mentioned previously, Eya3 is thought to co-ordinate the LP-regulated increase in thyrotrophin (TSH) production from melatonin-responsive cells in the PT, subsequently regulating seasonal changes in the hypothalamus (Dardente *et al*., 2010). Recent studies using the F344 rat model have revealed the photoperiod-dependent expression of the TSH binding partners, *TSHβ* and *CGA* in the PT region (Ross *et al*., 2011), further highlighting the similarities of this model with other species (Hanon *et al*., 2008; Nakao *et al*., 2008). However, the expression patterns of *Eya3* and *TSHβ* in relation to one another have not yet been investigated in the F344 PT and so it remains to be established whether this system follows the sheep or quail model. In the former, the peak expression of *Eya3* upon LP stimulation precedes the expression of *TSHβ,* thus supporting the view that Eya3 is involved in the transcriptional activation of *TSHβ* (Dardente *et al.,* 2010) whilst in the quail PT, *Eya3* and *TSHβ* expression rise in parallel, suggesting a role for both components early in the photoperiodic induction pathway (Nakao *et al*., 2008).

In the present study, analysis of *Eya3* expression in the rat PT revealed a progressive rise in expression during the first day of LP exposure, following an acute switch from chronic SP-housing. These findings are consistent with the ovine studies, which revealed that *Eya3* expression is up-regulated in the PT, as part of an acute response to LP exposure, from as early as day one (Dupré *et al*., 2010). *Eya3* expression continued to act as a marker of LP activation in sheep following chronic (4-week) exposure, and this was also the case in the F344/NHsd rats, which also demonstrated LP-activated expression of *Eya3* following 5 weeks of exposure. Whilst it is not yet known whether Eya3 is involved in the seasonal regulation of prolactin, its importance in regulating seasonal pathways downstream of its activation in the PT, is becoming increasingly evident.

Eya3 is known to act not only as a transcriptional co-activator but also as an enzyme, potentially influencing gene expression in a bi-functional manner (Rebay *et al*., 2005). Its latter role as a tyrosine phosphatase involves autocatalytical dephosphorylation of itself and disruption of this function severely affects the ability of Eya3 to regulate developmental processes of many different tissues including the eye, in *Drosophila* and vertebrates (Rebay *et al*., 2005). In view of this dual regulatory potential, which includes a key enzymatic role in the DNA repair pathway (Lukas and Bartek, 2009), it is clear to appreciate how influential the long-day signal of Eya3 could be, in driving

the photoperiodic molecular response pathway. Intriguingly, *Eya* gene homologs have also been demonstrated in higher plants (Takeda *et al*., 1999), indicating the highly conserved nature of the Eya family. Whilst it remains to be investigated whether plant Eya might be involved in developmental processes, it appears to most likely act as a protein phosphatase rather than a transcription factor (Rebay *et al*., 2005). As the highly conserved nature of Eya3 is unfolding in various species together with its extensive regulatory potential, it seems that the evolutionary importance of this protein in photoperiodism is yet to be fully appreciated.

7.4 Photoperiodic regulation of *TAC1* gene expression in the brain

Following the discovery of high concentrations of the neurokinin SubP in the sheep PT (Skinner *et al*., 2009; Skinner, 2009) and the revelation of strong LP-activated expression of *TAC1*, the gene encoding neurokinins, in the same region of the pituitary (Dupré *et al*., 2010), it has been hypothesised that these compounds may serve the role of the elusive PRL-regulator 'tuberalin'. However, investigation of *TAC1* mRNA expression in the present study, revealed a lack of expression of this gene in the rat and hamster PT. The studies were carried out using PT tissue from animals that had been pre-exposed to either SP or LP and culled 4h into the light phase. These experimental conditions were previously used in the ovine studies which presented an LP-activation of *TAC1* mRNA expression using both microarray analysis and *in situ* hybridization, following both acute (1 day) and chronic (4 weeks) exposure of the animals to LP conditions. Nonetheless, these results were not matched by the findings of the present *in situ* hybridization studies, which report no expression of *TAC1* following either 1-day or 5-week exposure to LP. These contradictory findings in sheep and rodents potentially signify a divergence in the seasonal pathways of prolactin regulation. However, considering that the identity of tuberalin has yet to be firmly established, it remains to be concluded whether the mechanisms governing the seasonal regulation of PRL may be species-dependent.

