REGULATION OF OESTROGEN RECEPTOR mRNA IN RAT CENTRAL NERVOUS SYSTEM

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I was brought up by my grandmother,
Whose love I shall cherish
The rest of my life

For my father, my mother and my family

Declaration

The studies outlined in this thesis were undertaken by the author in the Laboratory of Neuroendocrinology, Department of Biomedical Sciences, University of Edinburgh, Medical School and the Laboratory of Molecular Endocrinology, Molecular Medicine Centre, Western General Hospital, University of Edinburgh under the supervision of Professors J A Russell and J R Seckl and Dr M C Holmes. All of the work described was performed by the author and with collaborations with others as indicated below.

i)   All the surgery was performed by myself, Prof John Russell and Dr Megan Holmes, except adrenalectomy which was performed by Miss Sharon Rossiter.

ii)   The sub-cloning of ER was undertaken with guidance from Dr Karen Chapman and subsequent supervision of Miss Val Lyon.

iii)   Dr Karen Francis prepared the animals for the pregnancy studies.

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Abstract

The gonadal steroid 17-β oestradiol (E_2) influences a variety of neural activities, not only those involved in aspects of reproductive physiology but also in other functions, including autonomic regulation and cognitive processes. The effects of sex steroid hormones are principally, although not exclusively, mediated by binding to their cognate intracellular receptors to regulate functions of their target cells. The expression of these receptors is an important determinant of the actions of steroid hormones. There are two principal oestrogen receptors (ER) characterised to date, ER-α and ER-β, the products of two distinct genes.

Using quantitative *in situ* hybridisation histochemistry we first described the distribution of neurones expressing the messenger ribonucleic acid (mRNA) moieties for both types of ER in rat brain. We then investigated whether changes in the expression of these transcripts occur in response to hormonal manipulations as well as physiological stimulation in two discrete regions: the hippocampus, involved in memory, behaviour and regulation of the hypothalamo-pituitary-adrenal (HPA) axis and the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, producing oxytocin and vasopressin, and autonomic regulation. Both ER-α and -β mRNAs were found to be expressed in the hippocampus, while only ER-β mRNA was localised in the PVN and SON.

In the hippocampus, changes in the expression of both mRNA transcripts following gonadectomy were found in a region- and time-specific manner and displayed a sexual dimorphic pattern. However, replacement of E_2 in ovariectomised rats did not restore the basal level of expression indicating that factors other than the ligand itself are involved in mediating effects of gonadectomy. Hippocampal neurones also express GR and MR, receptors by which adrenal steroid hormones act through. Because glucocorticoids might act by heterologous regulation of ER mRNA expression, the effects of decreasing or increasing corticosteroid secretion were studied.

None of the adrenal manipulations changed ER-α expression, but adrenalectomy decreased ER-β mRNA expression, only in CA1, and this was prevented by corticosterone replacement. Repeated stress for 72 h had no effect on either ER-α or -β mRNA expression in the hippocampus.

The expression of ER-β mRNA was found in regions containing oxytocin and vasopressin magnocellular neurones; expression in the PVN was low in the medial parvocellular neurones, projecting to the median eminence, but high in the ventral group of parvocellular neurones which project to brainstem and spinal cord.

In the SON the greater ER-β mRNA signal found in female than in male rats was abolished by gonadectomy, but not restored or altered in intact male rats by E$_2$ treatment. In the ventral parvocellular neurones, we found no sex difference in the expression of ER-β mRNA. Here, ovariectomy significantly increased the expression of ER-β mRNA. The expression of ER-β mRNA in both the magno- and parvo-cellular neurones was not changed at the end of pregnancy, when oestrogen secretion is maximal. In contrast, marked changes in ER-β mRNA expression were found in SON and PVN neurones, but not in the hippocampus, following manipulation of adrenal-corticoid secretion. First, bilateral adrenalectomy, removing gluco- and mineralo-corticoids, significantly increased ER-β mRNA expression in the SON but not in the ventral parvocellular neurones. The effect of adrenalectomy was partially reversed by replacement with corticosterone. Stimulation of the HPA axis by repeated stress did not alter expression of ER-β mRNA, except in the ventral parvocellular neurones where expression was significantly increased. Stimulating the magnocellular neurones by salt-loading markedly attenuated the expression of ER-β mRNA selectively in these neurones, and also in the ventral parvocellular PVN neurones.

The results indicate actions of E_2 via ER-α/-β in hippocampus, but only via ER-β in the PVN/SON; there is a weak, regionally-specific regulation of ER-α/-β by sex steroids. In contrast, ER-β in magnocellular neurones may be up-regulated by gluco- and mineralo-corticoid deficiency, and down-regulated by physiological stimulation of the neurones. This would alter any ER-mediated effects of E_2 on the neurones in these states.

CHAPTER 1   General Introduction

CONTENTS

CHAPTER 1:   **General introduction**

CHAPTER 2:   **Materials and methods**

<u>CHAPTER 3</u>: Comparative distribution of two types of oestrogen receptor mRNAs (-α *vs* -β) in rat brain

<u>CHAPTER 4</u>: Regulation of oestrogen receptor-α and -β mRNAs in the hippocampus

<u>CHAPTER 5</u>: Regulation of oestrogen receptor-β mRNA in the supraoptic and paraventricular nuclei

List of Figures

<u>List of Table</u>

List of abbreviations

ACTH	adrenocorticotrophic hormone
AD	Alzheimer's disease
ADX	adrenalectomy, adrenalectomised- (bilateral)
ANOVA	analysis of variance
bp	base pair
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CORT	corticosterone
CRF	corticotrophin releasing factor (hormone)
CX	castration, castrated-
d	day
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
ER	oestrogen receptor
ERE	oestrogen response element
E_2	oestradiol, 17-β
g	gram
GC	glucocorticoids
GR	glucocorticoid receptor
h	hour
HPA axis	hypothalamo-pituitary-adrenal axis
HPG axis	hypothalamo-pituitary-gonadal axis
HRT	hormone replacement therapy
H_2O	water
ISH	*in situ* hybridisation histochemistry
kg	kilogram

l	litre
LTD	long-term depression
LTP	long-term potentiation
M	molar
μg	microgram
μl	microlitre
MCN	magnocellular neurosecretory neurone
mg	milligram
min	minute
ml	millilitre
mm	millimetre
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
ng	nanogram
nmol	nanomolar
nt	nucleotide
OVX	ovariectomy, ovariectomised-
OXT	oxytocin
PCR	polymerase chain reaction
PVN	paraventricular nucleus
P_4	progesterone
RNA	ribonucleic acid
s.c.	subcutaneous
SF	sham-operated female
SM	sham-operated male
SON	supraoptic nucleus
SPA	scintillation proximity assay
SSC	saline sodium citrate
T	testosterone
Tris	tris (hydroxymethyl) aminomethane
VP	vasopressin

Chapter 1

Introduction

1.1.   Sex steroid hormones

The principal female sex steroids consist of three different oestrogenic compounds, oestradiol-17β (E_2), oestrone (E_1), and oestriol (E_3) as well as naturally occurring progestogens, principally progesterone (P_4). Of the three oestrogens, E_2 is the most potent and most abundant molecule, and is responsible for mediating most of the oestrogenic effects in the body.

In women, the circulating concentrations of these hormones fluctuate during the reproductive cycle. Before pregnancy, the main source of oestrogens is the E_2 produced by the granulosa cells (and to a lesser extent thecal cells) of the follicles in the adult ovaries (Hilf & Wittliff, 1975). Other sites of oestrogen production include cells in the corpus luteum where E_2 is produced during the luteal phase as well as the adrenal glands, although they do not produce E_2 in any significant amount. During pregnancy, however, the foeto-placental unit takes over as the primary synthesis site for oestrogens and produces E_3 in large amount from precursors derived from maternal and foetal adrenal glands. On the other hand, the role of E_2 becomes less significant in postmenopausal women when the ovary has ceased to produce oestrogens. Instead the major source of circulating oestrogens is E_1 which is formed from the peripheral conversion of androstenedione under the control of the enzyme aromatase. Formation of E_1 occurs in many peripheral organs but chiefly in the adipose tissue from where the majority of E_1 is derived. Indeed, obese menopausal women can have normal or even raised oestrogen levels compared to the premenopausal non-obese women (Laycock & Wise, 1996).

As for P_4, it is secreted primarily by the corpus luteum during the luteal phase of the reproductive cycle and by the foeto-placental unit during pregnancy. P_4 is not only important for its own endocrine actions but also for its role as a precursor molecule in the synthesis of steroids in tissues that produce them.

The chief male sex hormones, the androgens, are primarily synthesised by the interstitial (Leydig) cells in the testes with the remainder being produced by the adrenal glands. Androgens are presented in two molecular forms; testosterone (T) and 5-α dihydrotestosterone (DHT). The conversion of T into DHT is controlled by the 5α-reductase enzyme (Normington & Russell, 1992; Russell & Wilson, 1994; Selmanoff *et al.*, 1977), where DHT exerts its physiological role in peripheral androgen-dependent structures such as the development and function of prostate gland (Poletti *et al.*, 1998). Moreover, in the CNS, the cytochrome P450 aromatase (oestrogen synthase) enzyme converts T into E_2 (Lephart & Ojeda, 1990; Naftolin *et al.*, 1975) where it elicits most of its effects in this tissue (MacLusky & Naftolin, 1981; Roselli, Horton, & Resko, 1987; Roselli & Klosterman, 1998).

The biosynthesis of oestrogens begins with the formation of androgens from the common precursor cholesterol, which can be synthesised by endocrine cells from acetate or it can reach the cell following its transport in the blood associated with lipoproteins. In fact, all steroid hormones are derived from cholesterol and therefore consist of the cyclopentanoperhydrophenanthrene nucleus with varying side chains and groups conferring specificity to the different classes of hormones (Figure 1.1.).
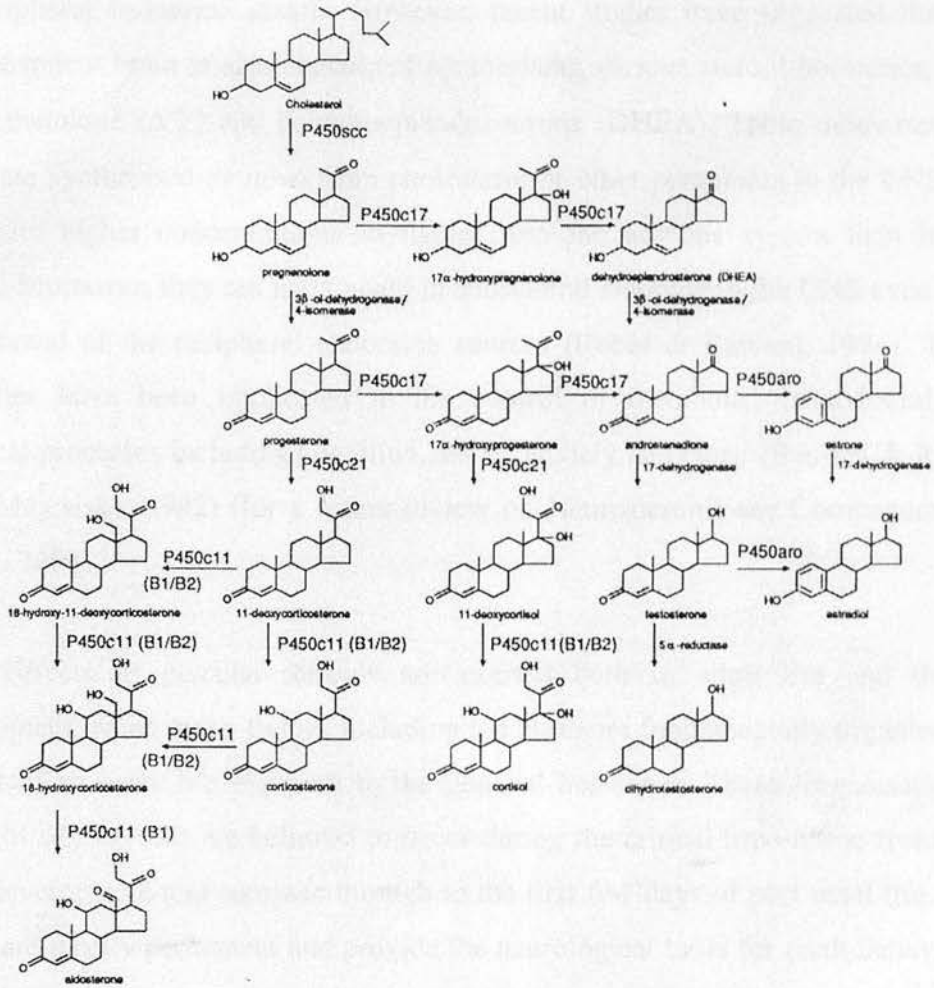
Figure 1.1. A schematic representation of steroid biosynthetic pathways. Some of the principle steroidogenic enzymes involved in the interconvertion of various steroids are indicated. Most of these enzymes, however, belong to a large family of P450 cytochromes that have multiple activities (Miller, 1988).

(Reproduced from Bolander, 1994)

Classically, the steroid hormones were believed to derive exclusively from the peripheral endocrine glands. However, recent studies have suggested that the CNS of rodent brain is also capable of synthesising various steroid hormones, such as pregnenolone (Δ^5P) and dehydroepiandrosterone (DHEA). These *neurosteroids*, which are synthesised *de novo* from cholesterol or other precursors in the CNS, are present in higher concentrations in tissue from the nervous system than in the plasma. Moreover, they are maintained in substantial amounts in the CNS even after the removal of the peripheral endocrine sources (Robel & Baulieu, 1994). These molecules have been implicated in the control of metabolic, behavioural and psychical processes including cognition, stress, anxiety and sleep (Baulieu & Robel, 1996; Majewska, 1992) (for a recent review on Neurosteroids see Compagnone & Mellon, 2000)

Effects of gonadal steroids are exerted both in adult life and during development, when many tissues including the brain are fundamentally organised as a result of an early life exposure to the gonadal hormones. These 'organisational' effects of sex steroids are believed to occur during the critical time-frame from late foetal development and continue through to the first few days of post natal life. The effects are mostly permanent and provide the neurological basis for adult behaviour. Later in life, however, sex steroid hormone exposure can also exert an 'activational' effect upon responsive neurones by a number of mechanisms e.g., by changing neurotransmitter function (Biegon & McEwen, 1982; Di Paolo, 1994) or affecting neuronal plasticity (Frankfurt, 1994). Often this results in an induction of a temporary alteration in the animal's behaviour. A prime example of these dual effects of sex steroids is evident in mammalian brain structures which are highly susceptible to circulating sex steroid hormone level. For instance, Phoenix *et al.* 1959 has shown that exposure of female rodents to androgens *in utero* permanently reduced their sexual receptivity which was persistent through to adulthood (Phoenix *et al.*, 1959). The permanence of such effects seems likely to occur as a result of an organisation effect on the nervous tissue rather than a mere temporary activation of the system. In contrast the induction in the short term of many well-established

behaviours such as maternal and sexual behaviours, can be induced experimentally by exposure of adult female rats to E_2 and P_4, the effects which are reversed upon removal of the hormone exposure (Bridges, 1984; Moltz *et al.*, 1970; Siegel & Rosenblatt, 1975).

Although generally regarded as sex hormones, gonadal steroids, in particular E_2, affects a wide range of physiological processes. This is reflected by the prominent expression of oestrogen receptor (ER), the mediator of E_2 action, found in many organs.

1.2.   Pathways mediating actions of oestrogens

Actions of steroid hormones are largely dependent on the receptors in cells in target organs. Although other cellular proteins are involved, it is the receptor that largely dictates the magnitude of steroid response. Pathways mediating actions of steroids are generally classified into two modes, although some other concepts have emerged in attempts to explain actions that deviate from both pathways (see below).

1.2.1. Genomic pathway

The major mode of steroid action is considered to be via the classical genomic pathway. Principally, the mechanism involves binding of the ligands to their cognate intracellular receptors, which function as ligand-dependent transcription factors. Steroid molecules are lipophilic in nature and so they diffuse readily across cell membranes and are retained in target cells by binding to their receptors. In the absence of hormone, each receptor monomer is sequestered with an inhibitory complex of heat-shock protein90 (hsp90) as well as other molecules (Smith, 1993; Smith & Toft, 1993). This receptor complex resides either in the cytoplasm or is loosely bound in the nucleus (this is the case for ER) (Parker, 1995). Once bound by ligand the receptor translocates into the nucleus and undergoes conformational

changes leading to dissociation of hsp90. The hormone-receptor complex then forms (homo-/hetero-) dimers and interacts sequence-specifically with a specific hormone response element (HRE) in the promoter region of target DNA to modulate the transcription process. The consensus oestrogen response element (ERE) is characterised by the inverted repeat of two half-sites palindromes 5′-GGTCAnnnTGACC-3′ separated by 3 bp (Klein-Hitpass *et al.*, 1988; Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986).

Such genomic events involve *de novo* protein synthesis and are characterised by a long latency and duration of action which usually takes up to hours or days to occur (Lee & Gorski, 1996). The process can be blocked by mRNA or protein synthesis inhibitors such as actinomycin D, purinomycin or cyclohexamide. Oestrogen receptor (ER) together with the receptors for various other sex and adrenal hormones constitute a large steroid-hormone receptor superfamily, which includes glucocorticoid (GR), mineralocorticoid (MR), thyroid hormone (TR), vitamin D metabolites (VD), retinoic acid (RAR), androgen (AR) as well as progesterone (PR) receptors (Keightley, 1998). Hormone receptor molecules are found in substantial quantities only in their target cells (Figure 1.2.).
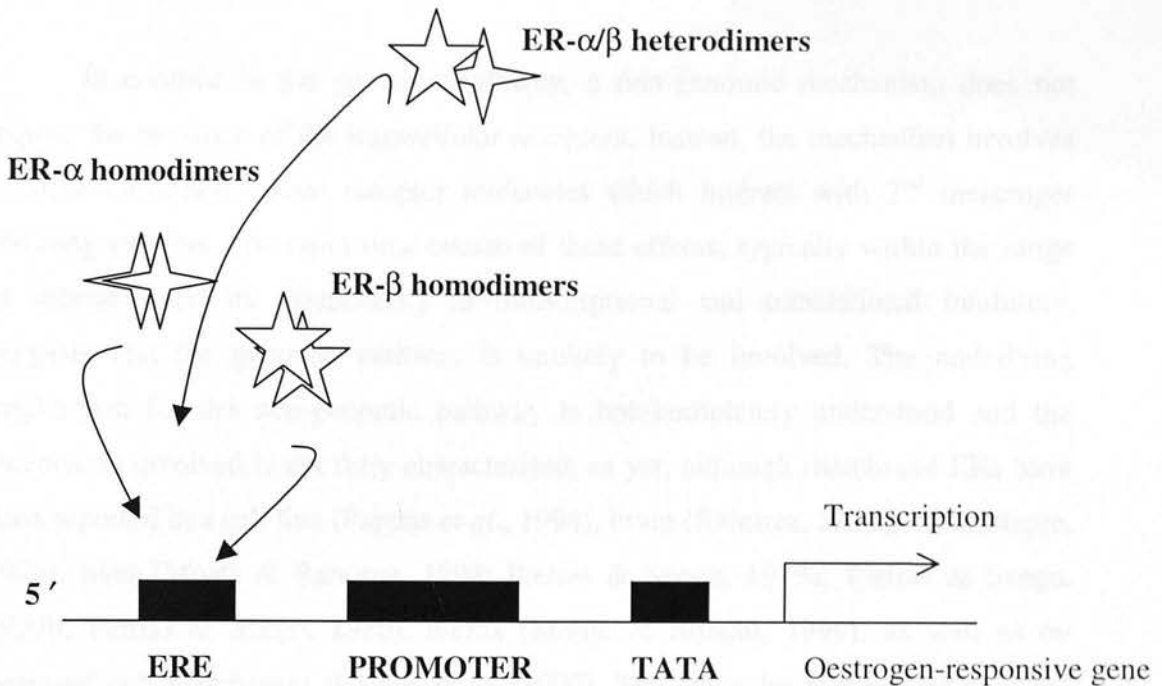
Figure 1.2. Proposed model for the oestrogen-signalling pathway suggests various alternative dimerisation states of the oestrogen receptors. Steroid hormone receptors are known to form predominantly homodimers and bind to the hormone response element to modulate the transcription of target genes (Beato, 1989; Kumar & Chambon, 1988). However, several *in vitro* as well as *in vivo* studies have shown that ER-α and ER-β can heterodimerise and that the activity of this complex is different from, and intermediate between that of the homodimers of either receptor subtype (in mouse (Pettersson *et al.*, 1997) and in human (Pace *et al.*, 1997)). Consequently this combinational approach could enhance the repertoire of possible target genes modulation by E_2. Indeed a study by Giguère *et al.* 1998 suggests that heterodimerisation is in fact the preferred state in the presence of both ER subtypes (Giguère, Tremblay, & Tremblay, 1998). Ultimately the response of genes to oestrogens would depend on the overall proportion of the ER-α or -β homodimers as well as the heterodimers in tissues that express both receptor subtypes. Alternative to the classic ERE, both ERs have recently been reported to be able to stimulate gene expression from promoters that contain the activating protein (AP)-1 site, the cognate binding site for the transcription factors Jun and Fos (Gaub *et al.*, 1990). Intriguingly the response of transcription via this AP-1 site appears to mediate an opposite effect between ER-α and -β in the presence of E_2. While ER-α exhibits dose-dependent transcriptional activation, ER-β, on the other hand, shows no effect or inhibits the stimulation in an antiestrogen-dependent fashion (Paech *et al.*, 1997). This suggests a novel route by which E_2 could modulate gene transcription in addition to the ER-ERE pathway.

1.2.2.   Non-genomic pathway

In contrast to the genomic pathway, a non-genomic mechanism does not require the presence of the intracellular receptors. Instead, the mechanism involves putative membrane-bound receptor molecules which interact with 2nd messenger coupling systems. The rapid time course of these effects, typically within the range of seconds, and its insensitivity to transcriptional and translational inhibitors, suggests that the genomic pathway is unlikely to be involved. The underlying mechanism for the non-genomic pathway is not completely understood and the receptor(s) involved is not fully characterised, as yet, although membrane ERs have been reported in a cell line (Pappas *et al.*, 1994), brain (Ramirez, Zheng, & Siddique, 1996), liver (Moats & Ramirez, 1998; Pietras & Szego, 1979a; Pietras & Szego, 1979b; Pietras & Szego, 1980), uterus (Monje & Boland, 1999), as well as on neuronal cell membranes (Clarke *et al.*, 2000). The study by Razandi *et al.* 1999 suggests that, perhaps, both the cell membrane and nuclear ER (both -α and -β) might originate from the same transcript, although the number of those membrane-bound ERs are markedly less than that found in the nuclear compartment (Razandi *et al.*, 1999).

In recent years the non-genomic action of steroids has gained a better appreciation. In particular, effects on neuronal excitability (Moss & Gu, 1999) have been intensely explored in the past couple of years. A detailed description of the mechanism for non-transcriptional pathway of oestrogen signalling is not intended as a primary aim in this thesis, thus only relevant points will be commented upon.

1.3. Nuclear oestrogen receptors

There are two oestrogen receptors (ER) identified and characterised to date. The first of the two ERs, named ER-α, was among the very first of the steroid receptors to be characterised and was cloned from many species during the late 1980s (for human (Green *et al.*, 1986; Greene *et al.*, 1986; Walter *et al.*, 1985), for rat (Koike, Sakai, & Muramatsu, 1987), for mouse (White *et al.*, 1987) and for chicken (Maxwell *et al.*, 1987). The physiological role of ER-α is well documented in many areas, particularly those related to reproductive function, and served as the basis for understanding the actions of oestrogens for a number of years. In contrast, the novel ER-β has only been recently cloned (Kuiper *et al.*, 1996; Mosselman, Polman, & Dijkema, 1996) and so relatively less is known about its role compared to that of the classic ER-α. The two receptors represent examples of steroid receptor molecules existing as two isoforms but encoded by two separate genes (Kuiper *et al.*, 1996). In the human, ER-β is located on chromosome 14 while the -α isoform is on chromosome 6 (Enmark *et al.*, 1997). Both ER-α and -β are able to stimulate the transcription of an ERE-transfected reporter gene in a dose-dependent manner (Cowley *et al.*, 1997; Kuiper *et al.*, 1997; Mosselman, Polman, & Dijkema, 1996; Watanabe *et al.*, 1997).

Although both receptors have been shown to be co-localised in a number of tissues including the central nervous system, the cellular distribution of the two forms of ER is rather distinct suggesting significant differences of each receptor subtype in conferring specific biological processes in a tissue- and/or cell type-specific manner (Brandenberger, Tee, & Jaffe, 1998; Byers *et al.*, 1997; Couse *et al.*, 1997; Kuiper *et al.*, 1997; Kuiper *et al.*, 1998b; Shughrue, Komm, & Merchenthaler, 1996; Shughrue, Lane, & Merchenthaler, 1997a; Shughrue *et al.*, 1998).

1	190	256	360	554	600

(1)

A/B	C	D	E	F

1	104	170	259	455	485

(2)

16.5	*97.0*	*28.9*	*59.7*	*16.7*
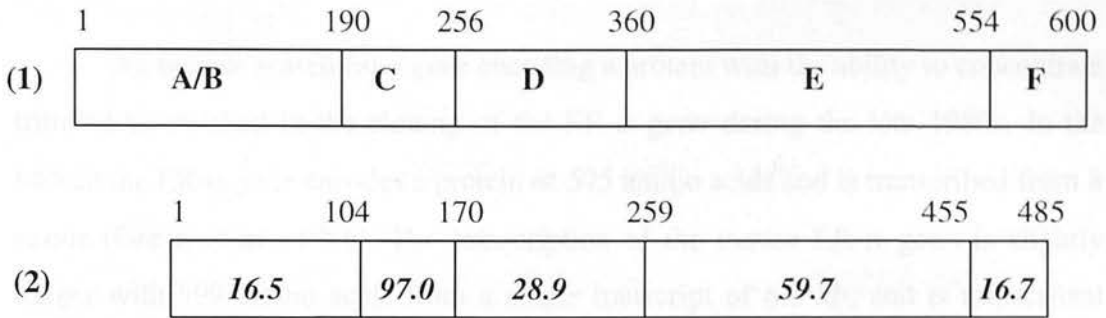
Figure 1.3. Schematic representation of the structures of rat ER-α (1) and ER-β (2) showing percentage (numbers in italics) of amino-acid identity between the two cDNAs. Unique to the nuclear receptor superfamily, their primary structure is characterised by amino acid sequence encoding four or five functional domains, A-F. The amino-terminal A/B domain is the most variable portion of the steroid receptor, both in length and sequence, and demonstrates only 16.5% homology between ER-α and -β. This domain contributes to the transactivation function of the receptor which activates target genes via the core transcription machinery (Horwitz *et al.*, 1996). The C region, denoted DNA-binding domain (DBD), is most conserved between different nuclear receptors including that of ER-α *vs* -β, with 97% homology between the two. Not surprisingly, both ERs interact with similar binding to a consensus DNA response elements (namely, ERE) located in the regulatory regions of the target genes. Apart from its role in the binding of the receptor to the DNA hormone response element, this region also contains a weak dimerisation activity. The ligand-binding domain (LBD), E region, is relatively conserved and possesses multiple activities but most importantly it is responsible for the receptor-specific ligand binding process. Although the amino acid encoding this domain of the two ERs shares *ca.* 60% sequence identity, each ER subtype binds to E_2 with nearly equal affinity and show a very similar ligand binding characteristics for a number of oestrogenic compounds (Kuiper *et al.*, 1997). The D region, also known as the hinge domain, is located between the DBD and the LBD. Its major function lies in its specific sequence for the nuclear localisation signal. The carboxy-terminal F domain is not present in all nuclear receptors and its sequence in ERs is unique. It is involved in modulating the expression of target genes. (Detail based on ER-α (Koike *et al.*, 1987) and ER-β (Kuiper *et al.*, 1996)).

1.3.1. Oestrogen receptor-α

An intense search for a gene encoding a protein with the ability to concentrate tritiated-E_2 resulted in the cloning of the ER-α gene during the late 1980s. In the human the ER-α gene encodes a protein of 595 amino acids and is transcribed from 8 exons (Green *et al.*, 1986). The transcription of the mouse ER-α gene is slightly longer with 599 amino acids from a single transcript of 6.3 kb, and is transcribed from 9 exons (White *et al.*, 1987). The rat ER-α clone with a complete open reading frame encodes 600 amino acid residues and shares 97% overall homology with the mouse ER-α protein sequence (Koike *et al.*, 1987).

The tissue distribution of ER-α indicates a relatively high degree of expression in the uterus, mammary gland, testis, pituitary, kidney and skeletal muscle and moderate to low expression in the epididymis, ovary and brain, although this depends on species, sex and techniques employed. (Byers *et al.*, 1997; Couse *et al.*, 1997; Cuhna *et al.*, 1991; Greco, Duello, & Gorski, 1993; Kuiper *et al.*, 1997; Pasqualini & Sumida, 1986; Shughrue *et al.*, 1996). Subsequent to the identification of the first ER-α many other splicing isoforms have been discovered. A recent investigation of the single human ER-α gene suggests a multiple promoter usage mechanism which gives rise to a total of 6 ER-α splicing transcripts, all of which encode a common receptor protein but containing a unique 5'-untranslated region (UTR) of their own. Furthermore each of these alternative splicing isoforms has been shown to exhibit a tissue-specific distribution and is subject to differential regulation by a number of transcription factors including their endogenous ligand, E_2 (Flouriot *et al.*, 1998). In rats, an isoform of the ER-α lacking exon four, which encodes part of the LBD, is expressed in the brain (Skipper *et al.*, 1993). Another report extended the finding and suggested possibly at least four other subtypes of rat ER-α mRNA. Again the expression of these alternative splicing isoforms is quantitatively different from one tissue to another, and they are present at different developmental stages (Kato *et al.*, 1998).

Although some of these splicing variants might not be translated into functional receptor protein or may be unable to bind ligand, they might function as important modulators of the tissue- and promoter-specific effects of E_2 behaving in either a dominant-negative or dominant-positive manner (Dotzlaw, Alkhalaf, & Murphy, 1992; Lehrer *et al.*, 1990; Murphy & Dotzlaw, 1989).

The role of ER-α is further highlighted by the generation of oestrogen receptor 'knock-out' (αERKO) mice in the early 1990s (Couse *et al.*, 1995; Lubahn *et al.*, 1993). This molecular technique in which the gene encoding a particular protein has been disrupted, resulting in a non-functional receptor molecule, has partially enabled the identification of function of those genes of interest (or rather the function of the *missing* genes). This approach is useful particularly in assessing altered behaviours as a result of the disrupted genes. Oestrogen receptor was traditionally thought to be critical for the survival of embryonal organisms, since no mutation in the ER gene that results in insensitivity to E_2 had been reported in human or laboratory animals, while critical roles of E_2 during embryonic development were demonstrated. However, this idea was challenged by the discovery of a man lacking functional ER in 1994 (Smith *et al.*, 1994). Analysis of the ER gene of this patient showed a point mutation of codon 157 resulting in a non-functional ER lacking both the DBD and LBD. This homozygous mutation of the gene results in a range of impaired functions in many systems, especially within peripheral tissues, leading for example to osteoporosis. Equally, the αERKO mice, although they are viable and show normal gross development compared to their wild-type litter mates, exhibit several abnormalities in adulthood. Of note, these mice are infertile and display various alterations in their sexual behaviours both in males and females, confirming the essential role of ER-α in reproductive physiology (Ogawa *et al.*, 1998a; Ogawa *et al.*, 1997; Ogawa *et al.*, 1998c). However, in the brains of αERKO mice, neurones in some structures including the preoptic, arcuate, bed nucleus of the stria terminalis and amygdala nuclei can still concentrate ^{125}I-oestrogen, the effect being competitively eliminated by E_2 but not by an ER-α selective agonist, $16\alpha IE_2$ (Shughrue, Lane, & Merchenthaler, 1999). Functionally, E_2 has been shown to up-

regulate the expression of the progesterone receptor (PR) mRNA in the medial preoptic area of αERKO mice (Shughrue et al., 1997b).

Collectively these findings highlight the critical roles of the oestrogen-signalling pathway in many tissues although the disruption in the ER-α gene did prove to be non-lethal. Indeed the rescue mechanism and the remaining oestrogenic activity seen in the knock-out mice might be due to compensation mechanisms by other molecules, with ER-β being one very likely candidate.

1.3.2.   Oestrogen receptor-β

The recent discovery of ER-β has provided an alternative nuclear receptor pathway by which oestrogenic compounds could mediate their effects upon certain tissues. The initial characterisation of the first reported ER-β cDNA, cloned from rat prostate, indicated that it is composed of 485 amino acids with a molecular weight of 54 kDa (Kuiper et al., 1996) and shows a high degree of sequence homology to that of ER-α, particularly in the DBD (see Figure 1.3.). The genomic clone for ER-β was subsequently cloned from many other mammalian species including human (Mosselman et al., 1996), mouse (Tremblay et al., 1997), gold fish (Tchoudakova, Pathak, & Callard, 1999), catfish (Xia et al., 1999), and cow (Rosenfeld et al., 1999). These homologs all have a similar relationship to ER-α. A number of initial investigations indicated high degree of expression of ER-β in the ovary and prostate, moderate expression in the oviduct, testis, bladder, uterus, brain and lung and little or no expression in the mammary gland (Couse et al., 1997; Kuiper et al., 1997; Kuiper et al., 1996; Shughrue et al., 1998).

In terms of ligand binding activity, ER-β was found to bind E_2 with a near-equal affinity to ER-α (Kd of 0.4 nM for -β and 0.1 nM for -α), with low affinity for testosterone, progesterone and corticosterone (Kuiper et al., 1997; Kuiper et al., 1996). Other potent ligands for ER-β are the phyto-oestrogens, such as coumestrol,

genistein and kaempferol, which compete more strongly with E_2 for binding to ER-β than to ER-α. These phyto-oestrogens possess the ability to stimulate transcriptional activity of both types of ER in the low nM range, at least *in vitro* (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998a).

Following the identification of the first ER-β clone, many isoforms of ER-β have subsequently been cloned, with at least 9 or 10 reported in the current literature (Warner, Nilsson, & Gustafsson, 1999). They appear to be alternative splicing isoforms of the first ER-β (ER-β1), some of which have extended N-termini and others with truncations or insertions in the LBD. ER-β2 mRNA contains an additional 54 nucleotides in the ligand binding domain of the first ER-β (for human (Hanstein *et al.*, 1999; Moore *et al.*, 1998), for rat (Maruyama *et al.*, 1998), and for mouse (Lu *et al.*, 1998). The insertion of extra amino acids in this splicing isoform leads to decreased binding to E_2. Hence ER-β2 has an 8-fold lower affinity for E_2 and requires a 100- to 1000- greater concentration of E_2 for transcription activation compared to ER-β1 or ER-α. ER-β2 coexists with both ER-α and ER-β1 in varying proportions in a variety of tissues. In the brain, ER-β2 is located in the cortex, hypothalamus and hippocampus, although it is 2 to 6-fold less abundant than ER-β1 (Petersen *et al.*, 1998). Despite its reduced ligand binding activity, however, ER-β2 can heterodimerise with both -α and -β1 and bind to an ERE. It appears to function as a negative modulator of oestrogens since it suppresses ER-α and ER-β1 mediated transcription in a dose-dependent manner (Maruyama *et al.*, 1998). Other ER-β isoforms include the isoform 1-5 from a human clone as well as ER-βcx (for mouse (Pettersson *et al.*, 1997), for human (Moore *et al.*, 1998; Ogawa *et al.*, 1998b). Whether all of the ER splicing isoforms identified thus far are translated into functional proteins and have any significant biological role is currently unknown.

In contrast to αERKO, homozygous mutant mice bearing non-functional ER-β (βERKO) develop normal sexual behaviour and are reproductively competent, although female βERKO have a reduced fertility capacity, i.e., fewer and smaller

litter sizes, presumably as a result of reduced ovarian efficiency. They have normal mammary development and lactate normally (Krege *et al.*, 1998).

1.4.   Regulation of oestrogen receptor expression

The concentration of hormone (ligand) and the number of their cognate receptor molecules determine the formation of active hormone-receptor complexes, which are related to the magnitude of the hormone response. Many physiological processes induced by E_2 result from changes in the expression of those oestrogen-responsive genes which are in large part mediated by the ER. As a reflection of the variety of processes controlled by E_2, ERs are found in many tissues. Their expression varies considerably among these tissues as well as within a single tissue. Clearly the expression of such important genes is subject to multiple control mechanisms to ensure that the appropriate amount of receptor is available in the correct cells at the specific time frame, both during early development as well as at later stages in life.

The regulation of ER has been intensively studied, both *in vivo* and *in vitro* and in many different models. The effects of a number of oestrogenic compounds on target tissues can be very different and a single compound can mediate agonist- or antagonist-like responses depending on the tissue. Aside from the difference in the distribution pattern of the two ERs, these complex mechanisms are governed by a number of other factors and occur at several levels (Keightley, 1998). These include; 1) nature of the ER, i.e., whether it is wild type or mutant, 2) differential receptor expression 3) the promoter organisation of the ER gene, 4) interaction of ER with co-activators and co-repressors 5) dimerisation status of the receptor (homo-/hetero-), 6) species/ developmental stage/ sex and tissue-/cell-context, and 7) differential ligand binding specificity.

The changes in ER mRNA expression might arise as a consequence of altered stability of the mRNA (Martin *et al.*, 1995) or changes at the level of transcription (Koritnik, Koshy, & Hoversland, 1995; Shupnik, Gordon, & Chin, 1989). In a number of cases, the primary regulator of expression of the receptor is the ligand itself. Previous studies by Flouriot *et al.* 1996 have shown that E_2 treatment *in vitro* induced rainbow trout (rt) ER mRNA accumulation in a hepatocyte culture system (which contains high level of ER) by inducing a 5-fold increase in the ER gene transcription rate as well as a 3-fold increase in the mRNA half life, indicating both transcriptional and post-transcriptional regulation of ER (Flouriot, Pakdel, & Valotaire, 1996). In another model study using a cancer cell line, Read *et al.* 1989 (Read, Greene, & Katzenellenbogen, 1989) demonstrated that treatment of T47D cells (which contain a low level of ER) with E_2 induced a 2.5-fold increase in ER mRNA levels after 48 h, while progestin exposure resulted in an 80% decrease in ER mRNA and protein. On the other hand, the response of ER in MCF-7 cells (which possess a high level of ER) following E_2 exposure depended upon the prior growth history of the cells. In cells previously exposed to a low oestrogen medium, treatment with E_2 caused little change in either ER mRNA or protein. In contrast, cells that had been maintained in medium with a high level of oestrogen responded robustly, with a 40% and 50% decrease in the mRNA and protein respectively, following E_2 treatment. This indicates that ER can be regulated by its own ligand (auto-regulation) and/or by another ligand (heterologous regulation). The response was evident both at the level of the mRNA and protein and dependent on the cell type context.

1.4.1   Regulation of ER in the brain

The regulation of ER has also been studied extensively *in vivo*, particularly in the CNS. A study by Shughrue *et al.* 1992 showed that the expression of ER mRNA increased at 72 h after ovariectomy in the mPOA, ARC, VMH and the magnitude of responses was quantitatively different among the three regions. This effect was not observed in castrated male rats (Shughrue, Bushnell, & Dorsa, 1992). On the other hand, a recent report on the expression of ER-β mRNA showed that gonadectomy

markedly attenuated the expression of ER-β mRNA in peripheral tissues, with the level of ER-β mRNA in the prostate reduced to non-detectable levels (Shughrue *et al.*, 1998). By using Western blot analysis, Zhou *et al.* 1995 reported the detection of ER-α protein in the mPOA of normal cycling female and male rat brain. Quantitative analysis showed an alteration in ER protein content over the oestrous cycle, with the highest level detected during met-oestrus, attenuated on di-oestrus, and lowest during oestrus and pro-oestrus thus reflecting the response of ER to the available circulating E$_2$ (Zhou, Shughrue, & Dorsa, 1995). A heterologous regulation of ER was also observed *in vivo* where P$_4$ has been shown to decrease the concentration of ER in the hypothalamus of ovariectomised rats (Blaustein & Brown, 1984). Some recent studies have compared the expression of ER-α and ER-β. Osterlund *et al.* 1998 have reported a differential regulation of both receptor transcripts in rat brain. In the hypothalamus treatment with a high dose of E$_2$ in OVX rats (total of 170 μg over 14 days) decreased ER-α mRNA in the ARC, VMH and posterolateral cortical amygdala nuclei, but increased ER-β mRNA in the ARC nucleus. In the medial amygdala nuclei, only ER-β mRNA level was altered by E$_2$ treatment. The effect was largely not seen with a low dose of E$_2$ (17μg) (Osterlund *et al.*, 1998). Similar to *in vitro* experiments, the responses of both ER transcripts *in vivo* are largely dependent on cell-type and exhibit region- and dose-dependent manner.

As previously mentioned, alternative pathways have been proposed to explain how ER action and expression is regulated. One mechanism is perhaps by interaction of ER with other transcription factors including its own splice variants. For instance, Wang *et al.* 1999 has found that human ER splice isoforms, ERΔ5 and ERΔ7, substantially decreased the response of wide-type ER (wtER) activity in a yeast system, perhaps by competition with wtER to bind to the ERE (Wang, Zeng, & Khan, 1999). Another pathway is suggested by a recent study by Alarid *et al.* 1999 which showed an autologous down-regulation of ER protein via a proteosome-mediated pathway that involved nuclear ER but apparently in the absence of protein synthesis and transcription (Alarid, Bakopoulos, & Solodin, 1999). Another study has suggested that long term activation of the protein kinase C signal transduction

pathway could inhibit ER function through activation of other cofactors which interact with ER (Martin *et al.*, 1995).

Thus the expression and action of ER is multi-level and subject to a complex hormonal regulation both by homologous (controlled by its own ligand, oestrogenic compounds) and heterologous (controlled by other hormone signals) mechanisms. Both types of regulation appear largely to follow the tissue- and cell-specific as well as dose-dependent manner in both the direction and the extent of modulation. The regulation of ER can occur at many levels including transcriptional, post-transcriptional, translational and post-translational mechanisms as well as via other mechanisms such as protein-protein interaction. The precise molecular mechanism of how these regulations might come about, however, remain largely undetermined.

1.5.   Hypothalamic paraventricular and supraoptic nuclei

The production and secretion of oxytocin (OXT) and vasopressin (VP) by the hypothalamic neurones in the paraventricular (PVN) and supraoptic (SON) nuclei are modulated by sex steroid hormones. Recent studies have demonstrated the presence of ER-β in these two nuclei, suggesting the potential direct regulation of the neurosecretory system by E_2. A full account of the relevant introductory material on this regulation of gonadal steroids in the PVN and SON will be discussed in the experimental chapter 5.

1.5.1.   The hypothalamo-pituitary connection

Many of the endocrine glands in the body are controlled by the pituitary, or hypophysis. The pituitary is, in turn, under the influence of the hypothalamus, which in effect means that most of the endocrine system is brought under the control of the central nervous system. The pituitary gland comprises two major lobes, one neural tissue and the other glandular. The posterior lobe, or neurohypophysis, develops as

an outgrowth from the hypothalamus. It serves as the storage site for two neuropeptides, OXT and VP, synthesised in the magnocellular neurones in the SON and PVN of the hypothalamus. OXT and VP are transported from the hypothalamus to the posterior pituitary in the axons of the magnocellular neurones that project through the pituitary stalk and terminate on capillaries in the neurohypophysis where the stored peptide is contained within these axon terminals. This pathway forms the hypothalamo-neurohypophysial tract. In response to certain stimuli exciting the magnocellular neurones, the two peptides, separately or together are released into the general circulation (Figure 1.4.).

The anterior lobe, or adenohypophysis, on the other hand, is composed of glandular tissue, and arises as an ectodermal invagination from the stomodeum. A number of trophic factors are produced from cells within the adenohypophysis including, among other molecules, corticotrophin (also known as adrenocorticotrophic hormone, ACTH) which controls the synthesis and release of glucocorticoids from the adrenal glands. The release of anterior pituitary hormones is regulated by the releasing hormones synthesised in neuroendocrine neurones located primarily in the periventricular zone of the hypothalamus, one of which is corticotrophin-releasing factor (CRF) from the PVN which, together with VP in these neurones, regulates the release of ACTH.

As opposed to the hypothalamo-neurohypophysial tract, there is no direct neural connection between the hypothalamus and the anterior pituitary. Instead these hypothalamic factors are released into a primary capillary network in the external layer of the median eminence, and thus enter the hypothalamo-hypophysial portal system whence they influence the release of adenohypophysial hormones (Figure 1.4.).
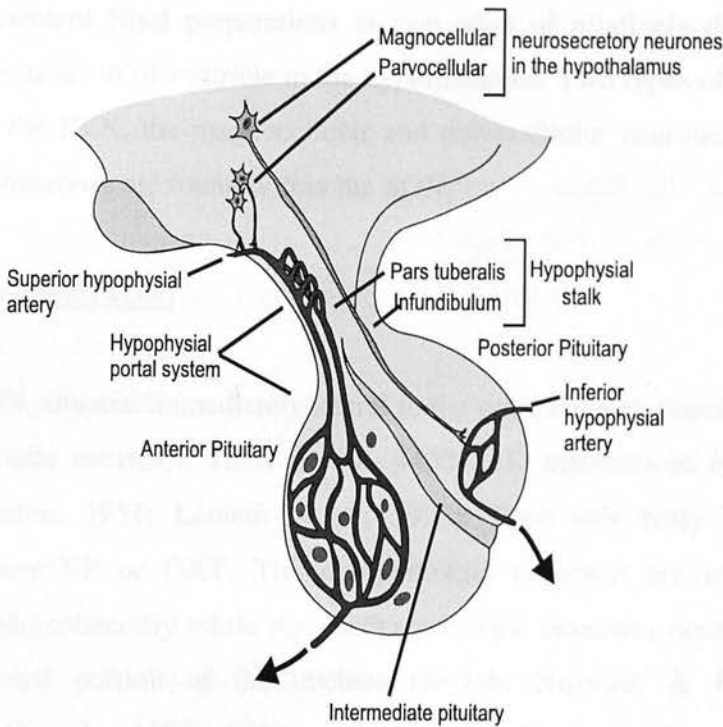
Figure 1.4. Schematic representation of the hypothalamus and pituitary. A schematic of a section through the hypothalamus and the pituitary showing several of the main anatomical components. The pituitary gland is connected to the hypothalamus through the hypophysial stalk. The posterior pituitary is connected to the hypothalamus via the infundibulum of the stalk, and it receives its blood supply from the inferior hypophysial artery. The anterior pituitary is connected to the hypothalamus by the pars tuberalis which contains, amongst other things, the veins of the hypophysial portal system. The portal system is supplied by the superior hypophysial artery in the hypothalamus, and the portal system provides the blood supply to the anterior pituitary gland (Adapted from Brown, 1994).

1.5.2.   The SON and PVN: Morphology and functional anatomy

The supraoptic (SON) and paraventricular (PVN) nuclei can be easily identified in standard Nissl preparations as two pairs of relatively discrete nuclei around the base of the third ventricle in the hypothalamus. Two types of neurones are present within the PVN, the magnocellular and parvocellular neurones, while only magnocellular neurones are found within the SON.

a) Supraoptic nucleus (SON)

The SON, situated immediately lateral to the optic chiasm, contain essentially only magnocellular neurones. There are *ca.* 4400-7000 neurones in each rat SON (Bodian & Maren, 1951; Léránth *et al.*, 1975). Each cell body in the SON synthesises either VP or OXT. The oxytocinergic neurones are predominantly located posterodorsolaterally while the vasopressinergic neurones occupy the more anteroventromedial portion of the nucleus (Swaab, Nijveldt, & Pool, 1975a; Vandesande & Dierickx, 1975). Although the two peptides are produced by separate neurones their relative abundance can change in response to certain stimuli, and under certain physiological conditions involving prolonged stimulation a few cells express both peptides (Kiyama & Emson, 1990). Although these magnocellular neurones may project collaterals to various central sites, the major axon outputs from the SON terminate in the neurohypophysis (Hatton, 1990; Sherlock, Field, & Raisman, 1975) where OXT and VP are secreted from the nerve terminals (Scharrer & Scharrer, 1954) into the peripheral circulation following specific stimulation (see Hatton & Li, 1998; Leng, Brown, & Russell, 1999).

Although these neurones synthesise OXT or VP, they can also produce and store some other neuropeptides, the amount of which can vary with the functional state. OXT-containing neurones have been shown to co-produce numerous other peptides, such as cholecystokinin (CCK), enkephalin as well as CRF, while VP neurones contain immunoreactivity for galanin, dynorphin, and a small amount of

CCK (Meister *et al.*, 1990). Following osmotic stress, in particular, a massive up-regulation of CRF mRNA and peptide expression occurs in the magnocellular OXT neurones (Dohanics, Kovacs, & Makara, 1990; Jessop *et al.*, 1989; Young, 1986).

b) Paraventricular nucleus (PVN)

The PVN is an important integrating site for behavioural, neuroendocrine and autonomic responses to various stimuli (Swanson & Sawchenko, 1983). These physiological responses are mediated by distinct subsets of intricately organised neurones found in this nucleus.

In contrast to the SON, the PVN represents a much more heterogeneous population of neurones in terms of the cytoarchitecture, chemical signatures and functional properties (Armstrong *et al.*, 1980; Swanson & Kuypers, 1980). Anatomically, the PVN can be divided into 8 distinct subdivisions, 3 of which are of magnocellular type while the rest are parvocellular. Each subdivision carries distinct neuroendocrine functions and is regulated differentially (Swanson & Kuypers, 1980). Functionally, neurones within the PVN can be classified into 3 groups: those that project to the posterior pituitary, those that project to the median eminence and those associated with the autonomic nervous system (the visceromotor cell group).

The posterior magnocellular PVN (MPVN) forms the largest group of neurones at the lateral edge of the nucleus. Like those in the SON, almost all of these large cells synthesise either OXT or VP and project their axons to the neural lobe of the pituitary (Fisher *et al.*, 1979; Sawchenko & Swanson, 1983; Swanson & Sawchenko, 1983). The anterior and medial MPVN largely consists of OXT neurones, *ca.* 400-600 in number (Sawchenko & Swanson, 1982).

On the contrary neurosecretory cells of the parvocellular (pPVN) origin send their axons to loci other than the neurohypophysis. The pPVN can be divided into 5 distinct subdivisions; periventricular, anterior, medial, dorsal and lateral parts (Swanson & Kuypers, 1980). The periventricular, medial and dorsal pPVN project to the median eminence (Swanson *et al.*, 1980; Wiegand & Price, 1980). Axons from dorsal, lateral and medial pPVN neurones also project to the dorsal vagal complex (Swanson & Kuypers, 1980), to the lateral medulla (Swanson *et al.*, 1980) as well as to the preganglionic sympathetic neurones in the spinal cord (Swanson & Kuypers, 1980) (Figure 1.5.).

Neurones in the parvocellular subdivisions of the PVN generally participate in functions other than those related to the neurohypophysis, although some of these cells have been identified to contain OXT or VP (Sawchenko & Swanson, 1982). Some of these small cells have been shown to express immunoreactivity and mRNA for CCK, neurotensin, dopamine, enkephalin, substance P, as well as CRF (Swanson & Sawchenko, 1983 for review). Likewise, the abundance of these peptide molecules varies among the separate subdivisions and their relative amount can change in response to various stimulations (Angulo, Ledoux, & McEwen, 1991; Sawchenko, 1987a). See more below.
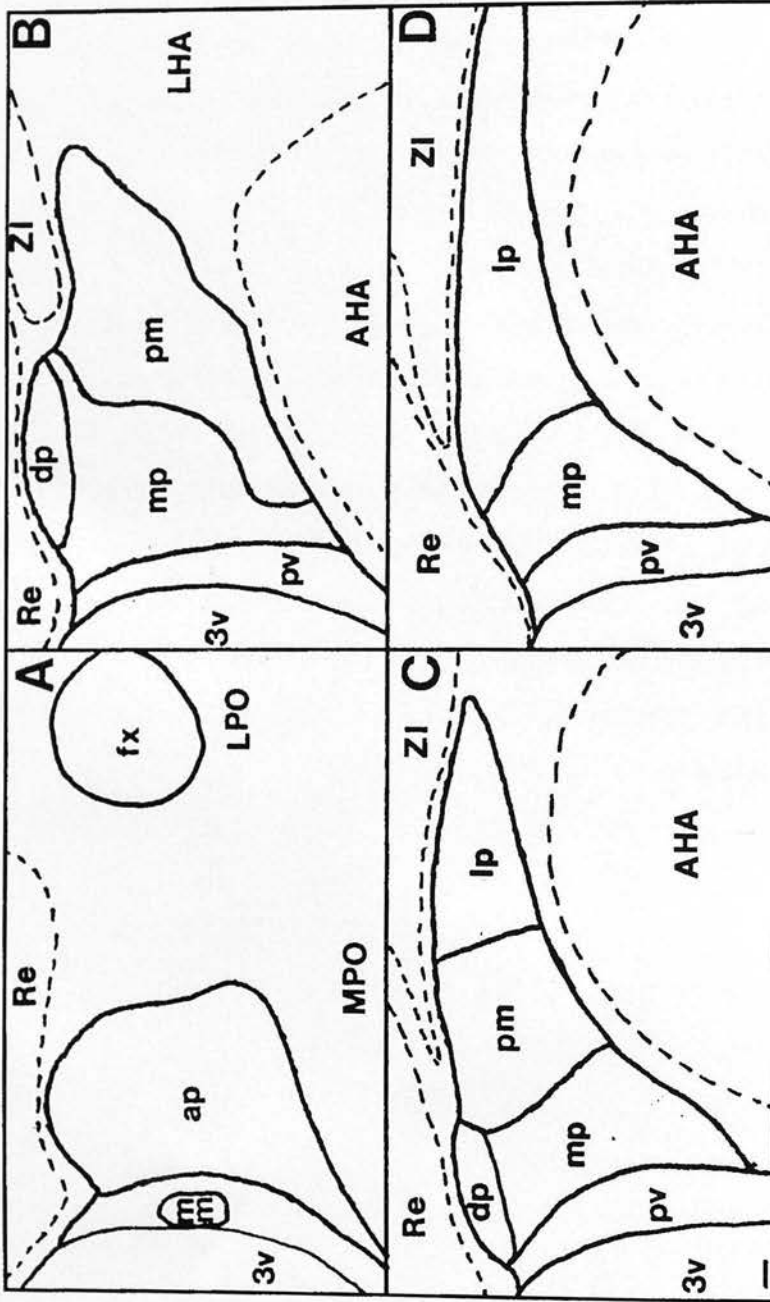
Figure 1.5. Schematic drawings of the cell group boundaries in and adjacent to the hypothalamic paraventricular nucleus of the rat

Legend 1.5. Schematic drawings of the cell group boundaries in and adjacent to the hypothalamic paraventricular nucleus of the rat

In the rat, the two paraventricular nuclei lie immediately lateral to the third ventricle in the midline of the brain. The PVN consists of three magnocellular parts and five parvocellular parts as described in the test. The cellular divisions within the paraventricular nucleus and the immediate surrounding areas are outlined in A-D from original brain sections and range from the rostral (A) to caudal (D).

ap:	anterior parvocellular part, PVN
dp:	dorsal parvocellular part, PVN
lp:	lateral parvocellular part, PVN
mm:	medial magnocellular part, PVN
mp:	medial parvocellular part, PVN
pm:	posterior magnocellular part, PVN
pv:	periventricular part, PVN

3V:	third ventricle
AHA:	anterior hypothalamic area
LHA:	lateral hypothalamic area
MPO:	medial preoptic area
PVN:	paraventricular nucleus
Re:	nucleus reuniens
ZI:	zona incerta
Fx:	fornix

(Reproduced from (Swanson & Kuypers, 1980))

1.6.   Functions of the magnocellular neurosecretory neurones: Oxytocin and
             Vasopressin

Oxytocin (OXT) and vasopressin (VP) are the two principal secretory products of the magnocellular neurosecretory neurones (MCN) in the SON and PVN. The peptides are similar to each other with respect to their chemical structures (Acher, 1980; Du Vigneaud, 1956; Gainer & Wray, 1994). Each comprises 9 amino acids (nonapeptides) with a carboxy-terminal amide and a disulphide loop between residues 1 and 6. OXT differs from VP by having the amino leucine instead of arginine or lysine (depending on species) and isoleucine instead of phenylalanine, otherwise they are identical (Figure 1.6.). The genes encoding the peptides show a remarkable similarity in both organisation and nucleotide sequence (Ivell & Richter, 1984; Schmale, Heinsohn, & Richter, 1983).

Position:	1	2	3	4	5	6	7	8	9

VP	Cys	Tyr	*Phe*	Gln	Asn	Cys	Pro	*Arg*	Gly-NH$_2$
OXT	Cys	Tyr	*Ile*	Gln	Asn	Cys	Pro	*Leu*	Gly-NH$_2$
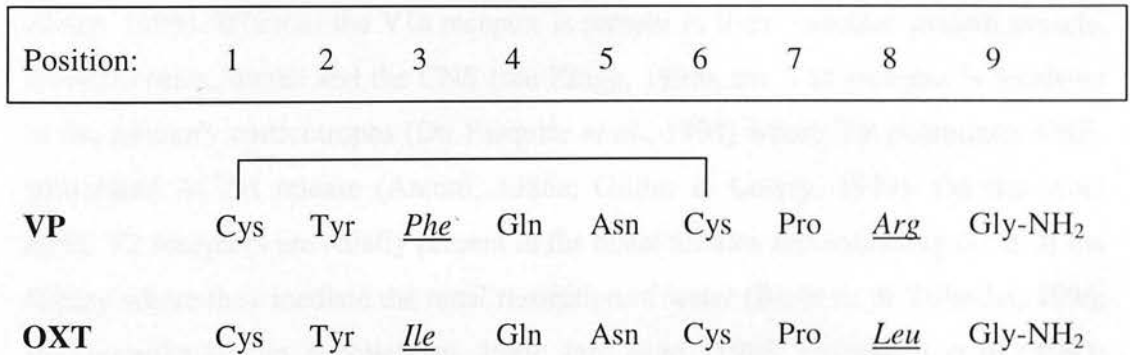
Figure 1.6. Sequences of vasopressin and oxytocin peptides. The two nonapeptides are highly related. They differ from one another by 2 residues only and both possess a disulphide-linked ring structure between residues 1 and 6.

Abbreviations:

Asn	=	Asparagine	Ile	=	Isoleucine
Arg	=	Arginine	Leu	=	Leucine
Cys	=	Cysteine	Phe	=	Phenylalanine
Gln	=	Glutamine	Pro	=	Proline
Gly	=	Glycine	Tyr	=	Tyrosine

The two peptides are initially synthesised as larger prohormone molecules which also contain neurophysin and, in the case of VP, a glycopeptide of unknown function. These complex molecules are incorporated into granules, which are stored in the neurohypophysis following axoplasmic transport from the cell bodies of the neurones of origin. The release of OXT and VP is elicited upon the arrival of action potentials at the nerve endings which depolarise the terminal membranes. As a result of depolarisation there is a prolonged opening of the voltage-dependent calcium channels which results in an influx of calcium and this subsequently triggers the exocytosis of the granule contents into the extracellular space, and diffusion into the bloodstream (Armstrong, 1995; Hatton, 1990).

Cellular effects of VP and OXT are mediated via G-protein coupled receptors. Various studies have, to date, identified the existence of three different VP receptors (V1a, V1b and V2) and one single OXT receptor (OTR), each coupled to a characteristic second messenger system (Barberis, Mouillac, & Durroux, 1998; Zingg, 1996). Whereas the V1a receptor is present in liver, vascular smooth muscle, adrenal cortex, uterus and the CNS (see Zingg, 1996), the V1b receptor is localised in the pituitary corticotrophs (Du Pasquier et al., 1991) where VP potentiates CRF-stimulated ACTH release (Antoni, 1986a; Gillies & Lowry, 1979). On the other hand, V2 receptors are chiefly present in the distal tubules and collecting ducts of the kidney where they mediate the renal resorption of water (Barberis & Tribollet, 1996; Burnatowska-Hledin & Spielman, 1989; Jard et al., 1988; Ostrowski et al., 1993; Tribollet et al., 1988a). On the other hand OTR are expressed within many peripheral tissues including the mammary gland (Soloff, Fernström, & Fernström, 1989), kidney (Schmidt et al., 1990), thymus (Elands, Resink, & De Kloet, 1990), the uterus and ovary (Fuchs et al., 1990) and testis (Bathgate & Sernia, 1994) as well as in the brain (Tribollet et al., 1988b) and anterior pituitary (probably on lactotroph cells) (Antoni, 1986a; Antoni, 1986b; Breton et al., 1995; Chadio & Antoni, 1993).

Although the two peptides are closely related, they have distinct functions. The primary role of VP in the periphery is to control osmotic balance by promoting

renal tubule reabsorption of water (hence VP is otherwise known as antidiuretic hormone) as well as to conserve blood pressure through vasoconstriction in certain vascular beds (Hatton & Li, 1998).

Peripheral OXT, on the other hand, is generally involved in reproduction-related events such as mediating the contraction of uterine smooth muscle at term and sustaining milk-ejection during lactation (Fuchs & Dawood, 1980; Higuchi *et al.*, 1985; Higuchi *et al.*, 1986; Jenkins & Nussey, 1991; Russell & Leng, 1998; Wakerley & Lincoln, 1973). Although it has been recently shown that parturition can continue without OXT, successful lactation, however, is critically dependent upon the presence of OXT. Without it contraction of the mammary myoepithelium can not occur resulting in an unrewarded suckling event even in the presence of a copious amount of milk in the mammary gland. This is evidently seen in the failure of transfer of milk in the oxytocin gene-knockout mice, an effect that is reversed by oxytocin injection (Nishimori *et al.*, 1996; Young *et al.*, 1996).