Despite the absence of *TAC1* expression in both the rat and hamster PT, this gene was expressed in other distinct areas of the brain. Perhaps the most striking finding was the activation of *TAC1* expression in the ependymal layer lining the third ventricle of the rat hypothalamus, under chronic (5 weeks) SP conditions, compared to a complete lack of expression under chronic LP. This region of the brain has previously been associated

with the seasonal regulation of thyroid hormone availability, body weight and reproduction (Barrett and Bolborea, 2012). The cells lining the region, are known as tanycytes and are considered to play an important role in mediating the PT-targeted actions of melatonin to the hypothalamus, ultimately leading to seasonal adaptations in physiology. The SP-activated expression of *TAC1* in the ependymal cells is therefore likely to be a downstream result of the melatonin-regulated action in the PT. Nevertheless, the significance of seasonal *TAC1* expression in this region remains to be investigated. Given the potential involvement of various *TAC1* protein products (neurokinins) in the regulation of hormone secretion from rat PD cells, it is possible that the seasonally-regulated expression of *TAC1* in this region of the hypothalamus is linked to the seasonal adaptations of the reproductive axis, or perhaps even of prolactin regulation.

The expression of PRL-Rs in the ependymal layer (Lerant and Freeman, 1998) encourages the idea of tanycytes being actively involved in the regulation of PRL, possibly receiving feedback information from the PD lactotrophs. It would therefore be interesting to investigate whether the expression of PRL-Rs in the ependymal region is also sensitive to photoperiod change, as this could reveal an important feedback pathway underlying the seasonal physiology of PRL. *PRL-R* mRNA expression has already been shown to be influenced by photoperiod in bovine mammary gland tissue, with a greater expression in SP-treated animals compared to those in LP (Auchtung *et al.*, 2003). This finding fits well with the consensus that generally speaking, PRL down-regulates the expression of its receptor (Goffin *et al.*, 2002), since LP animals have higher concentrations of circulating PRL compared to SP animals. Nevertheless, the consideration of a tanycytic feedback system in the seasonal regulation of PRL should be approached carefully, given the extensive work carried out using the hypothalamo-pituitary disconnected sheep model demonstrating that seasonal cycles in prolactin secretion are independent of hypothalamic regulation (Lincoln and Clarke, 1994; Lincoln and Clarke, 1995).

7.5 The role of neurokinins in the seasonal regulation of prolactin in the pituitary

Neurokinins have previously been shown to regulate prolactin secretion both directly, at the anterior pituitary level, and indirectly, through the modulation of different neurotransmitters at the hypothalamic level (Debeljuk and Lasaga, 2006). Furthermore,

they appear to exert both stimulatory and inhibitory actions on prolactin-regulating factors in order to influence prolactin release (Debeljuk and Lasaga, 2006). However, their potential role as seasonal prolactin secretagogues, was only recently suggested following extensive studies in sheep which revealed that neurokinins may be acting as the elusive 'tuberalins' driving the seasonal regulation of prolactin in the pituitary gland (Skinner *et al*., 2009; Skinner, 2009; Dupré *et al*., 2010).

In the present study, an *in vitro* luciferase reporter system was set up using dispersed primary pituitary cells derived from transgenic F344-Luc49 rats, to test the actions of three neurokinins, SubP, NKA and SubP(1-7), on prolactin promoter activity and also on prolactin release. The results revealed no effect of these substances on either the transcriptional activity of the promoter or on hormone release, and therefore do not support the idea that these neurokinins may be acting as prolactin-regulating factors in the rat pituitary.