Although OXT is synthesised in both sexes its principal biological effects are in females. Nonetheless, similar amounts of OXT (and VP) mRNA have been reported in the SON and PVN of male and female rats (Swaab, Pool, & Nijveldt, 1975b) suggesting that OXT might have additional roles beyond those confined to female reproduction. In rats, OXT stimulates *in vitro* the release of ACTH from the pituitary cells (apparently via VP receptor of V1b type) (Schlosser *et al.*, 1994). It also potentiates the ACTH response to CRF, both *in vivo* (Rivier & Vale, 1985b) and *in vitro* (Antoni, Holmes, & Jones, 1983; Gibbs *et al.*, 1984). OXT has been shown to be released from the median eminence into the portal blood system (Antoni, Fink, & Sheward, 1990; Tannahill *et al.*, 1991) and the concentration found here is substantially higher than that in the periphery (Gibbs, 1984), parallel to that of VP (Koenig *et al.*, 1986). This evidence supports the notion that perhaps OXT, in addition to its role in the periphery, can act as a releasing factor influencing ACTH secretion from the anterior pituitary.

Moreover, neurohypophysial OXT also appears to play a complementary role in the neuroendocrine stress-response. In the rat, an increase in plasma OXT concentration has been observed after stress exposures such as immobilisation and shaker stress, while plasma VP remained unchanged (Callahan *et al.*, 1992; Iványi, Wiegant, & de Wied, 1991; Lang *et al.*, 1983b). Interestingly, a study by Jezova *et al.* 1993 has shown that surgical denervation of the PVN by anterolateral knife cuts, leaving the SON-neurohypophysial connections intact, reduced the peripheral OXT increase following 30 min of immobilisation stress (Jezová *et al.*, 1993). This indicates that, at least in relation to immobilisation stress, the PVN is crucial for stress-induced OXT secretion and without the PVN the SON cannot preserve OXT release during stress. More recently Nishioka *et al.* 1998 also found that, in addition to an increase in plasma OXT concentration, central release of OXT in the PVN, measured by microdialysis (MD), is also increased in response to shaker stress. Similarly this central OXT increase also demonstrated site-specificity as such an increase in OXT release was not observed when MD probes were located outside the vicinity of the PVN (Nishioka *et al.*, 1998). This supports the notion that OXT is also a stress responsive hormone, specifically released from the PVN neurones (Callahan *et al.*, 1992; Iványi *et al.*, 1991; Lang *et al.*, 1983b; Onaka & Yagi, 1993).

Additionally, centrally released OXT can also act as a neurotransmitter where it mediates sexual, social and maternal behaviours (Argiolas & Gessa, 1991; de Wied, Diamant, & Fodor, 1993; Insel, 1992; Pedersen & Prange, 1979; Richard, Moos, & Freund-Mercier, 1991; Schumacher *et al.*, 1989) and exerts anxiolytic effects, as well as inhibiting food and salt appetite (Blackburn *et al.*, 1992; Stricker & Verbalis, 1987; Stricker & Verbalis, 1996; Verbalis *et al.*, 1995). Overall, OXT may serve multiple functions, in addition to its hormonal actions in the periphery, it acts in the CNS to modulate behaviour, peptide release as well as autonomic responses.

An additional role of VP is in the potentiation of CRF effects, acting as a co-secretagogue to stimulate the release of ACTH from the pituitary corticotrophs

(Rivier & Vale, 1985a). This is particularly applicable to its role under stressful stimuli. This is discussed more below.

1.7.   The magnocellular neurosecretory system and specific stimuli

Activation of the magnocellular neurosecretory neurones (MCN) occurs in response to various physiological stimuli including induced hyperosmolarity, cellular dehydration, a decrease in blood pressure, depletion of extracellular fluid volume as well as in response to suckling, causing milk let-down, and in parturition. It is the reversal of these conditions, e.g. rehydration, normalisation of blood pressure, which resets the system to the basal condition, suggesting the plasticity of the MCN (Hatton, 1997).

The response of the MCN to various physiological stimuli is a result of interactions of the intrinsic properties of the neurones with synaptic inputs from various afferent pathways, some of which are excitatory while others are inhibitory. These inputs contribute to the regulation and dictate the magnitude of the responses of the MCN. Numerous inputs of both peripheral and central origins, have been identified as projecting to the magnocellular neurosecretory system. Among the well-defined inputs are the ascending noradrenergic projections from the A2 neurones of nucleus tractus solitarii (NTS) which preferentially innervate the OXT neurones (Sawchenko, Arias, & Bittencourt, 1990; Sawchenko et al., 1988) as well as from the A1 cell group of the caudal ventrolateral medulla (VLM) which projects more extensively to VP neurones (Beroukas, Willoughby, & Blessing, 1989; Cunningham & Sawchenko, 1988; Day, Ferguson, & Renaud, 1984; Iwai, Ochiai, & Nakai, 1989). These two nuclei are noradrenergic in nature and generally associated with somatosensory and haemodynamic influences upon the release of neurohypophysial hormones, particularly in the cardiovascular regulation of VP release (Kannan & Koizumi, 1981).

A descending pathway influencing both types of magnocellular neurones arises from forebrain structures around the third ventricle collectively known as the 'osmoreceptor complex' which comprises the organum vasculosum of the lamina terminalis (OVLT), the subfornical organ (SFO), and the MnPO (median preoptic nucleus) (McKinley, Pennington, & Oldfield, 1996). The OVLT and SFO project directly to the SON and PVN and also indirectly via the MnPO (McKinley, Hards, & Oldfield, 1994; Oldfield *et al.*, 1994). This complex, as the name suggests, contributes essentially to the mechanisms regulating osmoresponsiveness of the OXT and VP neurones, as lesions in these brain sites result in a disrupted response of the magnocellular neurones to increased osmolarity (Hamamura *et al.*, 1992; Leng *et al.*, 1989).

1.7.1. Osmotic stimulation

As mentioned earlier, one of the well-established roles of VP is in controlling plasma osmolarity by acting via the V2 receptor to stimulate the reabsorption of water from the tubular fluid in the cortical and medullary collecting ducts in the kidneys. The plasma concentration of VP thus determines water balance in the body. This indirectly regulates the concentration of the osmotically actives solutes in the extracellular fluid, the most important molecule being sodium. The synthesis and secretion of VP is in turn linearly controlled by plasma osmolarity and, to a lesser extent, by blood volume and pressure. This effect is achieved through activation of central pathways with input to the hypothalamus (Chowdrey & Lightman, 1993). In rats, OXT is also natriuretic acting upon the kidney in conjunction with VP (Dicker & Heller, 1946; Verbalis, Mangione, & Stricker, 1991), although this is not its prime function.

Osmotic stimulation associated with salt loading provides a useful model to study the activity of the MCN. Both VP and OXT cells are osmoreceptors themselves, displaying the ability to sense and respond to changes in the osmotic pressure of their extracellular environment (Mason, 1980; Oliet & Bourque, 1993).

Increased osmolarity, induced by chronic salt loading by replacing drinking water with 2% NaCl for 14 d, induced a massive reduction in OXT and VP content in the neural lobe, while this increased both plasma OXT and VP concentrations and expression of their mRNAs in the SON and PVN (Burbach *et al.*, 1984; Van Tol, Voorhuis, & Burbach, 1987). Hyperosmolarity induced by water deprivation for 7 d also resulted in OXT depletion from the neural lobe (Young & Van Dyke, 1968). Conversely, hypoosmolarity, as found in hyponatraemia, suppresses the activity of the MCN and hence the secretion of VP/OXT (Hatton, 1997; Robinson *et al.*, 1990).

Although an increase in osmotic pressure directly induces a depolarisation of the magnocellular neurones, this effect is relatively small and is not sufficient to trigger an increase in spike activity. However, these magnocellular neurones constantly receive synaptic inputs from other neurones, some of which are excitatory while others are inhibitory, resulting in a constantly fluctuating membrane potentials (Leng & Brown, 1997). Only when the depolarisation exceeds this resting membrane potential do spikes occur. Thus, as previously described, the osmoresponsiveness of the magnocellular neurones depends on brain areas which provide synaptic inputs, as lesions in these areas can abolish the responses of magnocellular neurones to changes in plasma osmolarity.

Intriguingly, chronic hyperosmolarity induced by salt loading also modulates the expression and secretion of CRF in the PVN and SON. In particular the expression of CRF is predominantly reduced in the parvocellular system while the opposite effect is observed in the OXT-containing magnocellular neurones with a corresponding increase in neural lobe peptide content (Dohanics *et al.*, 1990; Imaki *et al.*, 1991; Imaki, Vale, & Sawchenko, 1992; Jessop *et al.*, 1989; Young, 1986). Both of these effects might contribute to the natriuresis and antidiuresis that are clearly needed upon salt loading; the diminished output of the HPA axis could serve to dampen a posited weak glucocorticoid inhibition of VP secretion (Raff, 1987), while increase in CRF content in the neural lobe could exert a paracrine effect upon stimulation of VP release (Alzein *et al.*, 1984).

Thus the response of CRF in salt-loading provides an opportunity to examine co-ordinate and differential effects on two closely-linked neuroendocrine cell types simultaneously.

1.7.2. Oxytocin system in pregnancy and parturition

Oxytocin has a prominent role in the progression of parturition, being released in large amount during delivery in all placental mammals studies thus far (Russell & Leng, 1998). Parturition is a time of high demand for OXT such that almost 30% of the neurohypophysial OXT store would be depleted during the 60-90 min of parturition (see Leng *et al.*, 1999). Thus an accumulation of OXT prior to term is important in the progression of parturition. Stores of OXT in the neurohypophysis increase substantially during pregnancy, particularly shortly before term. This increase in the pituitary store of OXT is a result of both a restraining of OXT secretion from the neural lobe as well as a stimulation of OXT synthesis in the magnocellular neurones. During mid-pregnancy a reduction in OXT release is due to a restraint effect of an opioid peptide, dynorphin which acts via κ-receptors (Rusin *et al.*, 1997) by blocking voltage-gated calcium influx into the neurosecretory terminals (Rusin *et al.*, 1997) thus inhibiting the release of OXT from the pituitary. In late pregnancy this reduction in OXT secretion is mediated by an inhibitory effect of histamine on the electrical activity of the OXT neurones (Yang & Hatton, 1994) provided by cells in the dorsomedial tuberomammillary nucleus (Weiss, Yang, & Hatton, 1989). Apart from its role in inhibiting the release of OXT secretion, histamine also induces an increase in OXT synthesis during pregnancy as central administration of histamine has been shown to increase the expression of OXT mRNA in the SON and stimulate the expression of Fos in magnocellular neurones indicating that OXT gene is activated (Kjaer *et al.*, 1994a; Kjaer *et al.*, 1994b).

On the other hand, the increase in OXT production might be related to changes in the steroid environment especially with a rise in circulating E_2 during pregnancy as well as a precipitous decline in P_4 level immediately before term.

The importance of steroid hormones upon the magnocellular system in pregnancy and parturition was part of our current study and is discussed in detail in Chapter 5.

There are multiple hormonal changes associated with pregnancy, in particular those associated with osmotic balance and plasma volume homeostasis. In rats, from about day 12 of pregnancy until term, relaxin is secreted in a large amount from the ovary to prepare the birth canal for parturition (Sherwood et al., 1980). In addition relaxin also acts upon the circumventricular organs such as the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT); neurones in these loci project their axons directly to the OXT and VP cells in the hypothalamus. Systemic administration of relaxin has been shown to excite both OXT and VP cells as well as increase OXT and VP secretion (Way & Leng, 1992). Thus an increase in VP cell activity under the influence of relaxin results in antidiuresis and plasma volume expansion. This process allows an essential adaptation to cope with an increase in vascular needs of the developing placenta and foetus during pregnancy. However, the consequent increase in blood volume then exerts a negative feedback and inhibits the VP cells. Overall there is no gross chronic activation of VP cells in pregnancy, even in the presence of large relaxin content. The OXT cell activity, on the other hand, is maintained throughout late pregnancy, presumably to sustain OXT synthesis required for the ensuing parturition (Leng & Brown, 1997).

When parturition begins the OXT cells are activated and demonstrate synchronised bursts of very high frequency action potentials, intervened by a brief period of silence (Summerlee, 1981), similar to those occurring during the milk-ejection reflex (Wakerley & Lincoln, 1973). Such a burst induces a release of ca. 1 mU of OXT as a bolus into the general circulation, resulting in the progression of parturition. This pattern of OXT cell electrical activity is only seen during parturition and suckling. Following other stimuli, including increased osmolarity, OXT is released continuously as a result of a maintained increase in the background non-burst activity of OXT neurones. Thus continuous OXT release provides an effective means for the natriuretic role of OXT while pulsatile OXT release is efficient only

for functions in suckling and parturition. These dual modes of release enable OXT cells to perform two physiological functions independently yet simultaneously.

1.8.   Functions of the parvocellular PVN: autonomic responses and regulation of the HPA axis

The PVN comprises both magnocellular and parvocellular neurones, each with a characteristic topographical distribution. These neurones express different peptides, and have different functions, and respond differently to a range of inputs. The magnocellular PVN contains OXT and VP neurones as in the SON. The parvocellular PVN comprises two principal groups of neurones: those projecting to the median eminence and concerned with neuroendocrine stress mechanisms, and those projecting to the brainstem and spinal cord influencing autonomic responses.

1.8.1.   Role of oxytocin neurones in cardiovascular regulation

The notion that the PVN is one of the co-ordinating centres controlling autonomic responses stems from retrograde labelling studies in which injection of fluorescent dye, true blue, into the spinal cord or the dorsal vagal complex labels cells primarily in the parvocellular subdivision of the PVN, in particular those concentrated in the lateral and medial zone (Sawchenko & Swanson, 1982; Swanson & Kuypers, 1980). The chemical signature here is chiefly OXT localised mainly in the ventral parvocellular PVN neurones (Swanson, 1987). Lesions of the PVN resulted in a large decrease of OXT content in the spinal cord (Hawthorn, Ang, & Jenkins, 1985; Lang *et al.*, 1983a). These neurones project directly to sympathetic preganglionic neurones (SPN) in the dorsal vagal complex and spinal cord (Conrad & Pfaff, 1976; Saper *et al.*, 1976), and are involved in autonomic regulation by the PVN, particularly in cardiovascular responses such as defence against plasma volume expansion (Coote *et al.*, 1998; Gilbey *et al.*, 1982). Previous studies have indicated a critical role of the PVN in cardiovascular homeostasis. Lesion of the PVN attenuates the development of hypertension in Dahl salt-sensitive rats (Goto *et*

al., 1981) as well as in spontaneously hypertensive rats (Ciriello *et al.*, 1984) and reduces the renal vascular response to volume load (Gilbey *et al.*, 1982; Lovick, Malpas, & Mahony, 1993). Previous studies have shown that the OXT paraventriculospinal pathways also mediate the tachycardic response to stress (Callahan *et al.*, 1989; Morris *et al.*, 1995). Collectively these findings suggest a role of parvocellular OXT PVN neurones in mediating the cardiovascular responses.

1.8.2.   PVN and the regulation of the HPA axis

The PVN has a pivotal role in mediating stress reactivity via the regulation of the hypothalamo-pituitary-adrenal (HPA) stress axis. This HPA axis is primarily responsible for the control of secretion of glucocorticoid (GC) hormones which, upon release into the systemic circulation, mediate a wide range of adaptive responses in many organ systems (Antoni, 1986a). The major GC in human and non-human primates is cortisol and corticosterone in rodents, with the majority being produced by the adrenal cortex (Mason, 1950; Nelson *et al.*, 1951; Reich, Nelson, & Zaffaroni, 1950). These are 21-carbon steroid compounds synthesised from cholesterol. The hydroxy group at carbon-11 is required for their therapeutic efficacy (Kendall, 1941; Olson, Thayer, & Kopp, 1944).

GC actions are devised for preserving homeostasis under stressful conditions. GC initiates arrays of physiological actions essential for immediate survival while suppressing non-vital processes. Nonetheless these effects are largely catabolic serving to consume resources. They are particularly deleterious and can lead to severe damage, especially when the secretion of GC is prolonged and exaggerated. GC excess has been shown to be associated with many neurological conditions, in particular depression and memory disorders (Davis *et al.*, 1986; Lupien *et al.*, 1999). These effects are likely to occur at the level of the hippocampus, however, and will be discussed in details in the following section (see below).

The HPA responses to stress are initiated primarily by neurones located in the medial parvocellular division of the PVN which synthesise chiefly, among other neuropeptides, CRF and VP (Antoni, 1986a; Whitnall, 1993). Upon release following stress the two peptides act in concert to stimulate the secretion of ACTH from the anterior pituitary corticotrophs which in turn initiates the production of glucocorticoids from the adrenal cortex (Antoni, 1986a).

The CRF-containing neurones in the PVN receive numerous afferent projections from various sources (Sawchenko & Swanson, 1985). The catecholaminergic neurones of the brainstem located principally in the A2 and C1, C2, and C3 cell groups in the caudal medulla have been shown to innervate the parvocellular PVN (Cunningham, Bohn, & Sawchenko, 1990; Cunningham & Sawchenko, 1988). The CRF neurones also share, in common with the magnocellular neurones, afferents arising from the lamina terminalis (Miselis, Shapiro, & Hand, 1979; Saper & Levisohn, 1983; Sawchenko & Swanson, 1983) as well as from the NTS and the ventrolateral medulla (VLM), although in the latter case at least, the particular aspects of the NTS and the VLM that project to those two groups of neurones are partially distinct (Cunningham et al., 1990; Cunningham & Sawchenko, 1988). These pathways provide the mediators of the potent reflex control of the stress-related neuroendocrine neurone output by visceral sensory stimuli. Other afferent inputs to the PVN comprises the several components of the limbic area including the amygdala, the septal nuclei and the hippocampus which are involved in conveying the emotional and cognitive stimuli upon the CRF-rich zone of the PVN. This is, however, mediated indirectly via the bed nucleus of the stria terminalis (BNST), the key integrative centre for the limbic region, which receives substantial direct inputs from the amygdala, septum and hippocampus (Swanson, Kohler, & Bjorklund, 1987) and reciprocally provides a direct projection to the PVN (Sawchenko & Swanson, 1983).

The sensitivity of CRF expression towards stress exposure appears to be dependent upon the type of stress used in the study. Previous studies have

demonstrated that chronic stress increases CRF mRNA expression (Kiss & Aguilera, 1993; Makino, Smith, & Gold, 1995; Mamalaki *et al.*, 1992) in the parvocellular PVN. However, other studies have failed to find any up-regulation of CRF mRNA (Harbuz *et al.*, 1992). In addition to CRF, neurones in the medial parvocellular portion of the PVN are also capable of synthesising VP which is released into the median eminence via the portal blood to regulate the secretion of ACTH from the anterior pituitary (Antoni, 1986a; Gillies & Lowry, 1979; Yates *et al.*, 1971) via V1b receptors (Antoni, 1984; Baertschi & Friedli, 1985; Du Pasquier *et al.*, 1991). The expression of VP is low under basal conditions, but is manifest following either adrenal steroid withdrawal (Kiss, Mezey, & Skirboll, 1984; Young, Mezey, & Siegel, 1986a) or chronic stress (Herman *et al.*, 1989a; Lightman & Young, 1988). Following ADX in rats *ca.* 70%-90% of the parvocellular CRF immunoreactive neurones exhibit immunoreactivity for VP (Kiss *et al.*, 1984; Sawchenko, Swanson, & Vale, 1984) and contain the mRNA for VP prohormone (Wolfson *et al.*, 1985). At the level of the median eminence 95% of CRF-containing axons and nerve terminals contain VP following ADX (Whitnall, Smyth, & Gainer, 1987). Indeed there is evidence suggesting that during repeated or chronic stress there is a shift in the modulation of pituitary adrenal activity from CRF to VP (Hashimoto *et al.*, 1988; Scaccianoce *et al.*, 1991). In a recent study, exposure to repeated immobilisation for 14 d in rats resulted in an increase in VP mRNA content which was greater than that seen for CRF mRNA (Makino *et al.*, 1995).

Similar to CRF, there appears to be a stress-specificity in the response of VP to various stimuli. The responses appear to vary from no activation or a small increase (Darlington, Barraclough, & Gann, 1992; Harbuz *et al.*, 1994; Lightman & Young, 1988) to a more substantial increase (Bartanusz *et al.*, 1994; Herman, 1995), indicating that this may reflect differences in the stress paradigm used.

A distinction between the regulation of VP gene expression in neurones in the parvocellular PVN and that of the magnocellular PVN is their differential sensitivity to glucocorticoids. VP and VP mRNA expression in the parvocellular subdivision is

under the negative control of glucocorticoids (Davis *et al.*, 1986b; Sawchenko, 1987b; Sawchenko *et al.*, 1984; Wolfson *et al.*, 1985). On the other hand, VP mRNA in the magnocellular neurones is generally insensitive to changes in circulating glucocorticoids (Young, Mezey, & Siegel, 1986b) even though corticosteroid treatment inhibits, while its withdrawal increases basal and stimulates VP secretion from the neural lobe (Healy *et al.*, 1985; Papanek & Raff, 1994).

2.1. Hippocampus

Recent epidemiological studies have demonstrated an association between increased risk in cognitive impairment and the loss of ovarian hormones after menopause. Moreover, in non-demented women E_2 has been shown to enhance cognitive performance, while E_2 replacement therapy in postmenopausal women is associated with a reduced incidence of neurodegenerative disorder, especially Alzheimer's disease. Collectively, the evidence suggests a neuroprotective role of E_2.

Learning and memory are essentially governed by structures within the limbic system, one of which is the hippocampus. Its role in maintaining cognitive functions, particularly those aspects related to spatial memory (Eichenbaum, Otto, & Cohen, 1992), as well as its involvement in a number of neurological disorders, such as senile dementia of Alzheimer's type, has led to extensive studies on the regulation of this structure. The synaptic organisation and functional characteristic of its neurones are also among one of the most extensively studied areas in neurobiology (Skrede & Westgaard, 1971; Yamamoto & McIlwain, 1966).

2.1.1. Anatomical organisation of the hippocampus

The hippocampus proper can be divided into four regions, designated CA1–CA4 which derived from the Latin 'cornu Ammonis' or Ammon's horn. The more general term describing the hippocampal formation includes the dentate gyrus, the subiculum, and the entorhinal cortex, in addition to the CA regions. Cells in CA1 and

CA3 are large pyramidal neurones which occupy most of the hippocampal proper. The CA2 area, a morphological transitional zone, is perhaps a trivial distinction and, in some species, is indistinguishable from CA1 and CA3 that it is often ignored. In rats the approximate number of CA1 pyramidal neurones is around 250,000 while that in the CA3 is 160,000 and the dentate gyrus contains *ca.* 1,000,000 granule cells (Squire, Shimamura, & Amaral, 1989). Both the hippocampus and the dentate gyrus are three-layered cortices. In the hippocampus, these comprise the polymorphic layer (*stratum oriens*), the pyramidal layer (*stratum pyramidale*) and the molecular layer (*stratum radiatum* and *stratum lacunosum-moleculare*). The dentate gyrus consists of a polymorphic layer (*hilus*), a granular layer (*stratus granulosum*), and a molecular layer (*stratum moleculare*). The molecular layer of the dentate is a continuation of that of the hippocampal molecular layer (see Brown & Zador, 1990).

The principal organisation of the hippocampal neuronal connectivity is generally known as the *tri-synaptic* circuit. Fibers of the perforant path from the entorhinal cortex synapse onto granule cells of the dentate gyrus. Granule cells in the dentate gyrus send their mossy-fibers and synapse onto CA3 pyramidal neurones. The pyramidal cells in the CA3 region then project their axons to and innervate the CA1 pyramidal cells via the Schaffer collaterals. These three synapses display the unique unidirectional progression of excitatory pathways and define the tri-synaptic circuit. However, the current understanding of such connectivity is considerably more complex than this simple closed loop (see Brown & Zador, 1990).

2.1.2. The involvement of the hippocampus in HPA axis negative feedback: correlation with cognitive function

In the preceding section we have mentioned the role of glucocorticoids in mediating the stress response to pervasive stimuli. As mentioned previously, prolonged exposure to glucocorticoids is particularly detrimental, thus organisms are equipped with a system to limit the extent of glucocorticoid secretion. This is achieved via a co-ordinated negative feedback loop mediated by glucocorticoids

themselves. The effects of glucocorticoids are mediated by two types of corticosteroid receptors; 1) type I, also known as the mineralocorticoid receptor (MR) (Arriza *et al.*, 1987; Patel *et al.*, 1989) and 2) type II, or glucocorticoid receptor (GR) (Hollenberg *et al.*, 1985; Miesfeld *et al.*, 1984). While aldosterone, corticosterone and synthetic mineralocorticoids bind to MR with very high affinity (Kd ~ 0.5 nM), corticosterone bind to GR with a 10-fold lower affinity (Kd ~ 2-5 nM). Thus MR are largely occupied under basal nadir corticosterone levels while GR become increasingly occupied at the peak of the diurnal rhythm and during stress (Reul, van den Bosch, & de Kloet, 1987; Spencer *et al.*, 1990).

This negative feedback mechanism occurs at several levels but is principally mediated by the brain, with the major sites of such feedback mechanism being the hypothalamus and the pituitary. Additionally higher central loci such as the hippocampus (see more below), the amygdala and the bed nucleus of the stria terminalis (Casada & Dafny, 1991; Cullinan *et al.*, 1995; Gray *et al.*, 1993), the septum (Dobrakovová *et al.*, 1982; Seggie, Uhlir, & Brown, 1974) as well as the medial prefrontal cortex (Diorio, Viau, & Meaney, 1993) also play the prominent roles in regulating this negative feedback. Other sources include the brainstem, predominantly although not exclusively, the noradrenergic, adrenergic (Plotsky, Cunningham, & Widmaier, 1989) as well as serotonergic projection pathways (Feldman, Conforti, & Melamed, 1987; Saphier & Feldman, 1989; Sawchenko *et al.*, 1983). Local hypothalamic integrative systems involving the medial preoptic area as well as other hypothalamic nuclei known to provide afferent inputs to the parvocellular PVN also contribute to the regulation of the HPA axis activity (Herman, Prewitt, & Cullinan, 1996). The end point of the negative feedback is to facilitate the termination of stress response by inhibiting the secretion of CRF (and VP) from the hypothalamic neurones and ACTH from pituitary corticotrophs, ultimately preventing excess glucocoticoids being released into the peripheral circulation.

Considerable evidence shows the importance of hippocampus as one of the crucial sites for the HPA axis feedback regulation (Dallman *et al.*, 1987; Keller-Wood & Dallman, 1984; McEwen, De Kloet, & Rostene, 1986; Plotsky, 1987). Lesions in the hippocampus are associated with increased glucocorticoid levels under basal and stress conditions (Feldman & Conforti, 1976; Sapolsky, Krey, & McEwen, 1984; Wilson *et al.*, 1980), reduced suppression of ACTH following glucocorticoid administration (Feldman & Conforti, 1976) and elevated hypothalamic CRF and VP mRNA expression (Herman *et al.*, 1989b). Application of corticosteroid to the hippocampus suppresses the stress response, whereas receptor antagonists elevate basal glucocorticoid levels and block negative feedback. Indeed the hippocampus contains the largest accumulation of both GR and MR mRNA as well as protein in the brain (Herman, 1993; Jacobson & Sapolsky, 1991; Reul & de Kloet, 1985). However, the apparent lack of significant direct projections of the hippocampus to the hypophysiotrophic neurones of the PVN (Cullinan, Herman, & Watson, 1993; Sawchenko & Swanson, 1983) suggests that any interactions with neurones responsible for ACTH release are likely to be indirect, requiring at least one intermediary synapse.

The hippocampus plays a pivotal role in learning and memory processes. It is also particularly vulnerable to disruptive events such as seizures (Sloviter, 1983), head trauma (Lowenstein *et al.*, 1992), and ischemia (Hsu & Buzsáki, 1993) as well as degenerative changes accompanying ageing and neurodegenerative disorder such as Alzheimer's disease and depression (de Leon *et al.*, 1988; Pasquier *et al.*, 1994). In fact, both conditions are associated with impaired memory and hypercortisolism (Davis *et al.*, 1986a; Dinan, 1994) indicating a link between elevated levels of glucocorticoids and hippocampal damage.

There is an 'inverted U-shaped' relationship between glucocorticoids and the hippocampal spatial memory (Diamond *et al.*, 1992; Kerr, Huggett, & Abraham, 1994). Thus low glucocorticoid levels or MR agonists facilitate memory processes by increasing long-term potentiation (LTP) (an electrophysiological correlate of

memory) while reducing after-hyperpolarisation (AHP) (which negatively correlates with spatial learning). On the other hand high level of glucocorticoids or GR agonists reduce LTP and increase AHP leading to hippocampal damage and memory impairment (Diamond, Fleshner, & Rose, 1994; Joëls & de Kloet, 1992).

Extensive studies have shown that chronically elevated glucocorticoid levels, such as those occur following stress, are detrimental to hippocampal neurones, increasing their vulnerability to a variety of insults (Landfield, Waymire, & Lynch, 1978; Sapolsky, 1985; Sapolsky, Krey, & McEwen, 1985). On the other hand Landfield *et al.* 1981 have shown that adrenalectomy with low dose of corticosterone replacement from middle-age prevented hippocampal neurodegeneration and cognitive impairment at later stage in life. Thus hippocampal atrophy and impaired cognitive function can be prevented by keeping glucocorticoid levels low (Landfield *et al.*, 1978).

The cellular mechanism underlying the effect of glucocorticoids might involve the plasticity of the hippocampal neurones. High levels of glucocorticoids produced by daily injection of corticosterone induce atrophy in the apical dendritic tree of CA3 pyramidal subfield leading to neurone loss (Woolley, Gould, & McEwen, 1990). Moreover, this atrophy is prevented by phenytoin, given prior to corticosterone treatment, thus implicating the involvement of the excitatory amino acids (EAA) in causing these dendritic atrophy (Watanabe *et al.*, 1992a). Similarly, application of restraint stress to the rats also produces the same pattern of CA3 dendritic atrophy (Watanabe, Gould, & McEwen, 1992b) and the effect is also blocked by phenytoin administration, confirming the involvement of EAA (Watanabe *et al.*, 1992a).

Interestingly, glucocorticoid depletion also adversely attenuates the hippocampus, although this predominantly affects granule neurones in the dentate gyrus (Gould, Woolley, & McEwen, 1991; Sloviter *et al.*, 1989) rather than the CA pyramidal neurones of Ammon's horn (McEwen *et al.*, 1992).

Conversely, the gonadal steroid E_2 has been shown to maintain the dendritic tree of neurones in the hippocampus. Thus removal of endogenous ovarian hormones leads to a decrease in the number and density of dendritic spines and axospinous synapses, specifically, in the apical dendrites of the CA1 pyramidal neurones (Gould *et al.*, 1990). This decrease can be prevented or reversed by E_2 treatment (Woolley & McEwen, 1992; Woolley *et al.*, 1997). Thus the hippocampus is subject to regulation of both the adrenal and gonadal steroid hormones.

3.1.   Aim of the thesis

Oestrogens have effects on neuronal phenotype and function. Apart from the classically described effects of oestrogens on brain resulting in changes in reproductive behaviour and physiology, they also affect regions that are important in memory and cognition as well as in homeostasis. The majority of oestrogen effects are mediated by the ER-α and -β, both of which are expressed in the brain. In order to understand how oestrogens could affect the brain, it is thus crucial to understand what governs the tissue-specific expression of those receptors and how they are regulated, particularly under varying physiological situations. In particular, the recent discovery of ER-β, in addition to ER-α, suggests additional pathways by which E_2 could exert its actions in the CNS.

Using *in situ* hybridisation histochemistry (ISH), we first determined the cellular distribution of the two types of ER mRNA in the laboratory rat brain, thus revealing the possible loci where E_2 could mediate its, probably, genomic actions in the brain.

We further investigated in detail the regulation of the ER mRNAs in two brain areas; the hippocampus and the PVN and SON, using quantitative ISH. A decline in cognitive functions is associated with the loss of ovarian steroids in postmenopausal women. Moreover, several epidemiological studies suggest that

oestrogen replacement therapy in postmenopausal women could potentially protect against Alzheimer's disease (AD) (for review see Henderson, 1997).

Since the hippocampus is involved in learning and memory and this area is also severely affected by AD, the known beneficial effects of E_2 on cognition might be effected in this brain structure. The underlying mechanism of E_2 action in the hippocampus, however, is not fully understood but might involve direct genomic action of E_2 via ER expressed here. Using a variety of hormonal (both sex- and adrenal-steroid) manipulations we examined the regulation of both types of ER mRNA expression within the hippocampus.

The hypothalamic PVN and SON have prominent roles in regulating fluid homeostasis and blood pressure via VP, and in the events of parturition and lactation by OXT, both being synthesised in magnocellular neurones in these nuclei. This neurosecretory system is also known to be modulated by sex steroid hormones. The discovery of ER-β and its high level of expression in these two nuclei suggests a possible direct genomic influence of E_2 upon the OXT/VP neurones. The expression of ER-β mRNA here was examined under the influence of changes in sex- and adrenal-steroid environments. In addition the regulation of the ER-β transcript was also determined under physiological manipulations associated with stimulation of neuronal activity.

This thesis is about investigating the regulation of the ER mRNAs within the CNS. Changes in the expression of such receptor transcripts, if translated into functional protein, will alter the sensitivity of the neurones to E_2, thus ultimately contributing to the impact E_2 has on the CNS.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Chemicals

Acetic anhydride	Sigma-Aldrich Company Ltd., UK
Acetone	BDH (Merck Ltd.), UK
Agarose	Gibco BRL (Life Technologies Ltd.)
Amberlite resin	BDH (Merck Ltd.), UK
Ammonium Acetate	Sigma-Aldrich Company Ltd., UK
Ammonium persulphate	Sigma-Aldrich Company Ltd., UK
Ampicillin	Sigma-Aldrich Company Ltd., UK
Boric acid	Sigma-Aldrich Company Ltd., UK
Bovine serum albumin	Sigma-Aldrich Company Ltd., UK
Calcium chloride ($CaCl_2$)	Sigma-Aldrich Company Ltd., UK
Cesium chloride (CsCl)	Gibco BRL (Life Technologies Ltd.)
Chloroform/isoamyl (24:1)	Sigma-Aldrich Company Ltd., UK
Citric acid	Sigma-Aldrich Company Ltd., UK
Corticosterone, 21-acetate	Sigma-Aldrich Company Ltd., UK
D-19 developer	Ilford Ltd., UK

Denhardt's 50x	Sigma-Aldrich Company Ltd., UK
Dextran sulphate	Sigma-Aldrich Company Ltd., UK
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich Company Ltd., UK
Dipotassium hydrogen orthophosphate Anhydrous (K_2HPO_4)	BDH (Merck Ltd.), UK
Disodium hydrogen orthophosphate Anhydrous (Na_2HPO_4)	BDH (Merck Ltd.), UK
DNA (testis salmon sperm)	Sigma-Aldrich Company Ltd., UK
DTT	Sigma-Aldrich Company Ltd., UK
EDTA (disodium dihydrate)	Sigma-Aldrich Company Ltd., UK
Enzymes and reaction buffers (all types)	Promega UK Ltd., UK
Ethanol	Sigma-Aldrich Company Ltd., UK
Ethidium bromide (aqueous solution)	Sigma-Aldrich Company Ltd., UK
Formamide	Sigma-Aldrich Company Ltd., UK
Gelatine	Sigma-Aldrich Company Ltd., UK
Glacial acetic acid	BDH (Merck Ltd.), UK
Halothane BP	Mackenzie & Co. Ltd., UK
Hybaid Recovery^{TM}	Hybaid Ltd., UK
Hydrochloric acid (HCl)	BDH (Merck Ltd.), UK
Isopropanol	BDH (Merck Ltd.), UK
Nucleotides	Life Sciences International, UK
Oestradiol, 17-β	Sigma-Aldrich Company Ltd., UK
Paraformaldehyde	Fisher Scientific, UK
Phenol/chloroform	Sigma-Aldrich Company Ltd., UK

Pico-fluor   Oxford pipettor

Poly-L-lysine   Sigma-Aldrich Company Ltd., UK

Potassium acetate   Sigma-Aldrich Company Ltd., UK

Potassium chloride (KCl)   BDH (Merck Ltd.), UK

Potassium dihydrogen

orthophosphate (KH_2PO_4)   BDH (Merck Ltd.), UK

Potassium hydrogen carbonate ($KHCO_3$)   BDH (Merck Ltd.), UK

Pyronin Y   Sigma-Aldrich Company Ltd., UK

RNase A (bovine pancreas)   Roche Diagnostics Ltd

Sodium acetate   Sigma-Aldrich Company Ltd., UK

Sodium azide   Sigma-Aldrich Company Ltd., UK

Sodium bicarbonate (Na_2CO_3)   BDH (Merck Ltd.), UK

Sodium chloride (NaCl)   BDH (Merck Ltd.), UK

Sodium citrate   BDH (Merck Ltd.), UK

Sodium dihydrogen orthophosphate

monohydrate ($NaH_2PO_4 \bullet H_2O$)   BDH (Merck Ltd.), UK

Sodium dodecyl sulphate (SDS)   Sigma-Aldrich Company Ltd., UK

Sodium hydroxide (pellets)   Sigma-Aldrich Company Ltd., UK

SPA reagent   Amersham, Life Science

Toluidine-Blue-O   Sigma-Aldrich Company Ltd., UK

Transcription buffer   Promega UK Ltd., UK

Triethanolamine   Sigma-Aldrich Company Ltd., UK

Tris base   BDH (Merck Ltd.), UK

Tryptone peptone   Difco Ltd., UK

Xylene	BDH (Merck Ltd.), UK
Yeast extract	Difco Ltd., UK
Yeast tRNA	Gibco BRL (Life Technologies Ltd.)

2.1.2. Radiochemicals

| ^{35}S-UTP | Amersham Pharmacia Biotech UK |
| ^{3}H-Corticosterone | Amersham Pharmacia Biotech UK |

2.1.3. Miscellaneous

Amfix fixative	H.A. West Ltd., UK
Coverslips	BDH (Merck Ltd.), UK
Cryostat	Bright Instruments Ltd., UK
DPX mountant	BDH (Merck Ltd.), UK
Hyperfilm β-max	Amersham Pharmacia Biotech UK
Lens tissue	Whatman Labsales Ltd., UK
Microscope slides (frosted)	BDH (Merck Ltd.), UK
Microtome blades	BDH (Merck Ltd.), UK
NICK column, Sephadex G50	Amersham Pharmacia Biotech UK
Photographic nuclear emulsion, NTB2	Anachem Ltd., UK
Spectrophotometre (UV-160A)	Shimadzu UK
Whatman paper 3 M	Whatman International Ltd., UK

2.2.   Methods

2.2.1. *In situ* hybridisation histochemistry

In situ hybridisation histochemistry (ISH) involves a reaction whereby a nucleic acid probe that has been labelled with a radioisotope, or otherwise detectable molecules, hybridises to target nucleic acid residing in a tissue section by means of hydrogen bonding of complementary base pairs. This technique allows the precise anatomical localisation and identification of individual cells that contain a specific nucleic acid sequence. Under appropriate conditions the specificity of the technique can be high, such that little or no detectable non-specific binding occurs.

ISH is particularly useful for the study of gene expression in the nervous system. Heterogeneous tissues such as the brain contain a myriad of different cell types, each of which produce a variety of proteins subserving different functions, and so they contain many mRNA species. An estimated 145,000 mRNA species can be found in mouse brain (Hahn, Van Ness, & Chaudhari, 1982) and the amount of each specific species varies vastly from neurone to neurone. Some of the rare mRNA molecules are specific to one cell type and contribute to only a small fraction of the cellular mRNA content. Detection of such a rare mRNA *in situ* is achievable due to the specificity of complementary base pairing between a nucleic acid probe and its mRNA target. The ISH technique generally involves 5 main steps including tissue preparation, probe labelling, hybridisation of probe to tissue, posthybridisation stringent wash and visualisation. The ultimate goal of ISH is to retain the target nucleic acids *in situ*, ensuring that they are not degraded by nucleases, to make probes that can effectively penetrate tissue and hybridise specifically and to preserve tissue morphology to enable subsequent identification of the mRNA of interest and its cellular localisation.

There are many alternatives available regarding types of probe, methods of labelling the probes and visualisation of the signal for ISH. Three main classes of probe are in current use:

1)   *Complementary DNA (cDNA) probes*; These probes are double-stranded, relatively easy to use and label, have high specific activity and thus give good amplification of the signal. However, because of the duplex nature of these probes they are not readily free to hybridise and so have to be denatured prior to use. The other disadvantage of using this type of probes is the reannealling of the two strands of the probes in a hybridisation solution, resulting in the reduction of the concentration of probe available for hybridisation (Cox *et al.*, 1984).

2)   *Oligonucleotide probes*; These probes are short (typically 15-50 bp in length) single-stranded DNA oligonucleotides which are quick and simple to use. The short length of the probes ensures a better access to target tissue sections. However, this also means that fewer labelled nucleotides are incorporated into each molecule of probe leading to a lesser sensitivity compared to that of nucleic acid probes. The relative ease of synthesising without involving the cloning procedures offers another attractiveness for these oligonucleotide probes.

3)   *Single-stranded RNA probes (riboprobes)*; These are single-stranded RNA molecules generated by *in vitro* transcription from a cloned cDNA that is introduced into a specifically designed plasmid transcription system (vector) which contains RNA polymerase promoters. This enables the transcription of either antisense (non-identical but complementary which will hybridise to the cellular mRNA thus used for detection of the mRNA of interest) or sense (identical and will not hybridise to cellular mRNA, therefore employed as a control) probes (Melton *et al.*, 1984). We chose to use riboprobes in our ISH studies because of the high stability of RNA-RNA hybrids compared to DNA-RNA hybrids. The longer length of RNA probes

relative to oligonucleotide probes allows the use of more stringent hybridisation conditions, leading to the highest sensitivity when detection of a single-stranded target like cellular mRNA is required. However, riboprobes have a tendency to hybridise to non-specific sites in the tissue sections therefore pre- and post-hybridisation treatments including the use of RNase are employed to reduce the background. The specificity of the hybridisation signal of an antisense probe is routinely determined by the use of a sense probe, which is conducted under an identical condition as that employed for an antisense probe. The lack of the hybridisation signal from the sense probe verifies that probe binding is a result of its base sequence and not its physical properties.

The stability of the hybrids formed, whether they are DNA-DNA, RNA-RNA, or DNA-RNA duplexes, is influenced by many factors, such as temperature, ionic strength, percentage of guanosine/cytosine (G/C) base pair and probe length (Wilkinson, 1992).

2.2.2. *In situ* hybridisation protocol

a) Slide preparation

During handling of microscope slides gloves were worn at all times to prevent RNase contamination. Prior to use, slides were washed in 0.2 M HCl, DEPC-treated water (0.1% DEPC in ultrapure water) and acetone for 3 min each. Slides were subsequently treated with subbing solution containing 0.03% Sodium azide and 15% gelatine and left to dry at 50°C. On the following day, slides were subbed in poly-L-lysine solution (100 mg in 500 ml DEPC-treated water) for 20 sec, washed in DEPC-water twice for 10 min and left to dry at 50°C. Slides were wrapped in aluminium foil and stored until use.

b) Tissue collection

At the end of the experiments the rats were killed by decapitation, their brains were removed, snap-frozen with powered dry ice and stored at −80°C. Coronal brain sections (10 μM thick) at various levels were cut using a cryostat (Bright) at −20°C. The sections were thaw-mounted onto gelatin and poly-L-lysine coated slides and stored at −80°C until processed for ISH. 3-4 brain sections were collected onto each slide. To identify the area of interest marker slides were stained with either 1% w/v Pyronin Y (Sigma) or 1% w/v Toluidine-Blue-O (Sigma). During tissue processing for ISH, gloves were worn at all times, including while handling the cryostat-cut sections on microscope slides to prevent contamination with RNase, a common concern for all types of ISH.

c) Tissue preparation and fixation

To achieve the best compromise between preserving tissue morphology, retaining the nucleic acid, and allowing easy penetration of probe to the target RNA, sections were fixed in cold freshly prepared 4% (w/v) paraformaldehyde in 0.1 M phosphate buffered saline (PBS, 20 mM NaH_2PO_4, 80 mM Na_2HPO_4, pH 7.4). Paraformaldehyde is an alkylating agent which cross-links nucleic acid and protein to form a network within the cells. As such, it provides good RNA retention as well as maintaining tissue morphology. Fixation was carried out at 0-4°C to inhibit endogenous ribonucleases. The fixation was immediately followed by 2 washes in 1 x PBS (diluted from 10 x stock solution (80 g NaCl, 29 g $Na_2HPO_4 \bullet 2H_20$, 2 g KH_2HPO_4, and 2 g KCl in 1 l DEPC-treated water) for 5 min each. To reduce the background activity the sections were then subjected to acetylation treatment which prevents the non-specific binding that normally occurs from binding of the probe to positively charged amino groups in the tissue. Slides were incubated for 10 min with 0.75 ml acetic anhydride (added prior to transfer of slides) in 300 ml of 0.1 M

Triethanolamine pH 8.0 (13.3 ml triethanolamine in 1 L DEPC-treated water), followed by another 5 min wash with 1 x PBS. The sections were then dehydrated through a series of alcohols (70%, 80%, and 95% ethanol made up with DEPC-treated water, 2 min each). The slides were then air-dried.

d) Prehybridisation treatment

To reduce the non-specific background binding which might occur during the subsequent hybridisation all tissue sections were incubated with the prehybridisation buffer (2 x stock containing 0.6 M NaCl, 10 mM Tris-HCl pH 7.5, 1mM EDTA pH 7.5, 1 x Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA and 0.1 mg/ml yeast tRNA). The purpose was to saturate sites in the tissue sections that might otherwise bind nucleic acids nonspecifically. The buffer was initially made up as a 2 x stock but was diluted 1:1 with deionised formamide (deionised using 10% amberlite resin) to provide sufficient to apply 200 µl of the hybridisation mix to each slide. The slides were placed flat in the humidified hybridisation boxes on 3 M Whatman paper soaked in box buffer (50% deionised formamide, 20% 20 x SSC and 30% DEPC-treated water) and incubated at 50°C for 2 h.

e) Hybridisation

Similarly, sufficient hybridisation buffer was made up to provide 200 µl per slide. The hybridisation mix is composed of 50% deionised formamide, 10 x 10^6 cpm/ml of the probe made up to the final volume of 200 µl with 2 x hybridisation buffer (10% dextran sulphate, 0.6 M NaCl, 10 mM Tris-HCl pH 7.5, 1mM EDTA pH 7.5, 1 x Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA and 0.1 mg/ml yeast tRNA). The mix was denatured at 70°C for 10 min, briefly cooled on ice and 1 M DTT (10 µl/ml of probe) added. The tissue sections were drained and

dried around the edges prior to the hybridisation mix being applied. 200 μl of the hybridisation mix was applied per slide. The slides were then placed in humidified hybridisation boxes, sealed and hybridised for 16 h (or overnight) at 50°C, after which the slides were subjected to posthybridisation washes.

f) Posthybridisation stringent wash

Following the hybridisation the slides were drained and washed 3 times in 2 x SSC (diluted from 20 x stock (3 M NaCl and 0.3 M Na$_3$Citrate pH 7.0) for 5 min each. They were then subjected to posthybridisation washing which was designed to reduce the background through a series of wash steps plus the use of nucleases that specifically degrade single-stranded nucleic acid, leaving only double-stranded hybrid intact. In particular, RNA probes tend to exhibit a degree of non-specific binding, which are most effectively reduced by the use of RNase (John, Birnstiel, & Jones, 1969). 200 μl of RNase buffer containing 30 mg/ml RNase A (RNase buffer composed of 10 mM Tris-HCl pH 7.5, 1mM EDTA pH 7.5 and 0.5 M NaCl in ultrapure water) was applied to each slide. The incubation lasted for 60 min at 37°C. Afterwards the slides were progressively washed with more stringency to remove non-specific adherence to tissue components, beginning with a 30 min wash in 2 x SSC at room temperature. Subsequently slides were washed in 0.1 x SSC at 60°C for 90 min followed by another wash in 0.1 x SSC starting at 60°C and allowed to cool to room temperature. The slides were then dehydrated in 50%, 70% and 90% ethanol in 0.3 M ammonium acetate for 2 min each. They were allowed to air dry then apposed against Hyperfilm-β max for 2 weeks.

g) Visualisation of hybridisation signal

Following film development, slides were dipped in photographic nuclear emulsion (NTB-2) diluted 1:1 with ultrapure H_2O, allowed to dry then stored in lightproof boxes at 4°C for 4-5 weeks before being developed. Slides were developed with D-19 diluted 1:1 with ultrapure H_2O for 5 min, briefly rinsed with H_2O then fixed in a 1:5 dilution of Amfix fixative for 5 min. Slides were then washed in H_2O for a further 5 min. All development steps were carried out at 15°C under safe light conditions. Following development, sections were counterstained with 1% w/v pyronin Y and coverslipped using DPX as mountant.

h) Quantification of autoradiographs

Anatomical identification of brain structures was based on Paxinos and Watson stereotaxic rat brain atlases (Paxinos & Watson, 1986; Paxinos & Watson, 1996). Autoradiographs were evaluated by measuring optical density of film images of regions of interest via a digital camera, or by counting silver grains over the profiles of individual neurones (x 40 objective), using a computer-aided image analyser (MCID-M4 (Version 3.0 Rev. 1.5), Imaging Research, Inc., Canada). For film measurements, optical density (units were given arbitrarily) was measured for 6-8 images of region of interest per animal. Silver grain counts were made over 8 neurones per section, and in 3-4 sections per region per rat. Background measurements were made over appropriate adjacent tissue with no evident expressing neurones and subtracted to obtain the net value of silver grain counts. The slides were coded so that the experimenter was unaware of the treatment of the rats at the time of evaluation. Animal means were calculated for each variable, and these values were used to calculate group means.

i) Statistical Analysis

Student's *t*-test or one-way Analysis of Variance (ANOVA or Kruskal-Wallis analysis on ranks), and post-hoc tests (Student-Newman-Keuls or Duncan's) were used (SigmaStat software) as appropriate to determine the statistical significance between groups. The alpha value was set at $P < 0.05$. Results are expressed as the group means±sem.

2.2.3. Restriction Endonuclease Digestion and Electrophoresis of DNA

DNA was normally digested using 5 unit (U) of restriction enzyme per 1 µg of DNA in 1 x restriction buffer (from a 10 x stock solution) and distilled H_2O (dH₂O) to the required volume. Digestion of plasmid DNA was routinely carried out at 37°C for 1-2 h. Digested DNA was visualised after electrophoresis through agarose gels. Typically a 1% w/v agarose gel was used to separate DNA fragments between 400 bp - 7 kb in size and was prepared by dissolving agarose in 1 x TBE (diluted from 10 x stock solution containing 108 g Tris base, 55 g Boric acid and 0.5 M EDTA pH 8.0 in DEPC-treated water) in a microwave oven. The melted agarose was allowed to cool down before adding 1-2.5 µl of ethidium bromide (10 mg/ml) and pouring the solution into a gel mould with a comb in place (the tooth size of the comb was dependent on the size of the DNA sample), and allowed to set. 2 µl of agarose loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 30% glycerol, Gibco BRL) was added to DNA samples before loading onto the gel. Gels were usually run with 1 x TBE at 50-80 mA with a 1kb DNA ladder (Gibco BRL) containing DNA fragments of 75 bp - 12 kb as a size marker, for as long as required to see sufficient separation of DNA fragments.

2.2.4.   Preparation of oestrogen receptor riboprobes

2.2.4.1. Oestrogen receptor-α (ER-α)

a) Subcloning of 367 bp PstI-EcoRI fragment of mouse oestrogen receptor-α cDNA into vector pGEM3

A plasmid encoding the full-length mouse ER-α cDNA, pMOR100 (White *et al.*, 1987), was generously provided by Dr K E Chapman, Molecular Endocrinology, MMC, Western General Hospital, Edinburgh, UK. In order to generate the riboprobe for ER-α, we first subcloned our required fragment into vector pGEM3 (Promega UK Ltd., UK) which carries RNA polymerase promoters, SP6 and T7, thus enabling the production of both 'sense' and 'antisense' riboprobes. The 367 bp PstI-EcoRI fragment from the pMOR100 cDNA was subcloned by ligation between the PstI site and EcoRI sites of pGEM3. This 367 bp fragment encodes part of the ligand-binding domain of the mouse oestrogen receptor-α. We chose this fragment of the cDNA because 1) it is highly conserved between rat and mouse (it shares 92% sequence identity with the rat ER-α cDNA) 2) it shows very limited similarity to other steroid receptors so that cross hybridisation between steroid receptors would not occur. The purified plasmid DNA was obtained and designated as pJS1.

Digests were set up as follows:-

1)   3 μg of pMOR100 was restricted with 10U of PstI and 10U of EcoRI in a total volume of 30 μl of 1 x restriction buffer (Buffer H (Promega) which contains 90 mM Tris-HCl, 10 mM MgCl₂, and 50 mM NaCl) to obtain the 367 bp fragment of interest.

2)   3 μg of vector pGEM3 was restricted with 10U of PstI and 10U of EcoRI in a total volume of 30 μl of 1 x restriction buffer (Buffer H) to give compatible ends to the obtained 367 bp fragment.

Digests were carried out at 37°C for 1 h and an aliquot of 1 μl of each digest in loading buffer was used to verify digestion on a 1% low melting point agarose gel with 0.5 x TBE and visualised under UV light at 365 nM wavelength in the presence of ethidium bromide. PstI-EcoRI digestion of pMOR100 yielded 4 fragments:- 1) 1400 bp PstI-EcoRI, 2) 170 bp PstI-PstI, 3) 367 bp PstI-EcoRI, 4) 3000 bp EcoRI-EcoRI while PstI-EcoRI digestion of pGEM3 yielded 2 fragments, a large fragment of 2832 bp with EcoRI/PstI ends and a 35 bp fragment.

The 367 bp fragment from pMOR100 was excised from the gel using a sterile scalpel as was the larger fragment from pGEM3, then DNA was extracted from the gel using a Hybaid DNA purification kit II (Hybaid Recovery[TM]), according to the manufacturer's instructions. DNA fragments were purified as follows. Before use 400 μl of resuspended silica gel matrix in binding buffer was added to a spin filter and mixed with the gel slice, then heated at 55°C for 5 min. After mixing, the tube was spun for 15-30 sec and the eluent discarded. The filter was washed with 500 μl of wash solution and DNA eluted using 12 μl of elution solution. Recovery was estimated by electrophoresis of 1 μl of recovered fragment. Equimolar amounts of vector and insert DNA were visually assessed and used for ligation.

The 367 bp restriction fragment from pMOR100 was ligated into pGEM3 in a 10 μl ligation reaction containing 1 μl of 10 x ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 1 mM DTT and 1 mM ATP pH 7.0), 3U of T4 DNA ligase and 0.5 μl of linearised pGEM3 and 7.5 μl of pMOR100 fragment. The ligation was carried out at 12°C overnight.

Escherichia coli strain HB101 cells were made competent based on the CaCl$_2$ method (described in Molecular Cloning, a laboratory manual, T. Maniatis 1982, p 250-251). 200 µl of competent cells were then mixed with 4 µl of ligation mix and stored on ice for 30 min. Cells were then heat shocked at 42°C for 1 min, then returned to ice for a further 2 min. The whole transformation mixture was plated onto an LB agar plate containing 100 µg/ml ampicillin and incubated at 37°C overnight. The positive control contained 1 µl of uncut pGEM3 (10 ng/µl), and the negative control contained only HB101 alone.

b) Plasmid DNA Minipreparation (Miniprep)

Single transformed bacterial colonies were transferred into tubes containing 2 ml LB broth (10 g Tryptone peptone, 5 g Yeast extract and 5 g NaCl in 1 l H$_2$O) and 100 µg/ml of ampicillin and incubated with shaking at 37°C overnight. Cells were pelleted, resuspended in 100 µl of GTE (0.05 M Glucose, 0.025 M Tris-HCl pH 7.5 and 0.01 M EDTA) then gently mixed with 200 µl of fresh 0.2 M NaOH/1% SDS and stored on ice for 15 min. Afterwards, 150 µl of 5 M K.Ac (147.2 g K.Ac and 57.5 ml Glacial acetic acid in 500 ml DEPC-treated water) pH 4.8 was added, mixed by vortexing before storing on ice for a further 15 min. Denatured chromosomal DNA and cellular proteins were removed by centrifugation at 13000 rpm for 5 min. The supernatant was tranferred into another tube then mixed with an equal volume of phenol/chloroform to remove residual protein. The mixture was vortexed then spun for 2 min. The top layer, containing the plasmid DNA was transferred to another tube and the DNA precipitated with 2 volumes of ethanol at room temperature for 5 min. After centrifugation for 5 min, the supernatant was removed and the pellet air dried then resuspended in 50 µl TE (10mM Tris-HCl pH 8.0 and 1mM EDTA pH 8.0) containing 1 µl of DNase-free RNase A and stored at -20°C.

To verify whether plasmid preparations contained the correct insert 10 μl of miniprep DNA was restricted with 5U of PstI and 5U of EcoRI in a total volume of 15 μl of 1x restriction buffer H. The digests were carried out as in section 2.2.3.1. Out of 16 miniprep DNAs 15 contained the correct DNA insert (> 93%). One of these was designated as pJS1.

c) Large Scale Plasmid DNA Preparation (Maxiprep)

A single bacterial colony was transferred into a tube containing 2 ml LB plus 100 μg/ml ampicillin and incubated overnight at 37°C. The overnight culture was diluted into 500 ml LB containing 100 μg/ml ampicillin, and grown at 37°C overnight. Cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C in a JA14 rotor in a Beckman J2-MC centrifuge. The obtained pellet was resuspended in 12 ml of GTE solution and mixed with 24 ml of freshly prepared alkaline/SDS (1 ml 0.2 M NaOH and 5 ml 1% SDS) then stored on ice for 5 min. 16 ml of cold 5 M K.Ac was added, mixed by flick and left on ice for a further 10 min before centrifuging at 6000 rpm for 10 min at 4°C in a JA14 rotor. The supernatant was strained through several layers of gauze into a clean 250 ml centrifuge pot, and precipitated with 32 ml of isopropanol at room temperature for 30 min. The plasmid DNA was recovered by centrifugation at 10000 rpm for 3 min at 4°C. The supernatant was decanted and the pellet was left to air dry at room temperature before being resuspended in 2.2 ml TE. Afterwards, 2.8 g of CsCl as well as 100 μl of ethidium bromide was added to the suspension. The resulting DNA/CsCl solution was then transferred into a 3 ml Beckman Quick Seal ultracentrifuge tube topped up with CsCl solution (100 g CsCl plus 100 ml TE). The tube was centrifuged at 70000 rpm for 17h at 20°C. Plasmid DNA bands were collected through the tube wall using a syringe and a 21 gauge needle. Ethidium bromide was removed from the plasmid DNA by repeated extractions with equal volumes of isopropanol until the pink colour disappeared. The DNA was dialysed overnight against 3 changes of TE.

The concentration of the plasmid was determined spectrophotometrically by measuring absorbance at 260 nm wavelength (1 OD_{260} = 50 µg/ml DNA).

2.2.4.2. Oestrogen receptor-β (ER-β)

A cDNA clone (EcoRI-AccI fragment) encoding approximately 400 bp of the 5' untranslated region (UTR) of rat oestrogen receptor-β (ER-β) subcloned into pBluescript KS (Stratagene) was kindly provided by Dr George G.J.M. Kuiper, Karolinska Institute, Sweden (Kuiper *et al.*, 1996). The clone was transformed into *E. coli*, and plasmid DNA prepared as described in section 2.2.4.1. The plasmid was designated as pBETA24.

2.2.4.3. Plasmid linearisation

Plasmids were linearised with appropriate restriction enzymes (as described in Table 2.1) to generate the antisense (non-identical but complementary which will hybridise to the cellular mRNA) or sense (identical and will not hybridise to the cellular mRNA) templates. The linearisation was carried out at 37°C for 1 h and confirmed by electrophoresis.

	Antisense	Sense
pJS1 (ER-α)	HindIII + T7	EcoRI + SP6
pBETA24 (ER-β)	EcoRI + T3	AccI + T7

Table 2.1. Diagram showing the restriction enzymes used to linearise plasmids and specific RNA polymerases used to generate antisense and sense riboprobes from pJS1 and pBETA24 accordingly.

2.2.4.4. Phenol/chloroform Extraction of the Plasmid

Protein was removed from templates using an equal volume of phenol/chloroform, the mix vortexed then spun at 13000 rpm for 3-5 min and the upper aqueous layer, containing the DNA, was carefully removed and collected into a fresh RNAse-free eppendorf. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the eppendorf, vortexed and spun as above. The top layer was

removed into another fresh RNAse-free eppendorf and ethanol-precipitated with 0.1 volumes of 3 M Na acetate (or 5 M NaCl) and 2.5 volumes of absolute ethanol. The mix was gently vortexed, placed on dry ice for no longer than 10 min then centrifuged for 15 min and the supernatant removed. The pellet was air dried at room temperature and resuspended in 20 μl of DEPC-treated H_2O. 1 μl aliquot of the DNA solution was electrophoresed on a 1% agarose gel to check for any contamination. The remaining template was aliquoted into fresh eppendorfs at a concentration of 1 mg/ml and stored at −20°C.