Despite past studies having demonstrated a capacity of neurokinins to affect the release of prolactin in the rat pituitary as potential secretagogues, this fact alone does not establish a physiological role for these substances as PT-derived prolactin-releasing factors (tuberalins). It was therefore essential that *TAC1* expression be investigated in the PT, the region considered to be responsible for translating the photoperiodic information received through melatonin signal duration. So far, the photoperiod-regulated expression of *TAC1* in the PT appears to be specific to sheep. The present study reports a lack of *TAC1* expression in both the rat and Syrian hamster PT, two species known to be sensitive to photoperiod in terms of prolactin release. These findings suggest that neurokinins are not synthesized in the PT, and are therefore unlikely to fulfil the role of a seasonal regulator of prolactin in rodents. Neurokinin synthesis has previously been shown in the distal region of the rat anterior pituitary, in somatotrophs and thyrotrophs (Brown *et al*., 1991; Arita *et al*., 1994). The present results indicate co-localization of neurokinin receptors in corticotrophs, gonadotrophs and folliculo-stellate cells. Whilst the absence of neurokinin receptors in lactotroph cells further complicates the potential of a tuberalin-related role, their expression on other PD cell types suggests a physiological role for neurokinins in the regulation of pituitary hormone release nonetheless. Whether their actions are mediated through paracrine communication or whether they directly influence the activity of the cells to which the

neurokinin receptors co-localize, it currently remains doubtful that neurokinins serve as the seasonally-regulated tuberalins in the rat pituitary.

Whilst the photoperiodic regulation of prolactin secretion in mammals appears to be an ancient system (Lincoln, 1999), it is not yet known whether the identity of tuberalin is also conserved in photoperiodic mammals. Size fractionation studies of PT-conditioned medium have suggested that bovine tuberalin has an estimated size of >30 kDa (Lafarque *et al.*, 1998) whilst the ovine tuberalin is believed to be <1kDa in size (Graham *et al.*, 2002). This discrepancy could potentially signify inter-species variability in tuberalin identity.

In view of the present findings in the rat, compared to previous findings in the sheep model with regards to the role of neurokinins as potential tuberalins, it is rather obvious that certain inconsistencies exist. Differences in the photoperiod-dependent expression of the *TAC1* gene in the PT, which is strongly induced by LP in sheep (Dupré *et al.*, 2010) but completely absent in the rat in both SP and LP, along with the prolactin-inducing capabilities of the *TAC1* products (neurokinins) in sheep primary pituitary cultures, but not in rat, may reflect a divergence in the pathways of the seasonal prolactin-regulating machinery in the two different species.

It should be noted that whilst both species appear to be sensitive to photoperiod, adapting their physiology accordingly, the amplitude of circulating prolactin up-regulation following chronic (4-week) LP exposure compared to SP, is considerably greater in sheep (15-fold; Dupré *et al.*, 2010) compared to the rat (1.7-fold; Chapter 3). This is possibly linked to the degree of seasonality and associated physiological requirements, which are not necessarily the same in the two species. Whilst sheep are known to be strongly seasonal and display photoperiod-dependent cycles of pelage moulting (shedding of wool), laboratory rats that are housed in environments of constant conditions are not commonly known to exhibit this characteristic. This would imply inevitable differences in the mechanisms governing this particular trait. In view of the role of prolactin in controlling seasonal hair growth, it could therefore be hypothesized that the underlying cause for the inter-species differences in the molecular pathways regulating prolactin, may simply be a reflection of the evolutionary divergence in physiological responses between species, possibly due to artificial

selection processes designed to optimise fecundity in domesticated and laboratory animals.

Investigation of gene expression in the Syrian hamster PT also revealed a lack of *TAC1* expression, similarly to the rat studies. However, the Syrian hamster is known to be a strongly seasonal rodent, displaying robust photoperiod-dependent alterations in physiology, including changes in fur density, fur re-growth and hair length (Paul *et al.*, 2007). If the *TAC1* gene is involved in regulating seasonal prolactin, including the subsequent changes in pelage, then it might be expected to display photoperiod-dependent expression in the PT of a seasonal hamster, similarly to sheep. In absence of this finding, it remains difficult to assign a role for *TAC1* in the seasonal PT-mediated regulation of prolactin in rodent species.