2.2.4.5. *In vitro* transcription of ^{35}S-UTP-labelled cRNA probes

We chose to use riboprobes for our ISH studies because of the higher stability of RNA-RNA hybrids compared to DNA-RNA hybrids. Since these probes are single-stranded and as such are not prone to the problem of reannealing, this leads to a greater amount of probe available for hybridisation. The longer length of RNA probes relative to oligonucleotide probes also allow the use of more stringent hybridisation conditions, leading to the highest sensitivity when it comes to detection of a single-stranded target like cellular mRNA.

1 μg of each of the linearised templates was incubated with 1.5 μl of mixed cold nucleotides (10 mM of ATP, CTP & GTP), 0.5 μl of 200 mM freshly-made DTT, 0.5 μl of RNase inhibitor, 4 μl of ^{35}S-UTP (specific activity 40 mCi/ 1 ml), 1 μl of the specific RNA polymerases (as described in Table 2.1.) in the presence of 5 x transcription buffer in a volume of ~10 μl. The reaction was carried out in an RNase-free eppendorf. The incubation was carried out at 37°C for T7 and T3, and 40°C for SP6 polymerases for approximately 1 h.

After the incubation 1 µl of RNase-free DNase was added to the reaction mixture and incubated for a further 15 min at 37°C. The reaction was stopped by placing the tube on ice. The newly synthesised probe was then purified through a Nick column, Sephadex G50. The column was first washed with 3 ml of TE, pH 8.0 in order to equilibrate the gel bed. The reaction mix was applied to the column followed by 400 µl of TE. The eluant, which contained little radioactivity, was discarded. A further 400 µl of TE buffer was applied to the column and the eluant collected, as this contained the labelled cRNA probe. Probes were stored up to 4 weeks at −20°C.

Incorporation of ^{35}S-UTP was measured in 1 µl of the probe in 1 ml of Pico-fluor in a β-counter. Probes generally gave between 2 to 5 x 10^5 cpm/ml. To assess quality of the probe 1 µl of the probe was added to 4 µl of loading buffer and electrophoresed on a 6 M Urea:5% Polyacrylamide gel at 10 mA until the first dye front reached the gel end. The gel was then apposed against the Hyperfilm-β max overnight and developed the following morning. An intact probe should yield only one discrete band on the film. A smearing of the band suggests a possibility of a degraded probe. These probes were discarded.

2.2.5. Preparation of corticotrophin-releasing factor riboprobe

A cDNA clone (BamHI-PvuII fragment; base 1525-2043) encoding approximately 518 bp spanning the intron/exon II boundary of rat corticotrophin-releasing factor (CRF) was subcloned into pBluscript KS (the cDNA clone was provided by Dr M C Holmes, Molecular Endocrinology, MMC, Western General Hospital, Edinburgh, UK) (Thompson, Seasholtz, & Herbert, 1987). The plasmid was linearised with either XbaI or HindIII at 37°C for 1 h to generate the antisense or sense templates, respectively.

Antisense riboprobe was generated by *in vitro* transcription in the presence of ^{35}S-UTP with T_3 RNA polymerase at 37°C for 1 h. (described in detail in section 2.2.4.5.)

2.3. *In vivo* studies

2.3.1. Animal maintenance

Male and female Hooded-Lister rats (obtained from Harlan-Olac UK limited) were employed in all of our studies except in the pregnancy experiment where Sprague-Dawley rats (Bantin and Kingman, Hull, UK) were used instead. The rats were caged in groups of 3 unless otherwise stated. They were maintained under controlled light:dark cycles with light on at 0700 h and off at 1900 h, ambient temperature at 18-20°C with rat chow and water available *ad libitum*. They were allowed a period of at least 2 weeks to acclimatise to their new surroundings prior to experimentation.

2.3.2. Surgery and experiments

2.3.2.1. Sex steroid manipulation studies (Gonadectomy and Sex steroid replacement)

Groups of male and female Hooded-Lister rats (180-220 g; n=5-6/group) were either gonadectomised (GDX) or sham-operated under 5% halothane anaesthesia. Male rats were bilaterally gonadectomised (castrated, CX) by a ventral incision through the scrotum followed by tying off the vas deferens. The testes were

then removed by cutting below the sutures. Female rats were ovariectomised (OVX) by bilateral dorsal incisions and removal of both ovaries.

While still anaesthetised, some gonadectomised rats were subcutaneously implanted with silastic capsules containing 17-β oestradiol (E$_2$). Sham-operated controls received vehicle-containing capsules. Silastic capsules were modified and prepared after Bridges (Bridges, 1984) by first sealing one end of the silastic tubing (Altesil High Strength Tubing, internal diameter 2 mm, external diameter 3 mm, and 1.5 cm in length) with silicone sealant, the capsules were autoclaved and subsequently filled with steroid. E$_2$ was dissolved in 100% ethanol and mixed (1:10) in oil vehicle (89.7% arachis oil, 10% benzylalcohol, and 0.3% cresol) to form a 15 mg/ml suspension which was used to fill the 1.5 cm capsule. All capsules were sealed with silicone sealant at the open end and, when the capsules were set, washed in 100% ethanol for 2 h and incubated in 0.1 M phosphate buffer saline (pH 7.4) at room temperature for 24 h prior to implantation.

2.3.2.2. Adrenal steroid manipulation studies

a) Adrenalectomy and Adrenal steroid replacement

Groups of male Hooded-Lister rats (180-220 g; n=5-6/group) were anaesthetised with 5% halothane and bilaterally adrenalectomised (ADX) through dorsal incisions and were given 0.9% NaCl to drink after the surgery. Control animals included rats that were sham-operated by making bilateral dorsal subcutaneous incisions under halothane anaesthesia. Sham-operated controls were injected with vehicle (corn oil) as were the ADX rats. Another group of rats was ADX and injected subcutaneously with 10 mg/kg of corticosterone (CORT) at 4 p.m. daily for 72 h. Concentration of CORT was 20 mg/ml dissolved in vehicle. Concentration of CORT replacement at around 4-10 mg/kg/day has been shown to

restore the physiological level of CORT. This was based on two sources of information; (1) studies of metabolic clearance in intact rats which suggests CORT production rates of 2.5 mg/rat/d (e.g. Waddell & Atkinson, 1994); (2) studies with steroid implants in ADX rats where the weight of implant at the end of the experiment is used to estimate release rate (Akana *et al.*, 1985; Meyer *et al.*, 1979).

b) Stress

The chronic stress model involved exposing rats to a cycle of various stress paradigms. A group of male Hooded-Lister rats (180-220 g; n=5-6) was subjected each day to forced water swim for 15 min at 4°C, 5% halothane exposure for 1 min in an air-tight box and immobilised in a restraint tube (a Perspex cylinder) for 60 min at different times for 3 days. The sequence of these stress paradigms was changed each day to prevent the adaptation of rats to the treatment as follows;

Day 1:   Immobilisation in restraint tube for 60 min (1000-1100 h)

             Forced water swim at 4°C for 15 min (1200-1215 h)

             5% Halothane exposure for 1 min (1600 h)

Day 2   5% Halothane exposure for 1 min (1000 h)

             Forced water swim at 4°C for 15 min (1200-1215 h)

             Immobilisation in restraint tube for 60 min (1600-1700 h)

Day 3   Forced water swim at 4°C for 15 min (1000-1015 h)

             Immobilisation in restraint tube for 60 min (1200-1300 h)

             5% Halothane exposure for 1 min (1600 h)

2.3.3. Bioassay of sex steroid replacement

Bioassay was used to verify the effectiveness of E_2 replacement in OVX rats by determining the weight of the uteri between the treatment and the vehicle groups. The uteri were removed post mortem. Data are expressed as uterus/body weight ratio and shown in Figure 2.1.

Uterus weight relative to body weight compared between ovariectomised females treated with E$_2$ and those treated with vehicle over 2 weeks
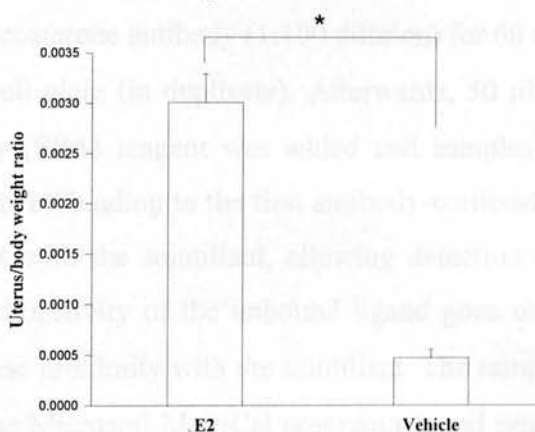
Figure 2.1. The uterus/body weight ratio from the ovariectomised rats treated with E_2 was significantly higher than those from rats treated with vehicle. The increase in uterine weight following chronic oestrogen treatment is expected to be a result of hypertrophy and hyperplasia of the myometrium and endometrium, and the stimulation of fluid secretion in the uterine horns. Asterisk indicates statistical significance between groups ($P < 0.0001$, Student's t-test).

2.4.   *In vitro* Assay

2.4.1. Corticosterone Radioimmunoassay

Total plasma corticosterone concentrations were measured using an in-house radioimmunoassay. Blood plasma was diluted 1:10 in borate buffer (163 mM boric acid, 81 mM sodium acetate, and 0.5% BSA, pH 7.4) and heated at 75°C for 30 min to dissociate steroid from protein in the samples. An aliquot of 20 µl of each of the corticosterone standards (0.625-320 mmol/l, Sigma) or the diluted sample were incubated with a mix of ^{3}H-corticosterone (total counts of around 10000 cpm) and rabbit anti-corticosterone antibody (1:100 dilution) for 60 min at room temperature in a flexible 96 well plate (in duplicate). Afterwards, 50 µl of anti-rabbit scintillation proximity assay (SPA) reagent was added and samples incubated overnight. The SPA reagent acts by binding to the first antibody-corticosterone complex, bringing it in close contact with the scintillant, allowing detection of the emission signal. In contrast, the radioactivity of the unbound ligand goes undetected because it is not brought into close proximity with the scintillant. The samples were quantified in a β-counter using the Microsoft MultiCal programme, and sample contents (expressed as nmol/l) estimated from the standard curve (Figure 2.2.). The sensitivity of the assay was 1.25 nmol/l and the intra- and inter-assay coefficients of variation were below 7% and 10%, respectively.
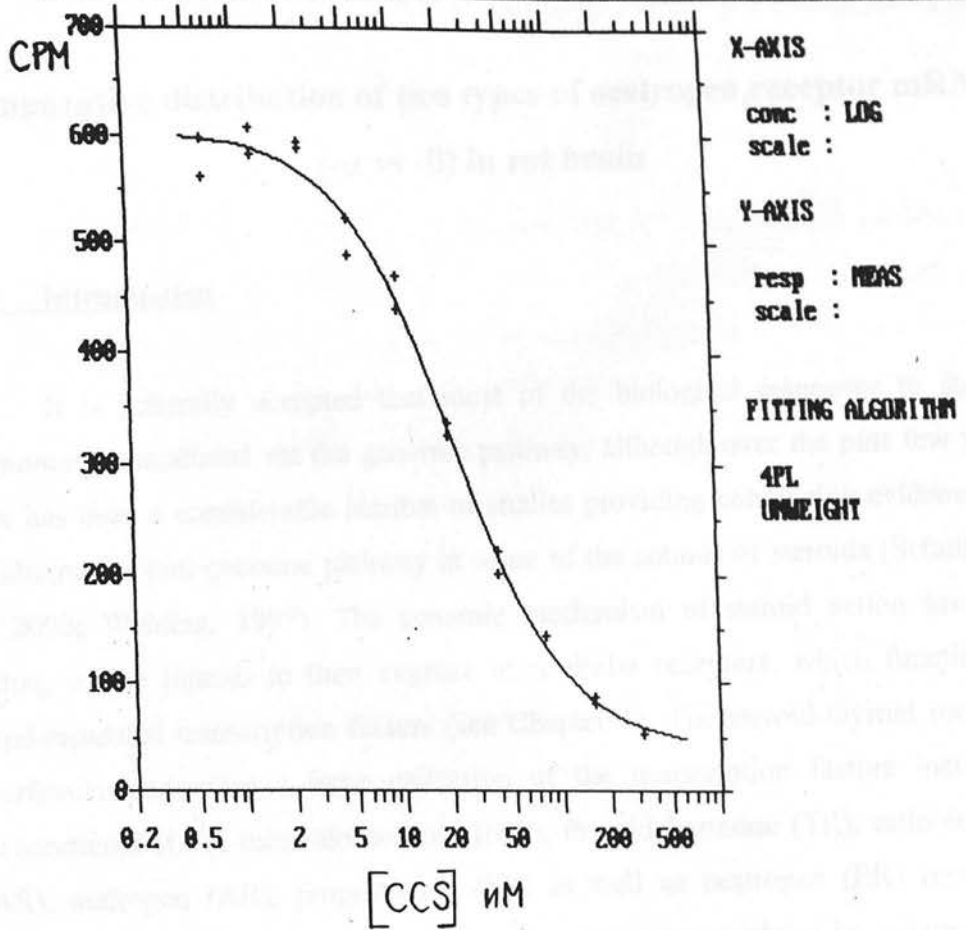
Figure 2.2. A representative corticosterone standard curve from the corticosterone radioimmunoassay. [CCS] = Corticosterone concentration, CPM = counts per minute

Chapter 3

Comparative distribution of two types of oestrogen receptor mRNAs (-α *vs* -β) in rat brain

3.1.   Introduction

It is generally accepted that most of the biological responses to steroid hormones are mediated via the genomic pathway, although over the past few years there has been a considerable number of studies providing convincing evidence for the alternative non-genomic pathway in some of the actions of steroids (Schmidt *et al.*, 2000; Wehling, 1997). The genomic mechanism of steroid action involves binding of the ligands to their cognate intracellular receptors, which function as ligand-regulated transcription factors (see Chapter 1). The steroid-thyroid receptor superfamily comprises a large collection of the transcription factors including glucocorticoid (GR), mineralocorticoid (MR), thyroid hormone (TR), retinoic acid (RAR), androgen (AR), progesterone (PR) as well as oestrogen (ER) receptors (Mangelsdorf *et al.*, 1995). These nuclear receptors are modular in construction, having four or five distinct domains, depending on the type of the receptor.

Oestrogens regulate a wide variety of neural processes ranging from organising sexual differentiation of the brain (Arnold & Breedlove, 1985), mediating various reproductive physiologies (Couse & Korach, 1999), controlling hypothalamic GnRH neuroendocrine function (Kalra & Kalra, 1983), modulation of mood and behaviour (Fink *et al.*, 1996) as well as its newly discovered role as a neuroprotective molecule in certain neurodegenerative diseases (Xu *et al.*, 1998). Such diversity of effects indicates that several areas of the brain are involved. Following the cloning of the first ER (ER-α) in the late 1980s (see Chapter 1) there was a general belief that only one ER existed, despite the commonality that many

receptors are expressed as multiple isoforms selective for a particular steroid (Tsai & O'Malley, 1994). In 1996, however, a second ER, designated ER-β, was cloned from a rat prostate cDNA library (Kuiper *et al.*, 1996). The two oestrogen receptor subtypes are products of two distinct genes, located in two different chromosomes, at least in the human (Enmark *et al.*, 1997).

Because of the essential role of the receptors for a hormone in mediating its action the distribution of the receptor is of key importance. Formerly, the identification of the sites of hormone action (e.g. oestrogens) relied on the use of systemically administered radiolabelled-ligand, which is concentrated by oestrogen-responsive cells, thus reflecting the cellular location of the hormone receptor binding site. This approach, however, relies on ligand-binding conformation and would not differentiate among many other receptor isoforms, some of which might not be able to bind ligand, but nonetheless possess the ability to modulate the transcription process and vice versa (see Maruyama *et al.*, 1998; Warner, Nilsson, & Gustafsson, 1999). The advent of elaborate molecular biology techniques over the past few decades has enabled the detection of a particular receptor, both at the level of transcription and translation by identifying the receptor mRNA or the protein, respectively. The biological role of the receptor is then assessed by various functional studies including at the behavioural level, plus the use of 'receptor knock-out' models whereby the receptor gene has been rendered functionally impaired. Since the initial cloning of ER-α over a decade ago a great deal of information regarding its localisation, functional properties and physiological relevance has been obtained. Despite the fact that ER-α mRNA or protein has been identified in most areas where oestrogens are known to exert their effects, there are some instances whereby oestrogens could evidently exert physiological actions in the absence of the classic ER-α mRNA or protein. One such system comprises the magnocellular neurosecretory neurones where the oxytocin gene is undoubtedly, although not necessarily directly, regulated by oestrogens (Van Tol *et al.*, 1988) (see more in Chapter 5). Moreover the retention of systemically administered ^{125}I-oestrogen in certain areas of brain, such as the medial preoptic area or the amygdala in oestrogen

receptor-α knockout mice (α-ERKO) (Shughrue *et al.*, 1997b) suggests that the effects of oestrogens might be accounted for by the newly discovered ER-β. Therefore this experiment was designed to investigate the comparative distribution of both types of oestrogen receptor in the rat brain.

Various techniques are available to approach this problem such as radio-ligand binding and autoradiography and immunocytochemistry. However, we chose *in situ* hybridisation histochemistry (ISH) because this technique allows a precise anatomical identification of cells that express the particular mRNA. This is particularly useful when dealing with tissue of such high anatomical heterogeneity such as the brain. This method can also be combined with some other techniques such as immunocytochemistry thus enabling the detection two or more moieties in the same tissue/cell simultaneously, although not used here. This technique is also quantifiable which enables 1) evaluation of relative expression of receptor gene between different types of neurones, or other cell types 2) determination of changes in receptor gene expression, reflecting the process regulating expression of the gene in different physiological states.

3.2.   Aims

By using semi-quantitative ISH, we designed and carried out a detailed comparison of the cellular distribution of two types of ER mRNAs (-α *vs* -β) in adult male and female Hooded-Lister rat brains. The distribution was confirmed in subsequent studies where the focus was on investigating the regulation of expression of the mRNA transcripts in selected brain regions (see Chapter 4 & 5).

3.3. Methods

3.3.1. Animals

Adult male and female Hooded-Lister rats (weighing between 180-220 g.) were obtained from a local supplier (Harlan-Olac UK limited) and allowed to acclimatise for at least 2 weeks prior to use. Rat chow and water were available *ad libitum.* Two rats of each sex were used in this study. The stage of the oestrous cycle in the female rats was not determined in this experiment. The rats were killed by decapitation, their brains removed, snap-frozen with powdered dry ice and stored at -80°C until used. Details of the preparation of ER-α and -β riboprobes as well as the *in situ* hybridisation protocols are described in detail in Chapter 2 (section 2.2.2.-2.2.4.)

3.3.2. Data analysis

Anatomical identification and nomenclature of brain structures was based on the stereotaxic rat brain atlases (Paxinos & Watson, 1986; Paxinos & Watson, 1996). The hybridisation signal was evaluated using a computer-aided image analyser (MCID-M4 (Version 3.0 Rev. 1.5), Imaging Research, Inc., Canada). Film autoradiographs and emulsion-coated sections were used. Film autoradiographs showed regional distribution of hybridisation, and in emulsion-coated sections hybridisation signal was visualised in dark-field as silver grains over neurones. The relative intensity of the hybridisation signal was measured as silver grain numbers overlying individuals neurones. Each mRNA transcript was hybridised in an adjacent series of anatomically comparable sections to compare the expression of both ER mRNAs, from rostral to caudal.

Abbreviations

3V	third ventricle
AhiAL	amygdalohippocampal area, anterolateral
ARC	arcuate nucleus
AVPV	anterior ventral periventricular nucleus
BNST	bed nucleus of the stria terminalis
CA1-CA4	Ammon's horn of the hippocampus
CP	choroid plexus
CVOs	circumventricular organs
DG	dentate gyrus
HIP	hippocampus
LC	locus coeruleus
LV	lateral ventricle
MeAD	medial anterodorsal amygdaloid nucleus
MePD	medial posterodorsal amygdaloid nucleus
MPN	median preoptic nucleus
mPOA	medial preoptic area
Opt	optic tract
OX	optic chiasm
Pe	periventricular hypothalamic nuclei
PVN, dp	paraventricular nucleus, dorsal cap
PVN, mpd	paraventricular nucleus, medial parvocellular dorsal zone
PVN, mpv	paraventricular nucleus, medial parvocellular ventral zone
PVN, pml	paraventricular nucleus, posterior magnocellular lateral zone
SFO	subfornical organ
SON	supraoptic nucleus
VMH	ventromedial hypothalamic nucleus

3.4.   Results

3.4.1. Specificity of the probes

Sense probes were included in the experiments for both ER-α and -β mRNAs. A sense probe has an identical sequence to the cellular mRNA of interest, and will not hybridise so serving as a negative control. All ISHs with sense probes were conducted under identical conditions to those for antisense probes. ER-α sense labelling was undetectable in all sections we examined (Figure 3.1.). For most regions showing ER-β hybridisation with antisense probe, there was no detectable labelling with the comparable sense probe. The exception was a degree of labelling of ER-β sense probe within the hippocampal formation, including the DG, although the intensity of the signal was less than that from ER-β antisense probe (Figure 3.2.). Certain CNS structures which contain high cellular and ribosomal RNA content tend to show such non-specific probe binding. These include the dentate gyrus (DG) and CA pyramidal subfields of the hippocampus, granule cell layer of the cerebellum, piriform cortex and some other regions that stain intensely with Nissl substance. This is especially true when using RNA probes since they appear to contain short sequences which hybridise to ribosomal RNA, even though corresponding DNA probe sequences do not recognise the RNA.

Additionally we also included tissue sections that were pretreated with RNase to eliminate mRNA from the tissue prior to hybridisation with antisense riboprobes for ER-α mRNA. No hybridisation signal was detected in any of the sections examined.

A   **B**

Figure 3.1. Representative film autoradiographs from *in situ* hybridisation histochemistry of 10 μM thick coronal rat brain sections hybridised with A) ^{35}S-labelled antisense riboprobe for ER-α mRNA and B) ^{35}S-labelled sense riboprobe for ER-α mRNA. The hybridisation signal from the ER-α mRNA antisense probe was detected in all subfields of the hippocampus (HIP) and in the arcuate (ARC) as well as the ventromedial hypothalamic (VMH) nuclei. The signal from the ER-α sense probe was undetectable in all regions examined.

A   **B**

Figure 3.2. Representative film autoradiographs from *in situ* hybridisation histochemistry of 10 μM thick coronal rat brain sections hybridised with A) ^{35}S-labelled antisense riboprobe for ER-β mRNA and B) ^{35}S-labelled sense riboprobe for ER-β mRNA. The hybridisation signal from the ER-β mRNA antisense probe was detected in all subfields of the hippocampus (HIP) and in the paraventricular nucleus (PVN). The expression was also detectable in the choroid plexus (CP). The hybridisation from the comparable sense probe was undetectable in most regions except in the hippocampus although the intensity of the signal was less than that from the antisense riboprobe.

In general there was no marked difference in terms of the distribution and the intensity of expression for either ER-α or -β mRNAs between intact male and female rats.

3.4.2. Distribution of ER-α mRNA

ISH with antisense riboprobe revealed an extensive distribution of ER-α mRNA throughout the rat brain although the majority of the labelled cells were localised within the diencephalon, in particular the hypothalamus as well as regions of the telencephalon that provide inputs to the hypothalamus.

In the telencephalon, the ER-α mRNA signal was detected as rostral as the piriform cortex (Pir) (Figure 3.3A). Labelling for ER-α mRNA was also seen in the bed nucleus of the stria terminalis (BNST) while the expression in individual cells was found to be weakly labelled (Figure 3.3C & 3.4A). Among the circumventricular organs (CVOs) the subfornical organ (SFO) expressed ER-α mRNA (Figure 3.3C). Analysis of the distribution revealed a considerable degree of expression of ER-α mRNA in the amygdaloid nuclei, although the expression was not uniformly distributed throughout the nuclei with heavily labelled cells found in the amygdalohippocampal, anterolateral portion of the nucleus (AhiAL) (Figure 3.6). Within the hippocampal formation, a moderate level of hybridisation signal was seen throughout the extent of Ammon's horn (CA1-CA4) and DG, with the most intense signal found within the CA1 and CA3 subfields (Figure 3.3I).

In the diencephalon, robust expression of ER-α mRNA was found within several structures of the periventricular zone of the hypothalamus, including the anterior ventral periventricular (AVPV) and the periventricular hypothalamic nuclei (Pe) (Figure 3.3E). Neurones intensely labelled for ER-α mRNA were also observed in the medial preoptic area (mPOA) (Figure 3.5A). In the caudal hypothalamus, a dense accumulation of the hybridisation signal was found within the arcuate nucleus (ARC), particularly in the lateral subdivision of the nucleus (Figure 3.8).

The ventromedial nucleus of the hypothalamus (VMH) also exhibited a marked ER-α mRNA hybridisation signal although the most heavily labelled neurones were found in the ventrolateral aspect of the nucleus (Figure 3.8 & 3.9).

Within the metencephalon, only a few weakly ER-α labelled cells were found in the locus coeruleus (Figure 3.10). There was no discrete labelling of ER-α of cells in the cerebellar cell layers.

3.4.3.   Distribution of ER-β mRNA

The present ISH study for ER-β mRNA revealed a different pattern of distribution from that of ER-α mRNA although both transcripts share a considerable degree of overlap within certain structures in the rat brain.

In the telencephalon a weak signal for ER-β mRNA was seen in the piriform cortex (Pir) (Figure 3.3B). Strong ER-β mRNA signal was evident over several cells in the bed nucleus of the stria terminalis (BNST) (Figure 3.3D & 3.4B). In the amygdaloid nuclei ER-β mRNA was widely distributed throughout the structure. A moderate level of ER-β mRNA expression was found in the medial anterodorsal (MeAD) nucleus with a gradual increase in the intensity of the signal towards the medial posterodorsal nucleus (MePD) (Figure 3.7). A low level of ER-β mRNA expression was found throughout the whole hippocampal formation including CA1-CA4 and DG, similar to the expression pattern found for ER-α mRNA (Figure 3.3J).

In the diencephalon ER-β mRNA containing cells were mainly localised in the hypothalamus, and particularly neurones in the periventricular zone were the most heavily labelled. Perhaps the most striking differences in the distribution of ER-α and -β mRNAs were found in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei (Figure 3.3H & J). Neurones within the PVN and SON,

completely devoid of the classic ER-α, expressed a high level of ER-β mRNA. In fact, the highest intensity of ERβ mRNA signal was seen in the PVN. At high resolution, ER-β mRNA was found to be differentially expressed among the discrete subnuclei of the PVN. The majority of heavily labelled cells were localised mostly in the parvocellular part of the nuclei, especially in the medial parvocellular part, ventral zone (mpvPVN) with little expression found in the dorsal zone (mpdPVN), while some heavily labelled cells were also found in the dorsal parvocellular part (dpPVN) (Figure 3.11). Within the magnocellular neurones, both in the PVN (pmlPVN) as well as the SON, the expression of ERβ mRNA was obviously less intense but nevertheless present throughout the rostro-caudal extent of the SON, and in both the dorsal (predominantly oxytocin-containing) and ventral (predominantly vasopressin-containing) portions of the nucleus. (Figure 3.12) Small accessory cell groups of magnocellular origin including the nucleus circularis (oxytocin neurones) also expressed ER-β mRNA (Figure 3.13).

Moderate expression of ER-β mRNA was found in the periventricular nucleus (Pe) (Figure 3.3F & 3.5B). Weak ER-β labelling was found in the medial preoptic area (mPOA) (Figure 3.5B). Similarly, in the arcuate nucleus (ARC) ER-β mRNA expression was weak whereas the ventromedial nucleus of the hypothalamus (VMH) showed no ER-β mRNA signal.

In the metencephalon, the largest concentration of ER-β mRNA containing cells was seen in the cerebellum, with the majority of the Purkinje cell layers being labelled (Figure 3.14).

One of the interesting findings in this present study was the exclusive expression of ER-β mRNA in the choroid plexus. We detected the signal in the choroid in the lateral ventricle, while the ER-α mRNA signal was completely absent from this structure as was sense control (Figure 3.3H & 3.2B).
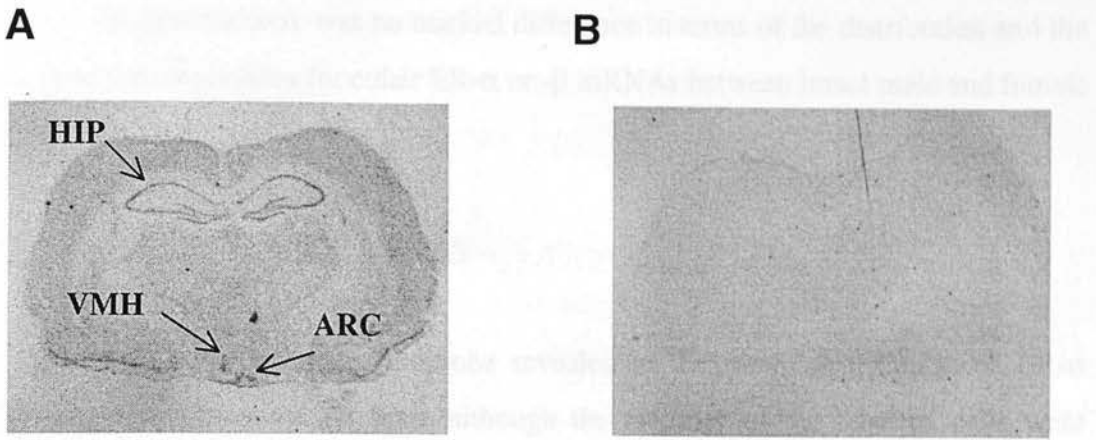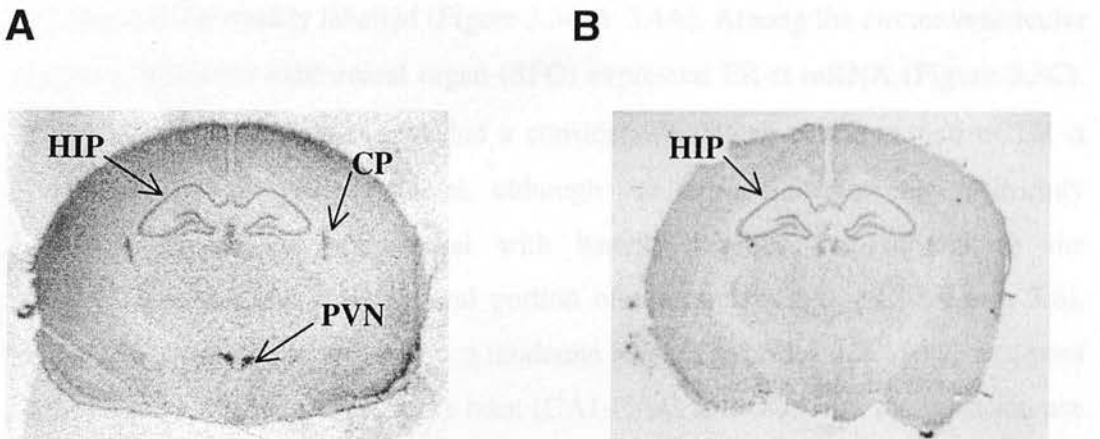
ER-α mRNA

ER-β mRNA
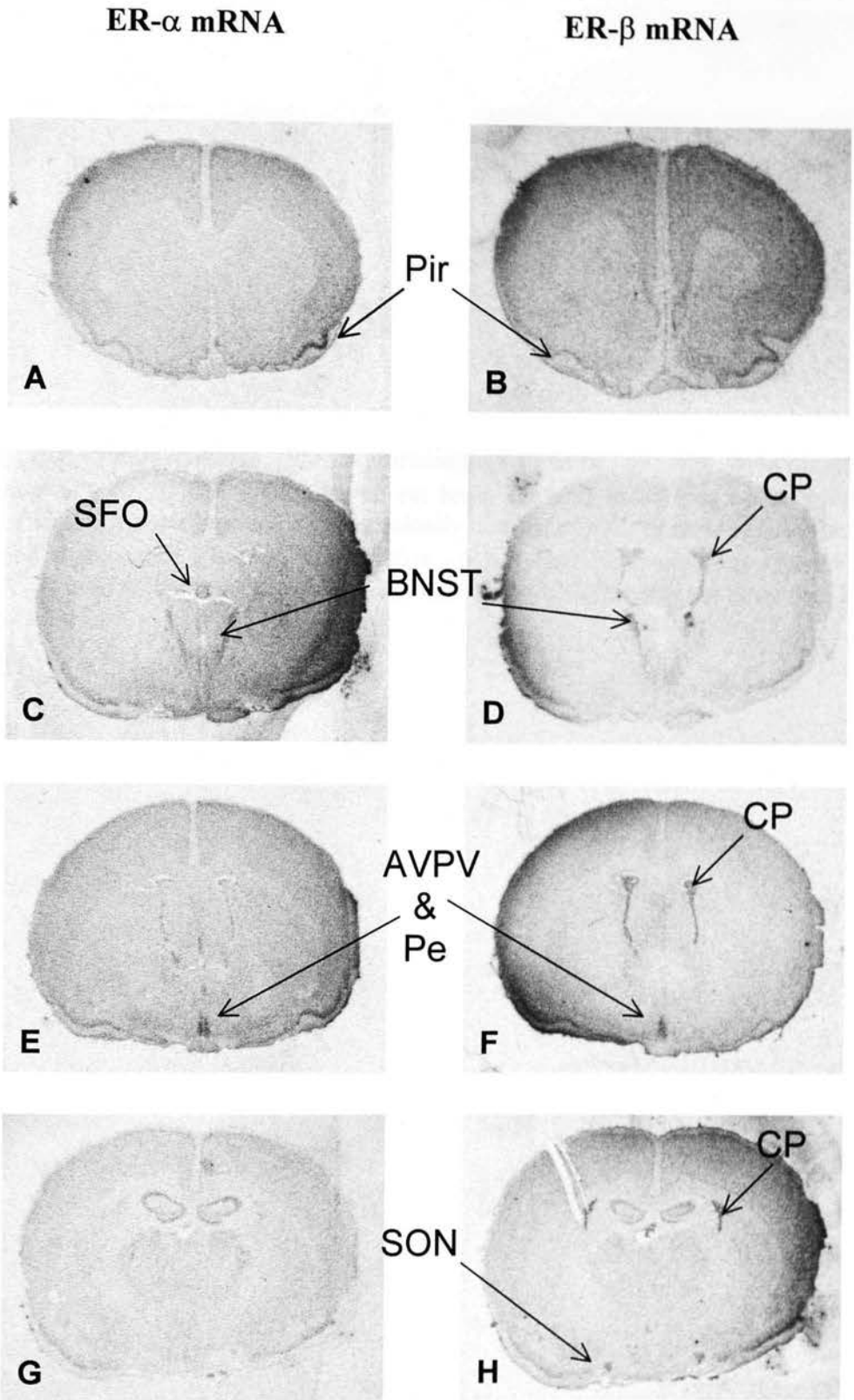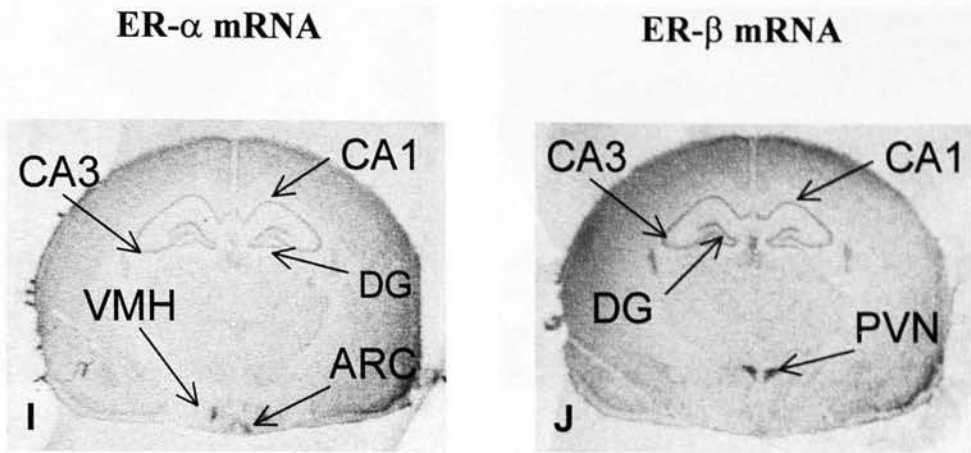
Figure 3.3.

Figure 3.3. Representative film autoradiographs from *in situ* hybridisation histochemistry of 10 µM thick coronal rat brain sections at various rostro-caudal levels. Pairs of autoradiograms of anatomically comparable sections hybridised with ^{35}S-labelled antisense riboprobes for ER-α mRNA (left hand column) and ER-β mRNA (right hand column). Abbreviations indicated for different brain structures are defined on p5.
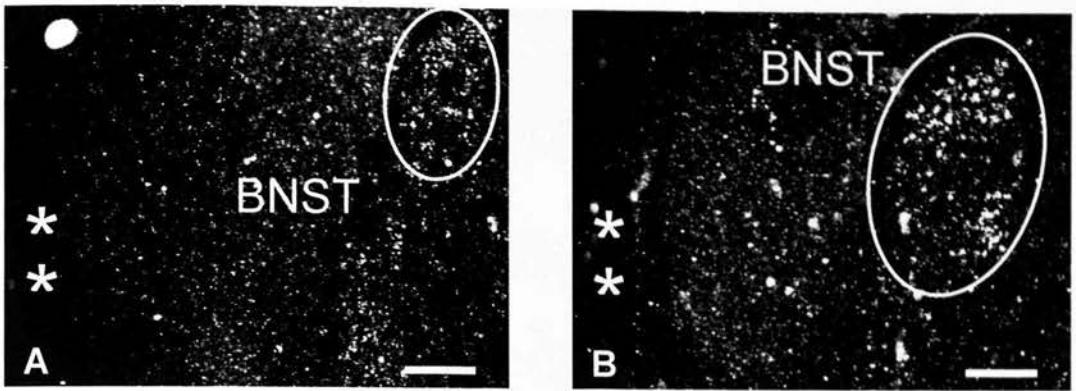
Figure 3.4. Dark-field photomicrographs showing silver grains over neurones hybridised with A) ER-α mRNA and B) ER-β mRNA in the bed nucleus of the stria terminalis (BNST). Asterisks indicate the medial septal nucleus. Scale bar = 100 μM.
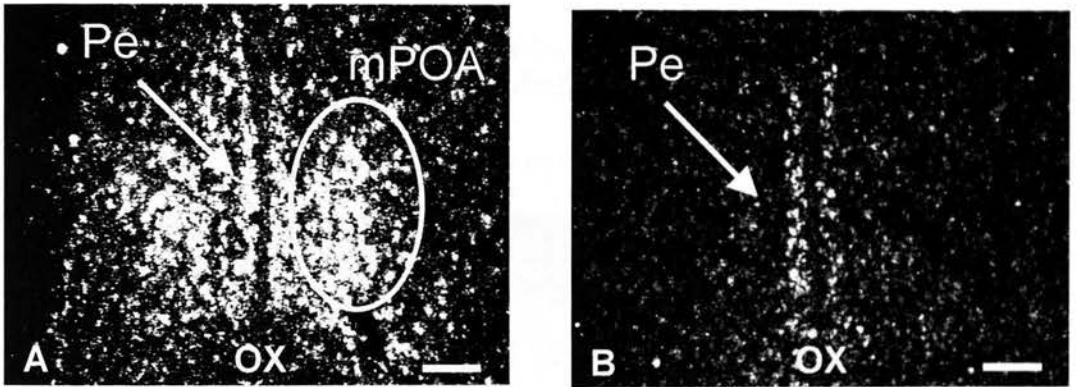
Figure 3.5. Dark-field photomicrographs showing neurones in the anterior hypothalamus hybridised with A) ER-α mRNA and B) ER-β mRNA. Intense hybridisation signal from ER-α mRNA was seen throughout the anterior ventral periventricular nucleus whereas signal from ER-β mRNA was restricted to a confined dorsal periventricular area (Pe). The medial preoptic area (mPOA) also contained a substantial number of neurones hybridised with the ER-α mRNA probe while a low density of ER-β mRNA signal was found in this region. OX denotes optic chiasm. Scale bar = 200 μM.
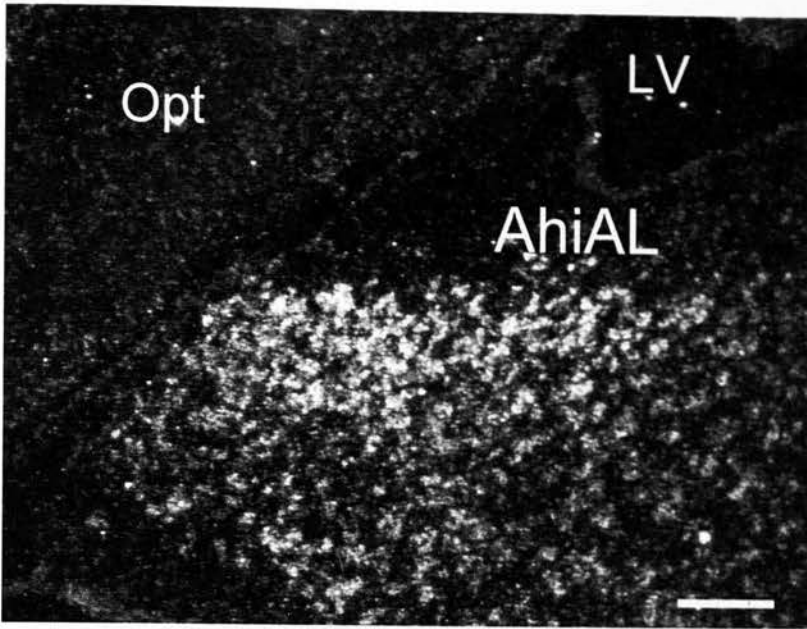
Figure 3.6. Dark-field photomicrographs showing neurones in the amygdala hybridised with ER-α mRNA. Strong hybridisation signal was localised in neurones in the anterolateral portion of the nucleus (AhiAL). Opt denotes optic tract and LV denotes lateral ventricle. Scale bar = 200 μM.
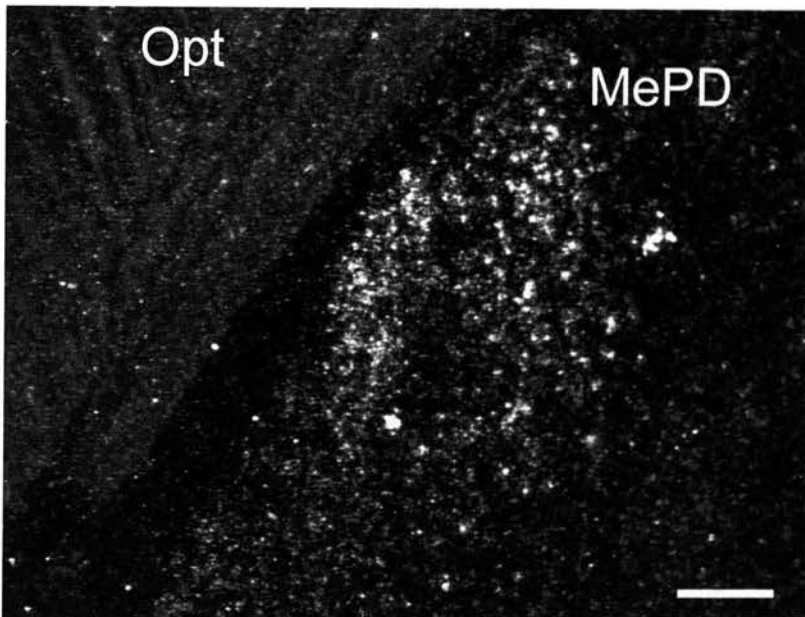
Figure 3.7. Dark-field photomicrographs showing neurones in the anterior amygdala hybridised with ER-β mRNA. The neurones strongest labelling for ER-β mRNA was found in the medial posterodorsal part of the nucleus (MePD). Opt denotes optic tract. Scale bar = 200 μM.
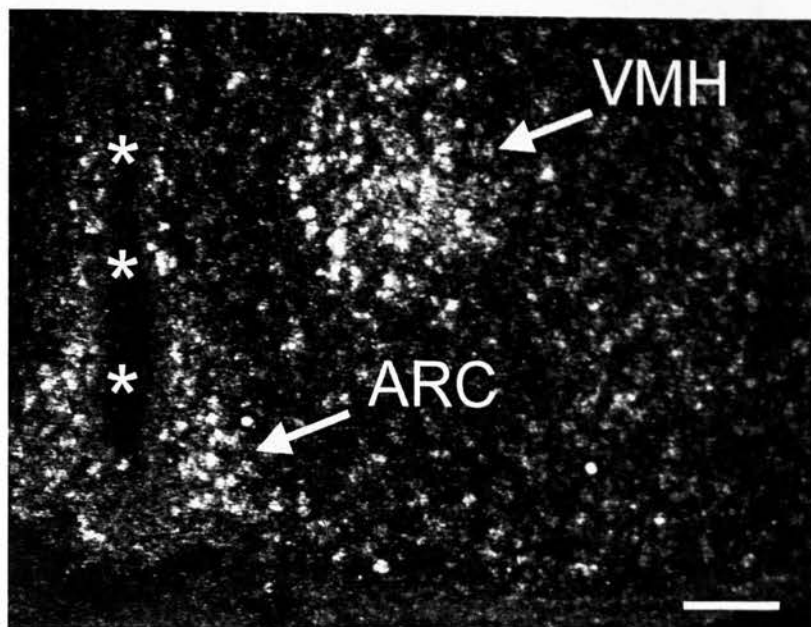
Figure 3.8. Dark-field photomicrograph exhibiting silver grains over neurones hybridised with an antisense riboprobe for ER-α mRNA in the arcuate (ARC) and ventromedial hypothalamic (VMH) nuclei. Asterisks indicate the third ventricle. Scale bar = 200 μM.
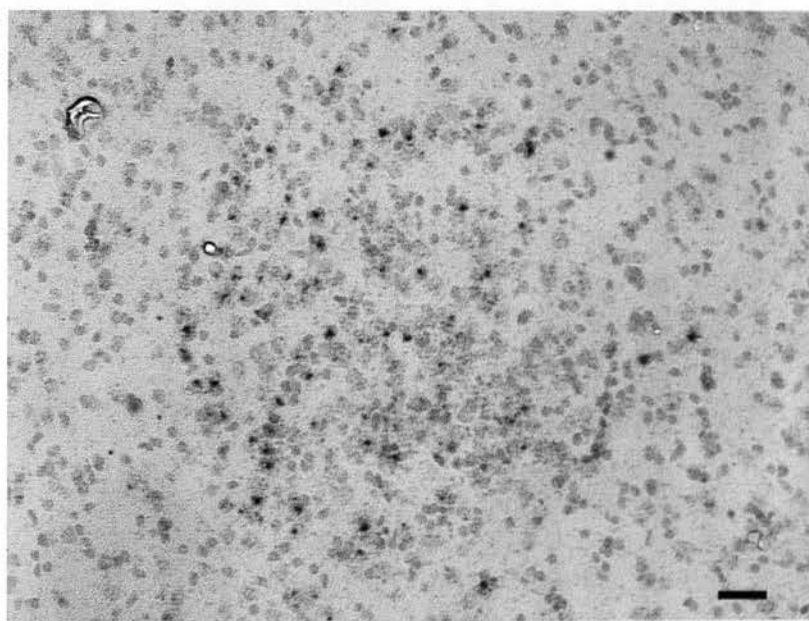
Figure 3.9. Bright-field photomicrograph exhibiting silver grains from *in situ* hybridisation signal for ER-α mRNA over neurones in the ventromedial hypothalamic nucleus. Scale bar = 50 μM.
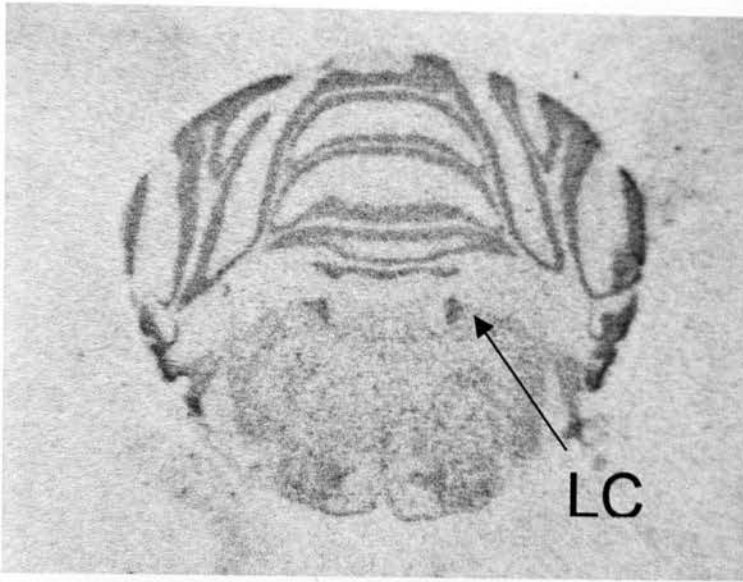
Figure 3.10. Autoradiograph from *in situ* hybridisation for ER-α mRNA antisense riboprobe on 10 µM thick coronal rat brain section. Hybridisation signal was detected in the locus coeruleus (LC) in the metencephalon.
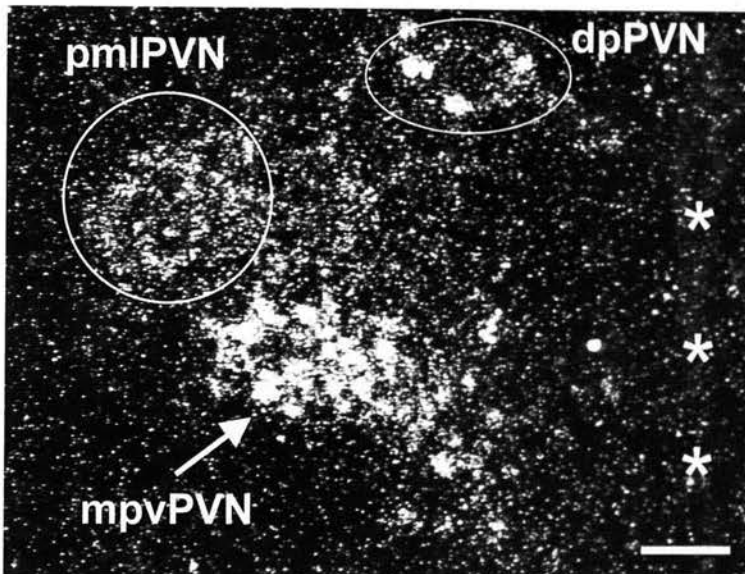
Figure 3.11. Dark-field photomicrograph showing silver grains over neurones hybridised with an antisense riboprobe for ER-β mRNA in the paraventricular nucleus (PVN). The hybridisation signal was expressed differentially in the different discrete subpopulations of the nucleus, with the majority of heavily labelled cells localised mostly in the parvocellular part of the nuclei, especially in the medial parvocellular part, ventral zone (mpvPVN). The posterior magnocellular part (pmlPVN) and the dorsal parvocellular part (dpPVN) also contained ER-β mRNA expressing neurones. Asterisks indicate the third ventricle. Scale bar = 100 µM.
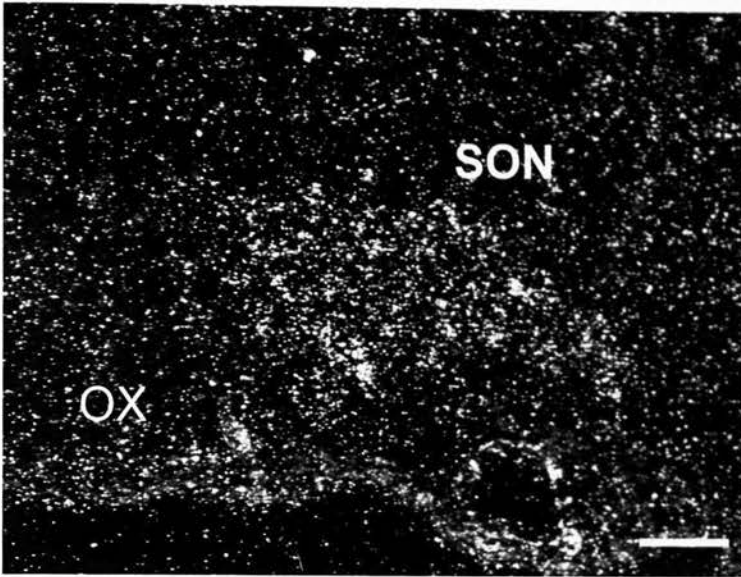
Figure 3.12. Dark-field photomicrograph showing silver grains over neurones hybridised with an antisense riboprobe for ER-β mRNA in the supraoptic nucleus (SON). The hybridisation signal was present in both the dorsal (predominantly oxytocin-containing) and ventral (predominantly vasopressin-containing) portions of the nucleus. OX denotes optic chiasm. Scale bar = 100 μM.
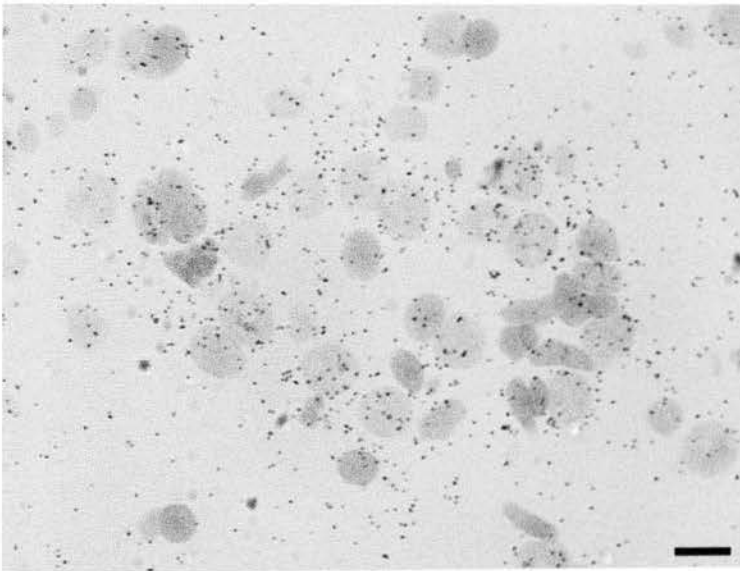
Figure 3.13. Bright-field photomicrograph showing silver grains over neurones in the nucleus circularis (accessory magnocellular neurones) hybridised with an antisense probe for ER-β mRNA. Scale bar = 10 μM.
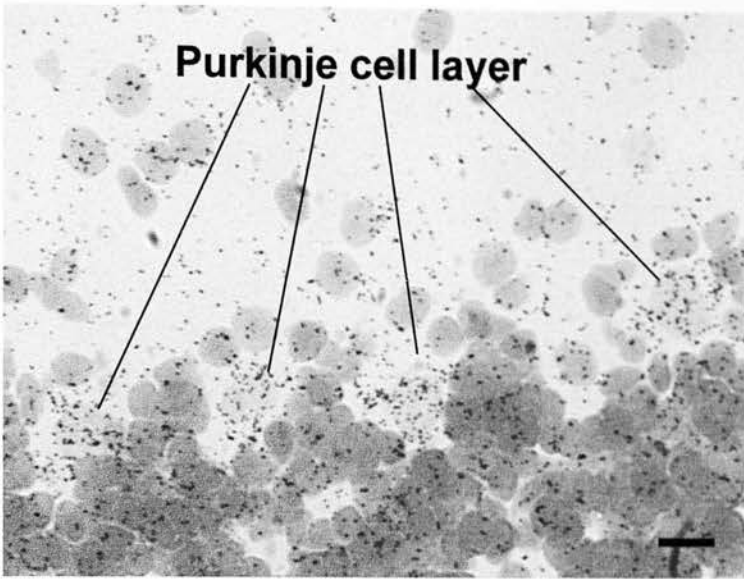
Figure 3.14. Bright-field photomicrograph showing silver grains over Purkinje cells in the cerebellum hybridised with an antisense probe for ER-β mRNA. Scale bar = 10 μM.

3.5. Discussion

The current ISH study indicates distinct distribution patterns for both ER-α and -β mRNAs, although they exhibit a considerable degree of overlap within certain CNS structures. By comparing the distribution of both ER mRNAs in adjacent sections both transcripts were present in the piriform cortex, the bed nucleus of the stria terminalis, the periventricular zone of the anterior hypothalamus, the amygdaloid nuclei, and the hippocampal formation. Exclusive expression of ER-α mRNA was found within the ventromedial hypothalamus, the subfornical organ and the locus coeruleus. The arcuate nucleus also expressed predominantly ER-α mRNA, with a barely detectable level of ER-β mRNA within this structure. In contrast ER-β mRNA, but not ER-α, was detected in the paraventricular and supraoptic nuclei, choroid plexus and the Purkinje cell layers in the cerebellum.

This pattern of expression corresponds to other recently published descriptions (Hrabovszky et al., 1998; Laflamme et al., 1998; Shughrue, Komm, & Merchenthaler, 1996; Shughrue, Lane, & Merchenthaler, 1997a). The possible functional significance of these patterns of ER-α/-β expression in these regions is discussed.

Our understanding of the roles of sex steroids in reproductive behaviour and the regulation of GnRH neurones arises from the study of gonadal steroid actions in the anterior and ventral hypothalamus. The anterior hypothalamus is a well-established target structure for oestrogens, and showed a characteristic distribution of ER-α mRNA, similar to a previous ISH study carried out by Simerly et al. 1990 (Simerly et al., 1990). The medial preoptic area (mPOA) and the ventromedial hypothalamus (VMH) are known to mediate several sexual behaviours including maternal behaviour and lordosis in female rats (Madeira & Lieberman, 1995) and the control of copulatory behaviour in males (McGinnis, Williams, & Lumia, 1996; Pfaus & Heeb, 1997). These areas expressed a strong hybridisation signal for ER-α

mRNA but a low level, or none, for ER-β mRNA, suggesting that it is primarily the ER-α subtype that mediates these effects. Similarly cells containing strong ER-α mRNA were seen in regions of the periventricular hypothalamus such as the anteroventral periventricular (AVPV) and the arcuate nuclei, regions thought to be involved in the control of gonadotrophin (GnRH) secretion (Lehman *et al.*, 1997), although the GnRH neurones themselves are not thought to possess either ER-α or -β (Herbison & Theodosis, 1992; Li, Schwartz, & Rissman, 1997; Shivers *et al.*, 1983; Shughrue *et al.*, 1997a) (although see Butler, Sjöberg, & Coen, 1999). Especially, the oestrogen-receptive neurones in the AVPV are proposed to be the critical site where oestrogens exerts its stimulatory effects upon GnRH release and synthesis (Herbison, 1998). In contrast the arcuate nuclei were only lightly labelled for ER-β mRNA and the signal was restricted to a defined area of the AVPA. This suggests that the feedback of ovarian hormones in controlling the release of GnRH might involve ER-α rather than ER-β subtype.

The limbic areas that share bidirectional connections with the anterior hypothalamus, such as the medial amygdala (MeA) and the bed nucleus of the stria terminalis (BNST) expressed both ER-α and -β mRNAs. The amygdaloid nuclei participate in a wide range of emotional behaviours related to anxiety, fear, sexual behaviour as well as learning and memory (Byrum, Ahearn, & Krishnan, 1999; Gallagher & Schoenbaum, 1999; Van Hoesen, Augustinack, & Redman, 1999). The patterns of distributions of both receptor mRNAs in the amygdala differed in the different subnuclei, suggesting that oestrogens could mediate these diverse physiological responses by means of both receptor subtypes via discrete populations of neurones. The BNST, serving as a relay site to various forebrain nuclei, also possesses both receptor mRNAs. Again the pattern of distribution shows subtle differences in terms of the localisation and the intensity of ER-α and -β mRNA expressed here. The BNST is a target of gonadal steroids known to be involved in the endocrine and behavioural control of reproduction (Emery & Sachs, 1976).

The subfornical organ (SFO), a circumventricular organ, involved in aspects of cardiovascular and body fluid regulation (Ferguson & Bains, 1996; McKinley, Hards, & Oldfield, 1994; Phillips, 1987) contained neurones expressing ER-α mRNA. This finding is supported by an earlier study that had shown the accumulation of oestradiol by SFO neurones (Pfaff & Keiner, 1973). Angiotensin II (ANGII) is the predominant circulating hormone regulating function of the SFO via ANG II AT_1-receptors (AT_1R) (Gutman, Ciriello, & Mogenson, 1988). A recent study has identified the colocalisation of both AT_1R and ER protein within the SFO (Rosas-Arellano, Solano-Flores, & Ciriello, 1999) suggesting that oestrogens could exert their effects, presumably via the ER-α receptor subtype, on SFO neurones thus modulating the cardiovascular response to ANG II.

The finding that both ER mRNAs, particularly ER-β, are distributed beyond hypothalamic neurones suggests that oestrogens might be involved in various other neural processes apart from the well-established reproductive functions. One of the interesting extrahypothalamic roles of oestrogens is the beneficial effects on the cognitive decline associated with ageing and various neurodegenerative diseases, particularly dementia of Alzheimer's type (AD) (see Henderson, 1997b). Although remaining inconclusive, several epidemiological studies in humans have suggested a potential role of oestrogen in improving cognitive function. For instance, Tang et al. 1996, have shown a significant delay in the onset and decrease in the relative risk of developing AD in postmenopausal women with E_2 replacement therapy (Tang et al., 1996). Greater cognitive impairment is associated with increased hypothalamic-pituitary-adrenal axis activity (HPA) (Heuser, Litvan, & Juncos, 1988) which results in a marked release of cortisol level, a phenomenon seen in the elderly and AD patients (Greenwald et al., 1986). Oestrogen replacement therapy in postmenopausal women has been shown to blunt stress-induced cortisol elevations (Lindheim et al., 1992) perhaps enhancing memory by diminishing deleterious effects of stress.