7.6 The role of photoperiod-regulated prolactin release in the rat pituitary

There is strong evidence in the literature implicating the seasonal regulation of prolactin secretion with seasonal-dependent changes in pelage condition, in many different species (Djungarian hamster: Duncan and Goldman, 1984; mink: Martinet *et al.*, 1984; Soay sheep: Lincoln and Ebling, 1985; blue fox: Smith *et al.*, 1987; red deer and Pére David's deer: Loudon *et al.*, 1989; cashmere goat: Lynch and Russel, 1990). In view of the fact that perhaps the primary role of photoperiod-dependent prolactin is to regulate seasonal hair growth, it is slightly perplexing that the laboratory rat, which displays seasonal rhythms in prolactin, does not appear to exhibit seasonal changes in pelage; or if it does, this is not a conspicuous event compared to other species (Fraser, 1931). As discussed above, the relatively minor amplitude of the difference in prolactin concentration between SP and LP in the rat is likely to be the underlying reason. Similarly, studies using different breeds of sheep have shown that the more domesticated breeds, which displayed the least seasonal changes in terms of moulting and growth of pelage, also had higher concentrations of circulating prolactin in the winter, compared to the wild breeds that had relatively reduced concentrations at that time (Lincoln, 1990). This suggests that the amplitude of the seasonal change in prolactin concentration (SP *vs* LP) is directly correlated with pelage condition, i.e. the greater the difference in PRL concentration between SP and LP, the greater the seasonal change in pelage. This is likely to explain why the F344/NHsd rats which display a 1.7-fold increase in PRL in LP conditions compared to SP (Chapter 3) do not exhibit

obvious changes in pelage, whilst in Soay sheep, a feral breed, there is a 60-fold increase in peak prolactin concentrations from natural winter to summer conditions and a clear seasonal cycle in pelage status (Lincoln, 1990).

Another possible explanation for the lack of noticeable pelage changes in the rat, could be that the effect of seasonal prolactin release on coat condition is also dependent on other contributing factors, which may not be in place in the laboratory rat. Potential proof of this concept comes from the hypothalamo-pituitary disconnection studies which demonstrate that whilst HPD-rams maintain robust photoperiod-dependent prolactin rhythms, they display continuous wool growth in SP and LP conditions and only partial LP-induced moulting, unlike the control animals which show the anticipated seasonal cyclicity in wool growth and moulting (Lincoln and Richardson, 1998). This is likely to be a result of the surgical disconnection of the pituitary from the hypothalamus, which results in a lack of functional communication between the two structures and inevitable dampening in the co-ordination of different physiological responses. This would suggest that whilst PT-mediated prolactin regulation is the main driving force in seasonal pelage cyclicity, it is perhaps not the sole contributing factor in this process.

Studies using rats have shown that other hormones, including the sex steroids, oestradiol and testosterone, and adrenal steroids, can have an effect on hair growth, by delaying the anagen stage, whilst gonadectomy and adrenalectomy advance it (Ebling, 1990). Furthermore, testosterone has been shown to have a suppressive effect on seasonal fur re-growth in hamsters (Paul *et al.*, 2007) and badgers (Maurel *et al.*, 1987). Whilst thermoregulation is one of the key outcomes of seasonal hair growth, ambient temperature is not considered to be a major driving force of seasonal prolactin rhythms (Curlewis, 1992). Nonetheless, in wild short-haired weasels, cold temperature was shown to affect the onset of moulting and the nature of the pelage change (Rust, 1962) suggesting that in certain species, temperature could have a contributing effect on the seasonally-induced pelage changes. Studies in different breeds of sheep have suggested that modification of the hair follicles and a lack of responsiveness of PRL receptors may also be factors contributing towards the apparent continuous wool growth and absence of moulting in domesticated sheep, which nonetheless display seasonal rhythms in prolactin, similarly to wild breeds (Lincoln *et al.*, 1990).

Intriguingly, prolactin and prolactin receptor expression have been demonstrated in a non-seasonal context during the developmental hair cycle of mice (Foitzik *et al*., 2003). Prolactin was visualised at both the mRNA and protein level, and was shown to directly affect the hair cycle by inducing premature catagen (regression stage) in skin hair follicles (Foitzik *et al*., 2003) and also to inhibit developmental fur growth waves in laboratory mice (Craven *et al*., 2006). In rats, prolactin receptor expression has been demonstrated in the skin using *in situ* hybridization (Ouhtit *et al*., 1993). Collectively, these studies suggest that prolactin has a direct role in regulating the non-seasonal developmental-cycling of the hair follicle.