Although the underlying mechanism of how oestrogens are implicated in the process of cognitive function remains unclear, it could be speculated that oestrogens might mediate their actions, in part, via the forebrain hippocampus. Given that both receptor mRNAs are expressed in all subfields of the hippocampus, they may confer oestrogen's action in a synergistic manner upon this limbic structure. The hippocampus plays a key role in certain aspects of learning and memory, particularly in the process of long-term potentiation (LTP) and depression (LTD), unique forms of synaptic plasticity that provide a possible biological basis for learning and memory (Malenka, 1994). The hippocampus is also one of the most vulnerable brain areas consistently affected by Alzheimer's disease. In animal studies oestrogens have been shown to increase dendritic spine density (Gould *et al.*, 1990a; McEwen, Tanapat, & Weiland, 1999) and synapses (Woolley & McEwen, 1992; Woolley *et al.*, 1997), particularly in the CA1 subfield of the hippocampus which might enhance learning and memory function (McEwen & Alves, 1999). A number of studies have shown the correlation between the process of LTP and synaptic plasticity in this area (Chang & Greenough, 1984; Lee *et al.*, 1980; Tsien, Huerta, & Tonegawa, 1996). LTP is also reported to vary across the oestrous cycle with the highest degree of potentiation during the afternoon of pro-oestrus when oestrogen levels reach their peak (Warren *et al.*, 1995). Cultured hippocampal neurones *in vitro* have been shown to be protected in the presence of oestradiol against a number of toxic agents including glutamate, $FeSO_4$ and amyloid β-peptide (Goodman *et al.*, 1996). The regulation of ER mRNA within the hippocampus especially in the context of HPA activity was of particular interest in this study and the subject of experimental investigation (see Chapter 4).

Exclusive expression of ER-β mRNA, without ER-α, was found within the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. While the PVN is comprised of a heterogeneous population of both magno- and parvo-cellular neurones, the SON consists of only the magnocellular type. A retrograde fluorescence tracing study conducted two decades ago by Swanson and Kuypers (Swanson & Kuypers, 1980) provided a detailed description of the organisation of

the subdivisions of the PVN and their projections. Based on this description we found a striking expression of ER-β mRNA within the medial parvocellular ventral zone (mpvPVN) and, to a lesser extent, the dorsal zone (mpdPVN). Neurones in the mpvPVN project to the autonomic brainstem and spinal cord while those in the dorsomedial PVN project to the median eminence. Thus the present findings suggest the potential role of oestrogens in mediating certain autonomic functions (see more in Chapter 5) via ER-β containing cells. Although the majority of cells intensely labelled for ER-β were localised within the parvocellular neurones, the hybridisation signal was nonetheless present in the magnocellular subdivision throughout the extent of the nuclei. Oestrogens have long been recognised as a modulator of the magnocellular vasopressin (VP) and oxytocin (OXT) neurones. The expression of OXT mRNA in the SON varies across the oestrous cycle (Van Tol *et al.*, 1988) and decreases following castration (Miller *et al.*, 1989). Replacement with oestradiol has been shown to increase the level of OXT mRNA in the SON and the anterior commissural nucleus in ovariectomised rats (Chung, McCabe, & Pfaff, 1991). The discovery of ER-β mRNA in the PVN and SON may provide an explanation of how oestrogens can regulate the VP and OXT genes where numerous previous studies have failed to identify the ER-α mRNA and protein in these sites (see more in Chapter 5).

Various laboratories have previously carried out histochemical studies to determine the distribution of the ER-β transcript and protein in the hypothalamus of the rat brain (Alves *et al.*, 1998; Hrabovszky *et al.*, 1998; Li *et al.*, 1997; Shughrue *et al.*, 1997a; Simonian & Herbison, 1997). Although our study on ER-β mRNA expression is largely in agreement with other findings subtle differences exist between studies. While our results revealed an intense ER-β mRNA signal within the parvocellular neurones, in particular in the medial ventral zone, Shughrue *et al.*, instead, reported a strong hybridisation signal in the magnocellular subdivision of the PVN (Hrabovszky *et al.*, 1998; Shughrue *et al.*, 1996). This difference might have originated from the difference in the riboprobes employed in this present and their studies. Our ER-β mRNA riboprobe was generated from the entire 5'- untranslated

region (5'UTR) of the rat ER-β cDNA, which is a long single stretch of nucleotide sequence of ~400 bp in length, whereas the riboprobe from Shughrue *et al.* was a mixture of two probes (Shughrue *et al.*, 1996), one of which corresponds to nucleotide bases 52-610, also encoded most part of the 5'UTR, and the other corresponds to bases 1809-2094 extending the 3'end of the rat ER-β cDNA. So the combination of their probe might have detected the expression of some other splicing transcripts in addition to the original ER-β mRNA. This perhaps explains the intensification of the hybridisation signal obtained in their study. Alternatively the difference in the degree of expression between their and our studies might have resulted from the difference in animal strain, hormonal status or from some other external factors such as housing condition, animal treatment or tissue handling, for instance.

Nonetheless, a recent immunocytochemical study by Alves *et al.* 1998 has demonstrated a distribution of ER-β protein in the paraventricular nucleus similar to our present ISH study (Alves *et al.*, 1998).

Apart from the PVN and SON, the cerebellar Purkinje cells, the only output element of the cerebellar cortex, is another example where ER-β is exclusively expressed. This provides a suggestion that oestrogens might be able to influence certain motor functions via this receptor subtype, although a functional study in the future is obviously required to ascertain this.

Lastly, finding ER-β mRNA within the choroid plexus is certainly intriguing although the function of oestrogens in this locus is currently unknown. It perhaps helps in the production of the cerebro-spinal fluid (CSF) or maybe is involved with regulating transport of peptides, such as prolactin or leptin (Bennett *et al.*, 1999; Pi & Grattan, 1998; Zlokovic *et al.*, 2000).

Conclusion

Although ISH studies have advanced understanding of where oestrogens could exert their diverse effects upon target organs this technique actually determines the receptor mRNA. Ultimately the crucial determining step in whether and where E_2 could mediate their biological effects requires the translation of their receptor mRNAs into the functional protein. Prior to the cloning of ER-β, numerous studies utilised various *in vivo* (Bettini & Maggi, 1991; Pfaff & Keiner, 1973; Stumpf, 1970) as well as *in vitro* techniques to determine oestrogen receptor binding sites (not protein as such). Shortly after the discovery of ER-β, specific antibodies were successfully generated against this new receptor. As a result, several laboratories have carried out immunocytochemical studies to detect ER-β protein especially in the nervous system (Alves *et al.*, 1998; Li *et al.*, 1997; Simonian & Herbison, 1997). Most of the literature available to date has yielded, more or less, a consistent result regarding the localisation of the protein. These are also in good agreement with our present ISH study.

Certainly the actions of oestrogens would not only depend on where their respective receptors are expressed but also on whether they would homo- and/or heterodimerise in a region-specific manner (Cowley *et al.*, 1997; Pace *et al.*, 1997). The differential ligand specificity for each ER subtype is also a factor contributing to the final action of oestrogens in target organs (Kuiper *et al.*, 1997; Paech *et al.*, 1997). The generation of recent ERKO mice, both ER-α and -β, has provided the opportunity to begin to partially dissect the functional significance of each receptor subtype and advance our knowledge of the oestrogen-signalling pathway (Couse *et al.*, 1995; Krege *et al.*, 1998; Lubahn *et al.*, 1993; Rissman *et al.*, 1999; Rissman *et al.*, 1997; Shughrue, 1998).

The overlapping but non-identical tissue distribution of both ER-α and -β transcripts indicates that discrete oestrogen-responsive neuronal populations are differentially regulated. This combination of different types of oestrogen receptor

and differing tissue distribution may be crucial in determining how the brain is likely to be affected by oestrogens. This is, however, not to discard the potential rapid non-genomic actions of this steroid all together.

The future development of selective agonists and antagonists of each receptor isoform may allow further dissection of E_2 function at each site throughout the CNS.

Chapter 4

Regulation of oestrogen receptor mRNA in the hippocampus

4.1. Introduction

The role of the ovarian steroid oestrogen was traditionally thought to be only involved in events related to reproduction and, in the brain, through actions predominantly mediated via certain neurones in the hypothalamus. However the past decade has witnessed revelation of other 'extra-hypothalamic' actions of oestrogen. One such interesting finding suggests that oestrogen could exert beneficial effects upon the cognitive decline associated with ageing and various neurodegenerative disorders, especially dementia of Alzheimer's type (AD).

The prevalence of dementia and AD doubles every 5 years after the population reaches the age of 65, so that nearly 50% of the elderly over the age of 85 may suffer from the condition (Evans *et al.*, 1989). AD is the most common neurodegenerative disease associated with ageing and affects as many as 4 million people in the United States (Evans, 1996). The total estimated cost for all AD patients in the United States alone was a staggering $67.3 billion in 1994 (Ernst & Hay, 1994) and has been increasing since.

Several findings suggest that oestrogen deficiency in postmenopausal women increases the vulnerability of selected regions of the central nervous system to the multiple factors contributing to the neurodegenerative changes associated with ageing and AD. Postmenopausal oestrogen replacement therapy reduces the risk of myocardial infarction (Ross *et al.*, 1989) and women with a history of myocardial infarction are more likely than other women to develop dementia (Aronson *et al.*, 1990). Several lines of evidence suggest that lower levels of circulating oestrogen are

associated with lower body weights in postmenopausal women (Meldrum *et al.*, 1981) and AD patients are thinner than control subjects (Berlinger & Potter, 1991). In ageing postmenopausal women, treatment with E_2 has been shown to promote memory function (Sherwin, 1997). Paganini-Hill and Henderson 1994 first demonstrated that E_2 might have a protective effect in reducing the risk of developing AD (Paganini-Hill & Henderson, 1994). In a more recent study, Tang *et al.* 1996 also show that E_2, given in the form of hormone replacement therapy following the menopause, is associated with a reduced subsequent risk of the individual developing AD (Tang *et al.*, 1996) suggesting that oestrogen could potentially serve as a neuroprotective molecule (for recent reviews see Fillit & Luine, 1997; Tierney & Luine, 1997).

There are many hypotheses attempting to explain actions of oestrogens in the brains of AD patients. AD is a progressive neurodegenerative disease which can be characterised by neuronal loss and accumulation of intracellular neurofibrillary tangles and extracellular senile plaques, particularly in the hippocampus and associated neocortex. Oestrogen has been shown to affect the metabolism of the amyloid precursor protein (APP). Deposition of insoluble β-amyloid in brain parenchyma is a distinctive feature in AD patients. In an oestrogen receptor-containing cell culture system, 17β-oestradiol at physiological concentrations increases the secretory metabolism of the soluble fragment of APP without increasing intracellular levels of APP, thereby reducing the deposition of β-amyloid and the formation of senile plaques (Jaffe *et al.*, 1994).

Neurochemical deficits involving cholinergic, serotoninergic, and monoaminergic neurones are present in AD patients. The cholinergic deficit is most pronounced in AD (in the basal nucleus of Meynert, which contains oestrogen receptors and from which the major afferent projections to the frontal cortex originate), and oestrogen stimulates cholinergic markers e.g. choline acetyl transferase, the enzyme responsible for the synthesis of acetyl choline, perhaps the most important neurotransmitter in cognitive function and memory (Gibbs, 1994).

Further evidence for the neuroprotective effects of oestrogens has been provided in cell culture work. Steroid hormones, including oestrogens, progesterone, and glucocorticoids, can directly affect neuronal vulnerability to excitotoxic, metabolic, and oxidative insults, suggesting roles for these steroids in several different neurodegenerative disorders. In particular, oestrogen protects while glucocorticoids endanger these cultured hippocampal neurones (Goodman *et al.*, 1996).

One of the interesting aspects of oestrogen action in the brain is its ability to reverse the hippocampal neuronal ageing and cell death caused by either inadequate or excessive levels of glucocorticoids. Glucocorticoids play a vital role in maintaining homeostasis under adverse conditions, i.e. 'stress'. Corticosteroids act by binding to and activating intracellular receptors (GR and MR) which are highly expressed in the hippocampus (McEwen, Brinton, & Sapolsky, 1988), indicating that this area is a major target for glucocorticoid action. Chronic stress or elevated glucocorticoid levels result in decreased dendritic branch points and decreased total dendritic length of apical dendrites in the CA3 region (Woolley, Gould, & McEwen, 1990b). Thus excessive corticosterone appears to be deleterious to the neurones in the hippocampus. On the other hand, inadequate levels of adrenal steroids also lead to degenerative changes within the hippocampus. An immunocytochemical study has shown shrinkage and cell death in the dentate gyrus (DG) following chronic adrenalectomy (Sloviter *et al.*, 1989). Massive loss of granular cells in the hippocampus within 3 days after adrenalectomy has also been reported by Gould *et al.* 1990 (Gould, Woolley, & McEwen, 1990b). All of these changes probably underlie the impairment of spatial memory performance. On the contrary, with oestradiol treatment, spatial memory performance is enhanced which might be due to the increased spine density, particularly on CA1 apical dendrites, induced by oestrogens (Gould, Woolley, & McEwen, 1991b; Woolley & McEwen, 1992; Woolley *et al.*, 1997). Thus the hippocampus shows structural changes after alterations in circulating levels of both oestradiol and corticosterone.

Moreover, the hippocampus is a key area of the brain responsible for integrating memory, mood and neural regulation of the hypothalamic-pituitary-adrenal, neuroendocrine stress, axis. This area is also a key site of neural degeneration in AD and in ageing. Many studies have investigated the effects of oestrogens in the brain, but most were done outside the region of the hippocampus. Evidence regarding effects of oestrogen in the ventromedial hypothalamus and medial preoptic area which are involved in copulatory and reproductive behaviour are well documented, as well as the neural control of gonadotrophin secretion which is mediated via the periventricular and arcuate nuclei (see Chapter 3). Relatively few studies have been carried out to determine oestrogen function in the hippocampus. Yet why do people with AD have neurone loss in this particular area and manifest memory impairment? Why are neurones in the hippocampus so sensitive to glucocorticoids and gonadal steroid hormones? Are there any interactions between the two types of hormones and what are they? And if there are any interactions, do they occur only in the hippocampus, or are any other areas in the brain involved as well? All of these questions have led to our interest in pursuing the effect of oestrogen in the brain, particularly in the area of the hippocampus.

From our ISH study we have shown that both ER-α and -β mRNAs are evidently expressed in all subfields of the hippocampus, including the pyramidal neurones in CA1 and CA3 as well as the granule cells in DG, suggesting that there might be an interaction between ER-α and -β in conferring the effects of oestrogens in these sites.

4.2.   Aims

Because the response of the target organ to a hormone is largely dictated by the expression of its receptor, knowledge of how the expression of the receptor is regulated is important. This is particularly appropriate in the hippocampus, the structure of which plays roles in learning and memory processes. Such study perhaps may give an insight into how some of the benefits of hormone replacement therapy upon cognitive function might be brought about. In this study we proposed to investigate whether and how ER-α and -β mRNAs are regulated in the hippocampus by various steroid hormone manipulations.

4.3.   Methods

We used a combination of hormone manipulations to study how ER mRNAs might be regulated in neurones in the hippocampus using quantitative ISH.

4.3.1.   Regulation of ER-α and -β mRNAs in the hippocampus by sex steroid manipulation

The regulation of steroid hormone receptors including ER-α and -β has been shown to be regulated by an autologous mechanism, in which the prime regulator is the ligand itself. However, the magnitude of response is also influenced by many other factors including cell-/tissue-type specificity. The regulation of ER-α and -β mRNA has not been extensively explored in the context of the hippocampus before. Thus we proposed to investigate whether such regulation occurs in this site.

4.3.1.1. Effect of acute *vs* chronic gonadectomy

To assess whether ER mRNAs can be regulated by sex steroids the endogenous source of sex hormones from both male and female rats was removed by bilateral gonadectomy. Adult male and random cycling female Hooded-Lister rats

were obtained from a local supplier (Harlan-Olac UK limited) and allowed to acclimatise to a 12 h light:dark cycle for at least 2 weeks prior to use. Rat chow and water were available *ad libitum*. Groups of male and female Hooded-Lister rats (n=5/group) were bilaterally gonadectomised (castration (CX) in males and ovariectomy (OVX) in females) under halothane anaesthesia and left to recover for 72 h or 2 weeks, prior to decapitation. Another set of rats was sham-operated as controls (sham-male (SM) *vs* sham-female (SF)) and killed at the same time as the experimental groups (animals were subjected to the same surgical procedures as the experimental group, except that the gonads were not removed). Surgical procedures are described in detail in Chapter 2, section 2.3.2.1.

The effectiveness of the surgery was assessed by quantifying the expression of ER-α mRNA in the medial preoptic area which was expected to increase 72 h after castration as previously shown (Lisciotto & Morrell, 1993).

4.3.1.2. Effect of exogenous 17β-oestradiol (E$_2$) in ovariectomised rats

A follow-up study was conducted, to determine whether the effect which long-term ovariectomy had on the expression of ER mRNAs could be reversed by replacement of sex steroid to the hormonally-deprived animals. Specifically to establish if the increase in ER mRNA expression in OVX animals could be accounted for by the loss of the major endogenous sex steroid, E$_2$. Two groups of adult random cycling female rats (n=6/group) underwent bilateral ovariectomy under halothane anaesthesia. While still anaesthetised, one group of rats was given a subcutaneous implant of a silastic capsule containing 15 mg/ml of E$_2$ in vehicle. The other group had a vehicle-containing implant as a control. (Details of steroid implant and surgical procedures are described in Chapter 2, section 2.3.2.1.) Rats were killed 2 weeks later by decapitation. The effectiveness of the steroid implant was assessed by bioassay, by determining the uterine wet weight per body weight ratio.

4.3.2. <u>Regulation of ER-α and -β mRNAs in the hippocampus by adrenal steroid manipulation</u>

In addition to the role of the hippocampus in cognitive function, it is also known to have a role in controlling the hypothalamo-pituitary-adrenal (HPA) axis, particularly on the negative feedback mechanism (Jacobson & Sapolsky, 1991). Considerable evidence suggests a modulatory role of sex steroid hormone upon the HPA axis and that oestrogen exerts an excitatory effect upon the activity of this axis. For example, both the basal and stress levels of ACTH and CORT are significantly higher in female rats in pro-oestrus when their production of oestrogen is at its peak (Bohler *et al.*, 1990; Carey *et al.*, 1995; Hiroshige & Wada-Okada, 1973; Raps, Barthe, & Desaulles, 1971). We sought to determine whether manipulating adrenal steroid hormone would affect the expression of ER mRNAs in the hippocampus, perhaps contributing to the effects oestrogen has upon the HPA axis. We elected to use male rats because we found no differences between males and females in patterns of ER-α or -β mRNA distribution in the hippocampus (see Chapter 3), while variations in E_2 exposure through the oestrous cycle are avoided (also testosterone is a precursor for E_2 and 5α-androstane-3β, 17β-diol, which has agonist activity at ER-β, in the male brain (Kuiper *et al.*, 1996).

4.3.2.1. <u>Effect of bilateral adrenalectomy and corticosterone replacement</u>

Three groups of adult male Hooded-Lister rats (n=5/group) were treated as follows;

1) Sham-operated plus subcutaneous vehicle (corn oil) injection

2) Bilaterally adrenalectomised (ADX) plus subcutaneous vehicle injection, given 0.9% NaCl to drink

3) ADX plus replacement of corticosterone (CORT) at a dose of 10 mg/kg in oil via subcutaneous injection daily (at 1600 h), given 0.9% NaCl to drink

All surgery was performed under halothane anaesthesia. Surgical procedures are described in detail in Chapter 2, section 2.3.2.2.a.

4.3.2.2. <u>Effect of stress exposure</u>

One group of adult male Hooded-Lister rats (n=5/group) was exposed to a cycle of stressors over 72 h (exposure to 5% halothane for 1 min; forced swim in cold water at 4°C for 15 min; restraint in a Perspex cylinder for 60 min: each once daily, in random order at 1000, 1200 and 1600 h). The other group of male rats was left undisturbed and served as controls. Details of the experimental procedures are described in Chapter 2, section 2.3.2.2.b.

Rats were killed by decapitation 72 h following surgery or after the beginning of stress exposure. The effectiveness of the treatments was confirmed by measurements of HPA axis parameters, including determining the expression of CRF mRNA in the dorsomedial parvocellular neurones of the PVN, plasma CORT concentration and thymus gland weight. Trunk blood was collected in tubes containing 5% EDTA and plasma separated by centrifugation and stored at -20°C for subsequent radioimmunoassay of plasma CORT concentration, described in Chapter 2, section 2.4.1. The thymus gland was excised and surrounding fat was removed, and the gland then weighed to assess tissue effects of integrated corticosterone secretion.

At the end of each experiment quantitative ISH (described in detail in Chapter 2, section 2.2.2.-2.2.4.) was employed to determine the expression of ER-α and -β mRNAs in the hippocampus. Autoradiographs were evaluated by counting silver grains over the profiles of individual neurones (x 40 objective), using a computer-aided image analysis system (MCID-4 Imaging Research, Canada). The relative amounts of ER-α and -β mRNA expression was determined in pyramidal neurones in CA1, CA3 and granule cells of the DG in the hippocampus. Silver grain counts were made over 8 neurones per section, and in 3-4 sections per region per rat.

Background measurements were made over adjacent tissue with no evident expressing neurones (adjacent to the hippocampal subfields) and subtracted to obtain the net value of silver grain counts. The slides were coded so that the experimenter was unaware of the treatment of the rats at the time of evaluation. Animal means were calculated for each variable, and these values were used to calculate group means. In addition some sections through the medial preoptic area in the acute gonadectomy (72 h) experiment were also hybridised with ER-α and -β mRNA and some through the dorsomedial parvocellular division of the PVN, from rats in the adrenal steroid manipulation study, were hybridised with an antisense riboprobe for CRF mRNA (described in Chapter 2, section 2.2.5.).

In each experiment Student's *t*-test or one-way Analysis of Variance (ANOVA) and post-hoc tests (Student-Newman-Keuls) (unless otherwise stated) were used (SigmaStat software) as appropriate to determine the statistical significance of differences between groups. The alpha value was set at $P < 0.05$. Results are expressed as the group means±sem.

4.4.   Results

In this study we employed quantitative ISH to determine changes in the expression of ER mRNAs in the hippocampus under the influence of gonadal and adrenal steroid hormone modulation. Analysis of the ISH autoradiographs revealed specific hybridisation signal from both ER-α and ER-β antisense riboprobes in all subfields of the hippocampus including pyramidal neurones in CA1 and CA3 and granule cells in the DG (Figure 4.1A.&B.). Control experiments were conducted as described in Chapter 3, section 3.4.1. which included the use of sense probe controls. The ER-α sense labelling was undetectable while there was a degree of hybridisation signal for ER-β sense probe in the hippocampus.
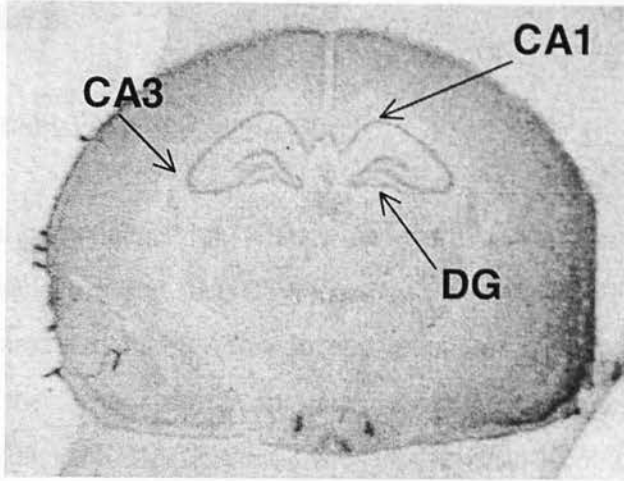
Figure 4.1A. Representative autoradiograph from *in situ* hybridisation histochemistry of tissue section hybridised with ^{35}S-labelled antisense riboprobe for ER-α mRNA to show the hippocampus
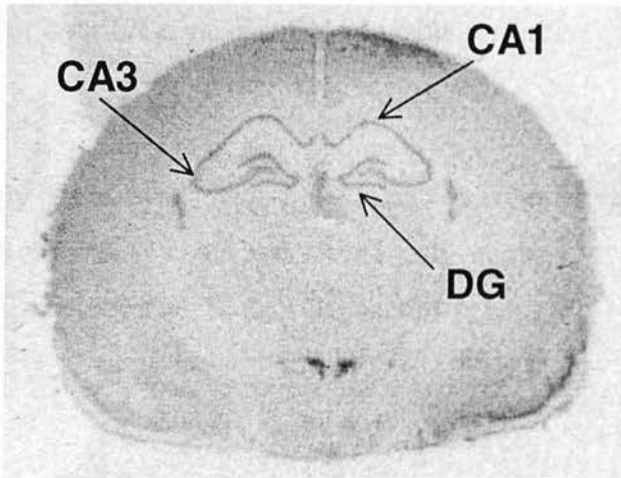
Figure 4.1B. Representative autoradiograph from *in situ* hybridisation histochemistry of tissue section hybridised with ^{35}S-labelled antisense riboprobe for ER-β mRNA to show the hippocampus

4.4.1. Regulation of ER-α and -β mRNAs in the hippocampus by sex steroid manipulation

4.4.1.1. Effect of acute *vs* chronic gonadectomy

At 72 h after gonadectomy (GDX) the expression of neither ER-α nor -β mRNA in the gonadectomised (CX/OVX) rats was significantly different from that in the sham-operated groups (SM/SF) in any region of the hippocampus (Figure 4.2.). In contrast, there was a significant increase in the expression of ER-α mRNA in the DG ($P < 0.05$, ANOVA, $P < 0.05$ Student-Newman-Keuls) and a significant increase in ER-β mRNA expression in the CA1 ($P < 0.0005$, ANOVA, $P < 0.05$ Student-Newman-Keuls) and DG ($P < 0.05$, ANOVA, $P < 0.05$ Student-Newman-Keuls) in OVX rats compared to these regions in the SF group (Figure 4.3.).

However, this increase in expression after GDX was not observed in males, as the expression of both ER-α or -β mRNA remained relatively unchanged after 2 weeks of CX.

Interestingly there was also a significant sex difference in the basal expression of ER-α mRNA which was found only in the 2 week gonadectomy experiment (Figure 4.3A.) but not in the 72h experiment.
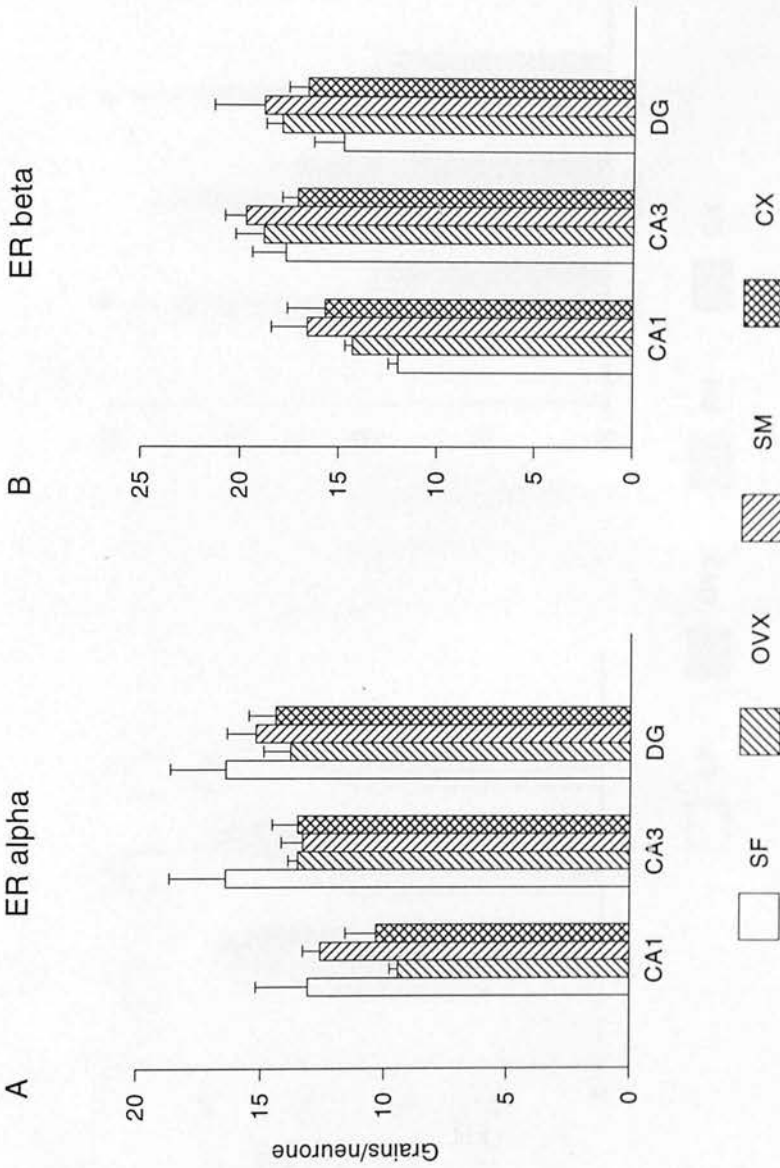
Figure 4.2. Effects of acute gonadectomy (72 h) on ER-α and -β mRNA expression in the CA1, CA3 and DG subfields of the hippocampus. Measurements of silver grain counts per neurone in autoradiographs from *in situ* hybridisations in the CA1, CA3 and DG subfields of the hippocampus. Following gonadectomy there was no significant change in the expression of either ER-α mRNA (A) or ER-β mRNA (B) between the gonadectomised and the sham-operated rats for either sex in any subfield of the hippocampus. Data were analysed by one-way ANOVA, n=5/group. (SF = sham-operated female, OVX = ovariectomised female, SM = sham-operated male, CX = castrated male)
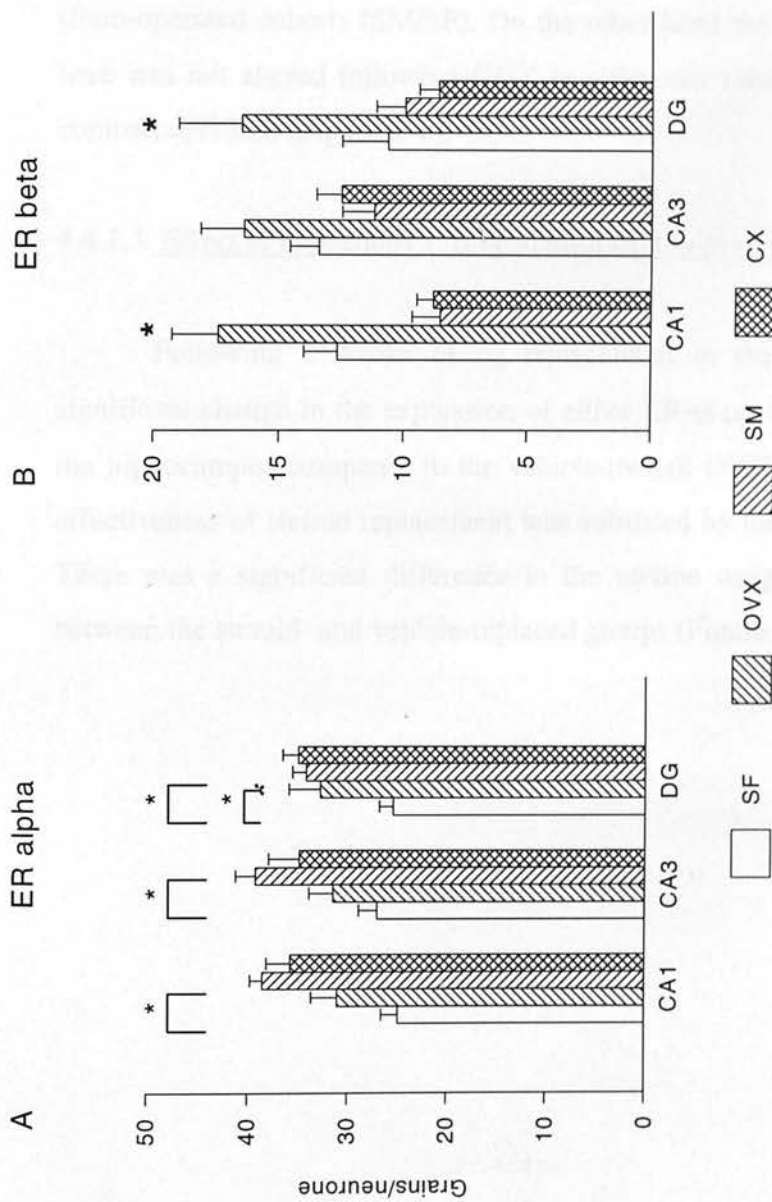
Figure 4.3. Effects of chronic gonadectomy (2 weeks) on ER-α and -β mRNA expression in the hippocampus. The expression of ER-α mRNA in DG was significantly increased in the OVX (A) while the expression of ER-β mRNA in CA1 and DG was significantly increased in the OVX (B), but not in the CX rats compared to the sham-operated controls (SF/SM) 2 weeks after gonadectomy. There was also a sex difference in the basal expression of ER-α mRNA in all subfields of the hippocampus examined (A). Data were analysed by one-way ANOVA, followed by Student-Newman-Keuls post-hoc comparison ($P < 0.05$). Asterisks indicate statistical significance between groups, n=5/group. (SF = sham-operated female, OVX = ovariectomised female, SM = sham-operated male, CX = castrated male)

4.4.1.2. <u>Effect of acute gonadectomy on ER mRNAs in the medial preoptic area (mPOA)</u>

Seventy two hours after CX in male rats the expression of ER-α mRNA in the mPOA was significantly increased ($P = 0.014$, ANOVA, $P < 0.05$ Student-Newman-Keuls) while this effect was not observed in OVX rats compared to their sham-operated cohorts (SM/SF). On the other hand the expression of ER-β mRNA here was not altered following GDX in either sex compared to the sham-operated controls (SF/SM) (Figure 4.4.).