In view of the discussed issues, it would be prudent if future studies included a definitive investigation into seasonal pelage changes in the F344/NHsd rat, in order to fully elucidate whether minor seasonal alterations in fur condition are taking place more evenly and over a longer period of time. This could provide a valid justification for the photoperiodic regulation of PRL in the F344/NHsd strain of rats, albeit reduced in amplitude compared to that of other more seasonal species (Table 7.1). Presumably, the ability to maintain seasonal prolactin rhythms in response to photoperiodic treatment, is of physiological importance in this laboratory rat strain, and given the multi-functionality of the PRL hormone with over 300 targets having been associated with its actions in vertebrates (Bole-Feysot *et al*., 1998; Freeman *et al*., 2000; Goffin *et al*., 2002), it would not be unreasonable to assume an additional non-pelage-related role for this seasonally-regulated hormone in the male F344/NHsd rat. Such reasoning is consistent with previous findings that indicate an involvement of seasonal prolactin release in male mammals, not only with changes in pelage but also in the control of gonadal activity, reproductive hormone release, sexual behaviour, growth and metabolism (Lincoln, 1989; Curlewis, 1992).

In conclusion, it appears that PRL most likely has a role in both the seasonal and non-seasonal regulation of hair growth cycles. The robust regulation of this hormone in strongly seasonal animals may reflect a heightened responsibility to adequately adapt their physiology in advance of the challenging environmental conditions. In addition, it is evident that a synergistic action of other hormones and additional environmental factors is often required in order to allow complete physiological adaptations in pelage to occur. In domesticated animals, the mechanisms controlling these alterations are likely to become redundant following long-term exposure to artificial conditions.

Whether seasonal prolactin secretion sub-serves any additional functions in the F344/NHsd rat, remains to be investigated.

7.7 Future directions

7.7.1 Investigating the *TAC1* promoter sequence across selected species

Collectively, the observations regarding *TAC1* gene expression in the rat, hamster and ovine PT suggest species variability in the regulation of the *TAC1* promoter. Analysis of the *TAC1* promoter sequences across different species could therefore be useful in identifying either highly correlated sequence fragments, or potential polymorphisms across transcription factor binding sites, which may reflect the species variability in photoperiod-dependent gene expression profiles. Sequences of the TAC1 promoter regions of various species, including the rat, can be retrieved from the Genebank database and subsequently aligned to potentially reveal variations of individual elements, which may be responsible for the differential regulation of this gene in various species.

7.7.2 Investigating non-neurokinin candidates as potential 'tuberalins'

In view of the findings of this thesis, it is not currently possible to assign a role for neurokinins in the rat pituitary as the PT-mediated tuberalins. It would therefore be of interest for future studies, to address the likelihood of other non-neurokinin candidates being involved in the process of seasonal prolactin regulation.

7.7.2.a VGF-derived peptides

The presence of VGF (non-acronymic) peptides has been demonstrated in pituitary lactotrophs of both rats and sheep (Ferri *et al*., 1995, Brancia *et al*., 2005), whilst a biologically active VGF-derived peptide, TLQP-21, has recently been shown to induce prolactin mRNA and protein expression in a GH3 rat pituitary tumour cell line (Petrocchi Passeri *et al*., 2012). GH3 cells present somatotroph and lactotroph phenotypes and thus are able to secrete and synthesize both growth hormone and prolactin (Boockfor and Schwarz, 1988), however TLQP-21 had no effect on growth hormone expression (Petrocchi Passeri *et al*., 2012). Furthermore, TLQP-21 induced a trophic effect on GH3 cells and contributed to their differentiation into a mammotrophic

phenotype, suggesting a neuroendocrine role for TLQP-21 on lactotrophs in the pituitary gland (Petrocchi Passeri *et al*., 2012).

In addition, *VGF* mRNA has been demonstrated early in the developing PT of the rat pituitary (Snyder *et al*., 2003), coinciding with the expression of the melatonin receptor, MT1 in this tissue (Johnston *et al*., 2003b). Nonetheless, a thorough investigation is required into the expression of *VGF* mRNA in the PT of the adult pituitary, in response to photoperiod treatment, in order to ascertain whether this gene could be involved in PT-mediated PRL-regulating mechanisms.

7.7.2.b Endocannabinoids

Although several studies have indicated tuberalins to be of neuropeptidergic origin, with neurokinins presented as the strongest candidates, there are others who describe the messengers as being of a lipidergic nature. Endocannabinoids fall into this category and were first investigated in relation to this action, in the Syrian hamster (Yasuo *et al*., 2010b). Gene and protein expression of the enzymes involved in endocannabinoid synthesis and degradation, were identified in the hamster PT, whilst photoperiod was found to control the synthesis of one major endocannabinoid, 2-arachidonoylglycerol 2-AG, whose concentration in the PT was found to be higher in LP animals compared to SP (Yasuo *et al*., 2010b). Furthermore, the cannabinoid receptor 1 (CB1), the main receptor for 2-AG, was found to be expressed in the PD region, mostly co-localized with the S100 marker, in folliculo-stellate cells. These findings are therefore in support of an endocannabinoid system regulating hypophyseal hormone release, through an indirect action on PD folliculo-stellate cells.

7.8 Concluding remarks

In summary, the present study confirms that the F344/NHsd rat displays marked photoperiod-dependent changes in physiology (Figure 7.1), thus providing a very useful rodent model for studying photoperiodism. The primary hypothesis examined in this thesis was that neurokinins are responsible for driving the seasonal rhythms of prolactin secretion in the rat pituitary. Whilst this theory has not been confirmed using this model, significant findings in the rat and hamster extend our current knowledge of both the conserved mechanisms underlying photoperiodism and also potential inter-species divergences. Overall, this thesis suggests that an integrative consideration of a range of

species, each displaying varying degrees of photoperiod-responsiveness, provides a deeper more meaningful analysis of the mechanisms driving not only the seasonal regulation of prolactin, but perhaps also of other photoperiod-sensitive biological functions.

SPECIES : (Strain/breed)	RAT (F344/NHsd)	HAMSTER (Syrian)	SHEEP (Soay)
Photoperiod-sensitive	√	√	√
LP-activated gene expression in the PT	*Eya3* ----	*Eya3* ----	*Eya3* *TAC1*
Photoperiod-dependent physiology (body weight, food intake, reproductive parameters)	√	√	√
Seasonal PRL release	√	√	√
PRL increase in LP relative to SP	1.7-fold [1] (5 weeks)	3.5-fold [2] (5 weeks)	15-fold [3] (4 weeks)
Seasonal hair growth / moulting	?	√	√

Table 7.1: Comparative analysis of the key markers associated with the seasonal secretion of PRL in three different photoperiodic species: the F344/NHsd rat, the Syrian hamster and Soay sheep.

The points presented in red font indicate results either obtained or confirmed in the present thesis. The PRL data are taken from: 1. The present study (Chapter 3); 2. Goldman *et al*., 1981; 3. Dupré *et al*., 2010.

Long Photoperiods

PT

⇧ Eya3

Tuberalin

Brain

PD

Other PD cell types

Lactotrophs

-Body weight
-Food intake
-Reproductive parameters
-Gonadal hormones (testosterone, oestradiol)

⇧PRL release

Seasonal hair growth

-Reproductive status
-Sexual behaviour
-Metabolism

Other environmental cues (e.g., temperature)

Figure 7.1: Proposed photoperiodic pathways downstream of LP activation, leading to alterations in the physiology of the F344/NHsd rat.

Upon LP activation, *Eya3* gene expression is up-regulated in the F344/NHsd rat PT. Whilst it is considered that Eya3 may be involved in the regulation of pathways controlling PRL release, the intermediate mechanisms remain to be identified. The current suggested model of prolactin regulation dictates that tuberalin is released from the PT to act in the PD region, altering the activity of lactotrophs, potentially through paracrine communication with other PD cell types, and ultimately increasing PRL release. The seasonal change in PRL is known to influence seasonal hair growth, however in the rat this is not a conspicuous event and may signify the requirement for other environmental cues. Furthermore, PRL is likely to be involved in non-pelage related mechanisms regulating reproductive status, and other aspects of physiology and behaviour. It is proposed that seasonal hair growth may be additionally influenced by the action of gonadal hormones. Adaptations of body weight, food intake and of reproductive parameters, are considered to be regulated via a separate PT-mediated signalling pathway to the brain, however the mechanistic links remain to be identified in the rat. The dashed arrows indicate suggested mechanisms that have not been confirmed using the present model. **PD**: Pars distalis; **PRL**: Prolactin; **PT**: Pars tuberalis. Figure by the author.

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