4.4.1.3. <u>Effect of exogenous 17β-oestradiol (E$_2$) in ovariectomised rats</u>

Following 2 weeks of E$_2$ replacement in the OVX rats, there was no significant change in the expression of either ER-α or -β mRNA in any subfield of the hippocampus compared to the vehicle-treated OVX controls (Figure 4.5.). The effectiveness of steroid replacement was validated by the increase in uterine weight. There was a significant difference in the uterine weight relative to body weight between the steroid- and vehicle-replaced groups (Figure 4.6.).
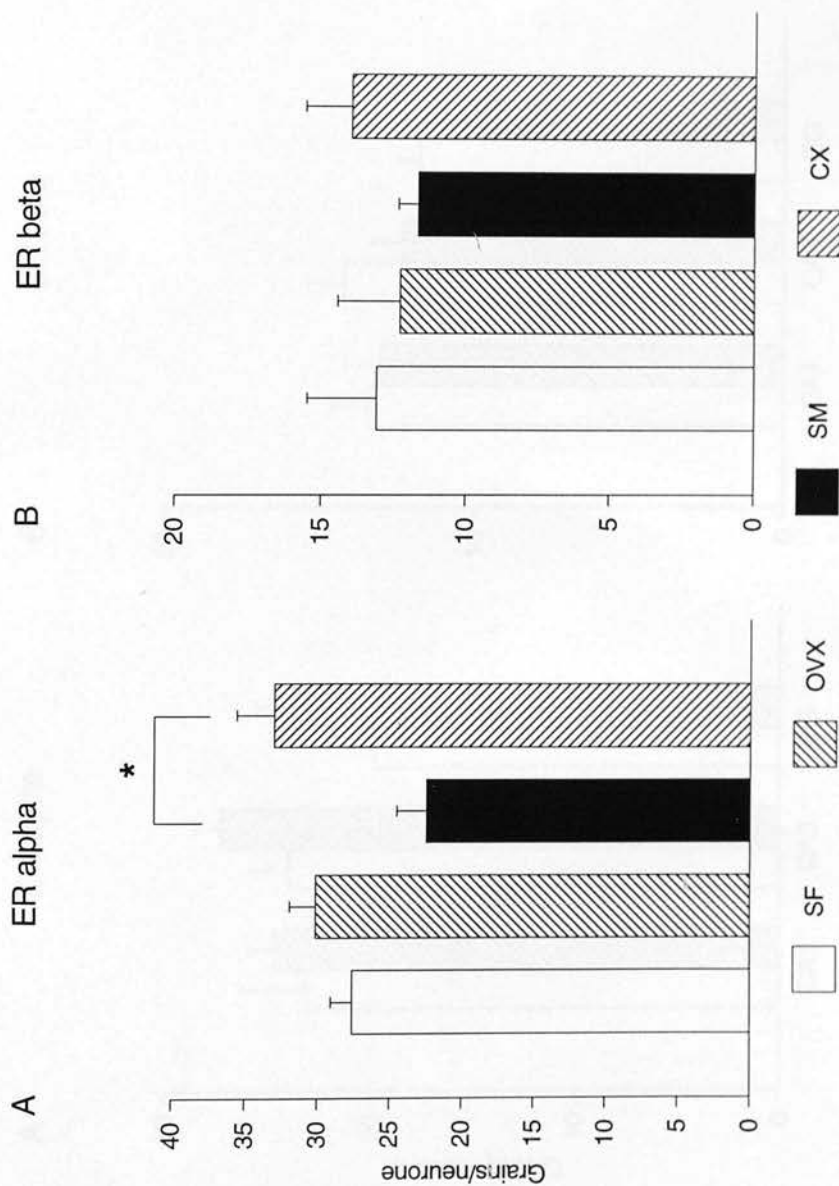
Figure 4.4. Effects of acute gonadectomy (72 h) on ER-α and -β mRNA expression in the medial preoptic area. The expression of ER-α mRNA was significantly increased in the preoptic area of castrated (CX) but not in ovariectomised (OVX) rats compared to the sham-operated controls (SM/SF). There was no change in expression of ER-β mRNA in either sex following gonadectomy. Data were analysed by one way ANOVA, followed by Student-Newman-Keuls post-hoc comparison ($P < 0.05$). Asterisk indicates statistical significance between groups. n=5/group.
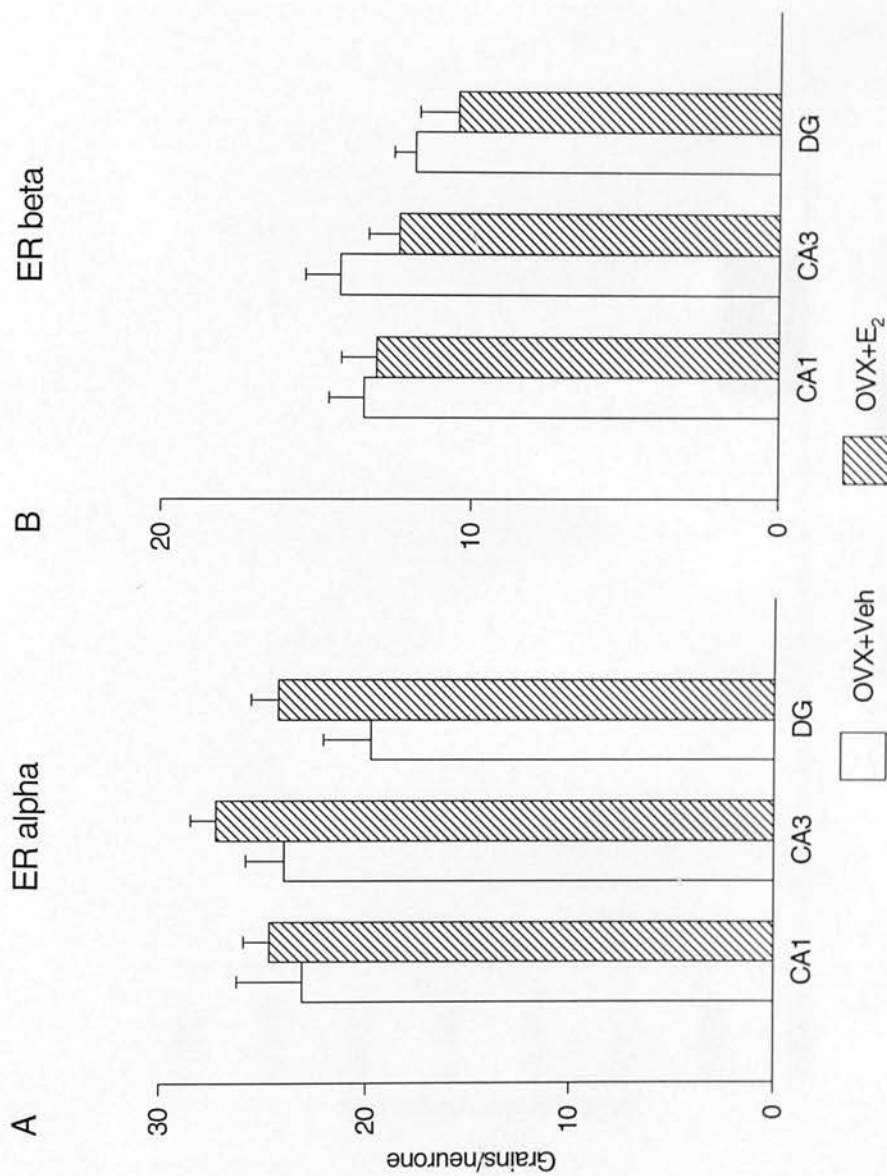
Figure 4.5. Effects of chronic 17-β oestradiol (E_2) treatment on the expression of ER-α and -β mRNA expression in the hippocampus of ovariectomised rats (OVX). Graph shows the relative expression of ER-α mRNA (A) and ER-β mRNA (B) in the CA1, CA3 and DG subfields of the hippocampus in OVX rats treated with E_2 (15 mg/ml s.c. implant) and those treated with vehicle over a period of 2 weeks. Data were analysed by Student's t-test, n=6/group. There was no significant difference between groups.
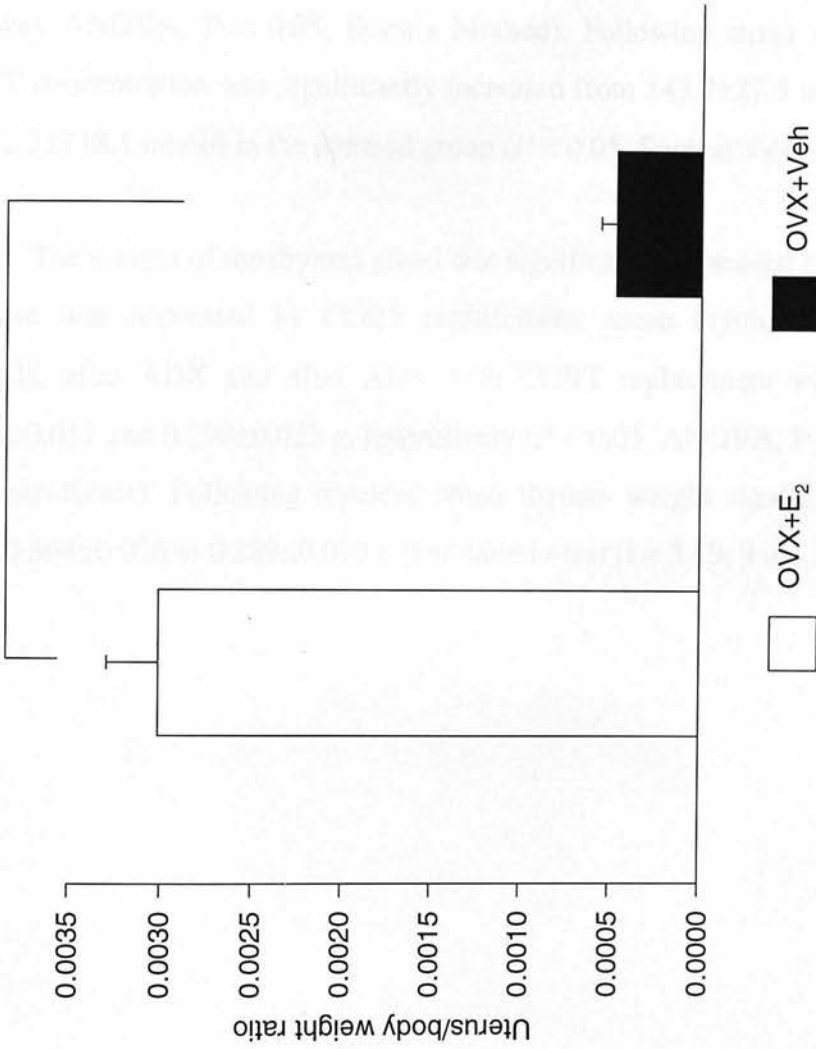
Figure 4.6. Graph demonstrates the uterus per body weight ratio compared between OVX females treated with E₂ and those treated with vehicle over 2 weeks. There was a significant difference in the ratio between two groups. Data were analysed by Student's t-test ($P < 0.0001$). Asterisk indicates statistical significance between groups, n=6/group.

4.4.2. Regulation of ER-α and -β mRNAs in the hippocampus by adrenal steroid manipulation

4.4.2.1. Plasma corticosterone concentration and thymus gland weight

The dose of CORT used increased plasma CORT concentration from 26.6±16.3 nmol/l after adrenalectomy to 2082±434 nmol/l; in sham-operated controls, plasma CORT concentration was 440±67 nmol/l ($P < 0.005$, Kruskal-Wallis one-way ANOVA, $P < 0.05$, Dunn's Method). Following stress exposure plasma CORT concentration was significantly increased from 143.7±27.5 nmol/l in controls to 452.2±118.1 nmol/l in the stressed group ($P < 0.05$, Student's t-test).

The weight of the thymus gland was significantly increased by ADX, and this increase was prevented by CORT replacement; mean thymus weight±s.e.m. in controls, after ADX and after ADX with CORT replacement was 0.325±0.023, 0.398±0.017 and 0.298±0.023 g, respectively ($P < 0.05$, ANOVA, $P < 0.05$, Student-Newman-Keuls). Following repeated stress thymus weight significantly decreased from 0.364±0.026 to 0.289±0.019 g (one-tailed t-test [t = 2.19, 9 df], $P < 0.05$).

4.4.2.2. Effect of adrenalectomy and corticosterone replacement

Seventy two h after bilateral adrenalectomy (ADX) there was no significant change in the expression of either ER-α or -β mRNA in any subfield of the hippocampus compared to those from sham-operated and ADX with CORT replacement groups. The exception was in the CA1 region where ER-β mRNA was significantly decreased following ADX (by 34.5%) and in which the basal expression was also restored by CORT replacement ($P = 0.022$, ANOVA, $P < 0.05$ Student-Newman-Keuls) (Figure 4.7.).

The expression of CRF mRNA in the dorsomedial parvocellular PVN neurones showed a substantial increase 72 h following ADX, while the expression significantly returned to the basal level upon CORT replacement ($P = 0.0148$, Kruskal-Wallis one-way ANOVA, $P < 0.05$ Dunn's Method) (Figure 4.8A.). The data were not normally distributed so non-parametric statistics were used.

4.4.2.3. Effect of stress exposure

There was no change in the expression of either ER-α or -β mRNA following 72 h of stress exposure in any subfield of the hippocampus (Figure 4.9.) and the expression of CRF mRNA in the medial parvocellular PVN was not evidently increased following stress exposure either (Figure 4.8B.).
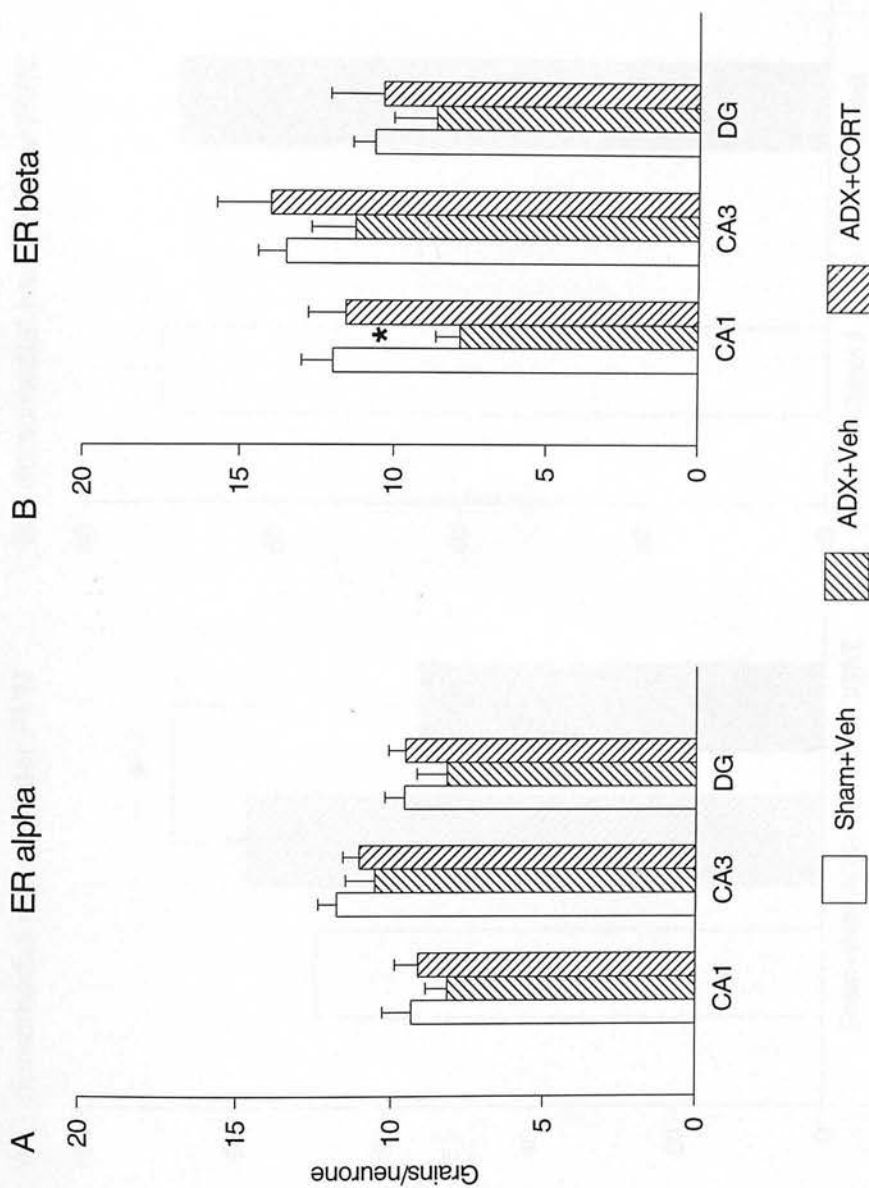
Figure 4.7. Effects of adrenal steroid manipulation on the expression of ER-α and -β mRNA expression in the CA1, CA3 and DG subfields of the hippocampus in male rats. Bilateral adrenalectomy (ADX) resulted in a significant reduction of ER-β mRNA in the CA1 subfield which was prevented by corticosterone (CORT) replacement (10 mg/kg s.c. injection at 4 p.m. daily for 72 h). There was no change in the expression of ER-α mRNA following (ADX) in any subfield of the hippocampus. Data were analysed by one-way ANOVA, followed by Student-Newman-Keuls post-hoc comparison ($P < 0.05$). Asterisk indicates statistical significance between groups, n=5/group.
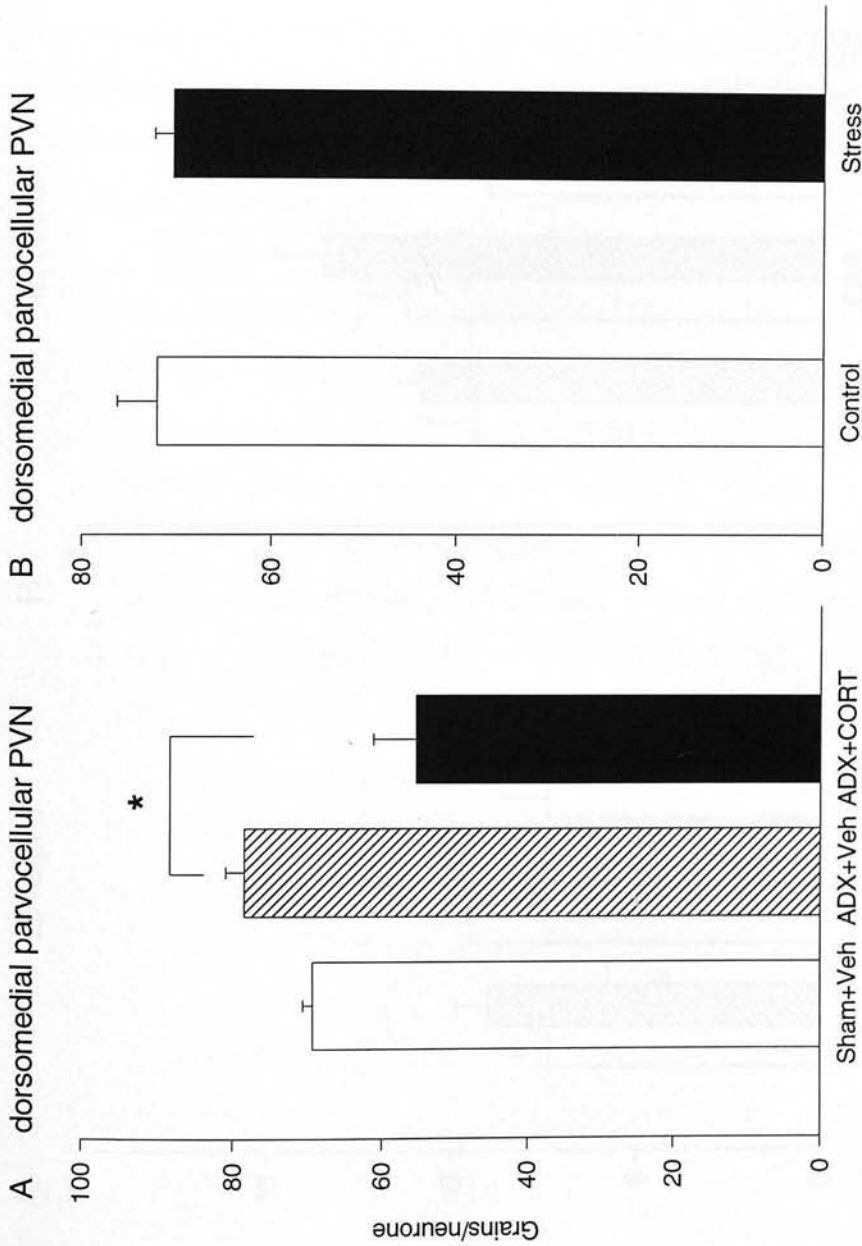
Figure 4.8. Effects of adrenal steroid manipulation on the expression of CRF mRNA in the dorsomedial parvocellular PVN in male rats. The expression of CRF mRNA was increased by ADX while corticosterone (CORT) replacement (10 mg/kg s.c. injection at 4 p.m. daily for 72 h) restored the basal expression in adrenalectomised (ADX) rats (A). The expression of CRF mRNA here in rats subjected to a cycle of various stress paradigms was not statistically significantly different from that in the controls (B). Data were analysed by Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method post-hoc (A, $P < 0.05$) or Student's t-test (B). Asterisk indicates statistical significance between groups, n=5/group.
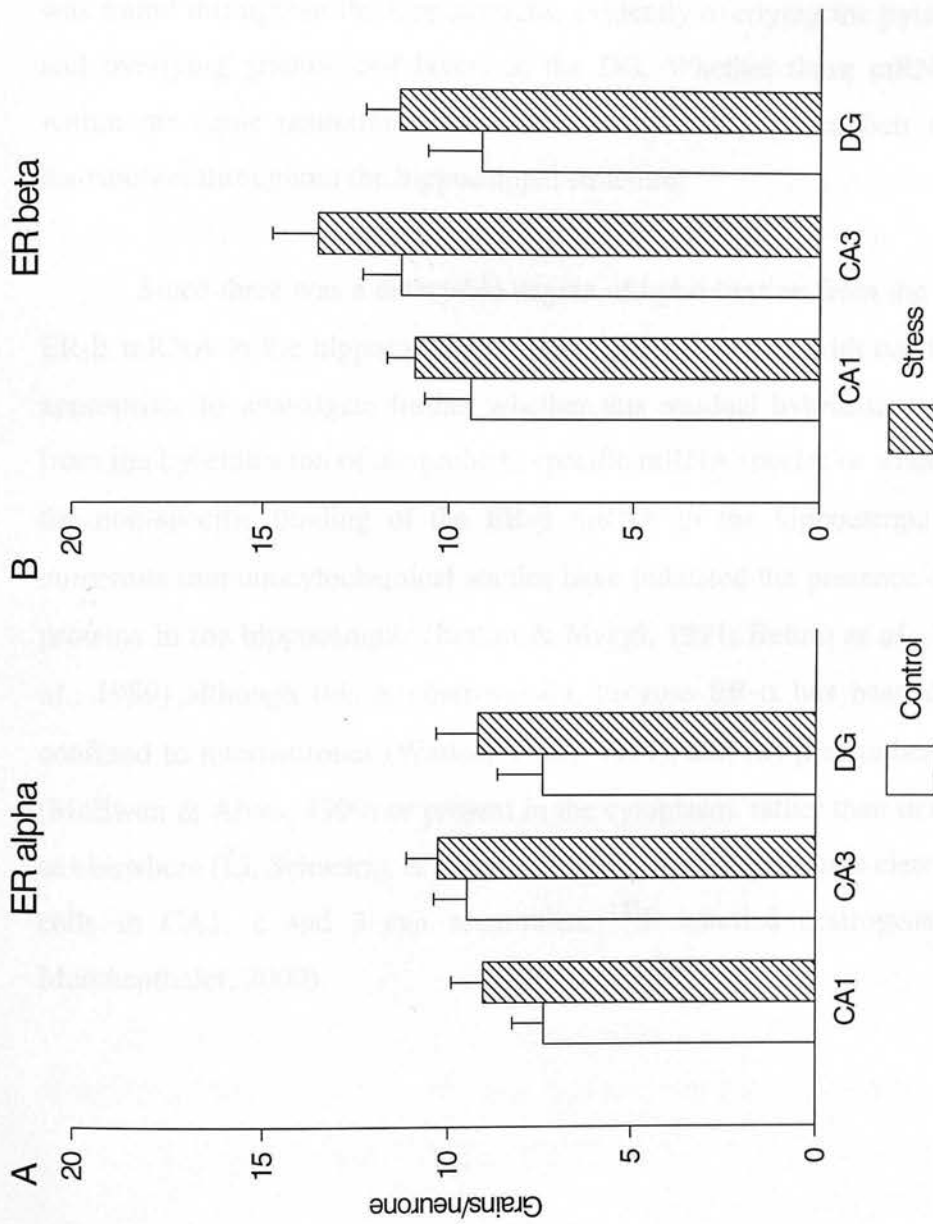
Figure 4.9. Effects of repeated stress (cold water swim, halothane, restraint tube exposure) on the expression of ER-α and -β mRNA in the CA1, CA3 and DG subfields of the hippocampus in male rats over 72 h. The expression of neither mRNA transcript from rats subjected to a cycle of various stress paradigms was statistically significantly different from that in the controls. Data were analysed by Student's t-test, n=5/group.

4.5.   Discussion

The distribution of ER-α and -β mRNA in the hippocampus has been previously described by others (Laflamme *et al.*, 1998; Osterlund *et al.*, 1998; Register, Shively, & Lewis, 1998; Shughrue, Lane, & Merchenthaler, 1997a; Simerly *et al.*, 1990). In the present study, specific expression of both ER-α and -β mRNAs was found throughout the hippocampus, evidently overlying the pyramidal neurones and overlying granule cell layers in the DG. Whether these mRNAs are present within the same neurones is unknown, but is likely given their near ubiquitous distribution throughout the hippocampal structure.

Since there was a detectable degree of hybridisation from the sense probe for ER-β mRNA in the hippocampus, results must be viewed with caution. It might be appropriate to investigate further whether this residual hybridisation signal derives from the hybridisation of the probe to specific mRNA species or whether this reflects the non-specific binding of the ER-β mRNA in the hippocampus. Nonetheless, numerous immunocytochemical studies have indicated the presence of ER-α and -β proteins in the hippocampus (Bettini & Maggi, 1991; Bettini *et al.*, 1992; Maggi *et al.*, 1989) although this is controversial, because ER-α has been described to be confined to interneurones (Weiland *et al.*, 1997), and ER-β as either non-detectable (McEwen & Alves, 1999) or present in the cytoplasm, rather than in neuronal nuclei as elsewhere (Li, Schwartz, & Rissman, 1997). Nonetheless, it is clear that pyramidal cells in CA1, 2 and 3 can accumulate [125]I- labelled oestrogens (Shughrue & Merchenthaler, 2000).

4.5.1. Regulation of ER mRNAs in the hippocampus by sex steroid manipulation

Changes in the expression of both ER-α and -β mRNAs that we observed within the hippocampus as a result of gonadectomy suggest that these transcripts can be regulated by gonadal hormones. The increase in the level of both ER mRNAs that occurred 2 weeks after the surgery and not at 72 h reveals the prolonged time course of the regulation of ER mRNA in the hippocampus, suggesting that the direction of hormonal regulation of such transcripts is according to a time-dependent manner. It is unlikely that a lack of response of ER mRNAs at 72 h after GDX was due to insensitivity of the method, as changes were found in ER-α mRNA expression in the medial preoptic area (mPOA). Castration for 72 h has been previously shown to induce a 2-fold increase in ER-α mRNA expression in the mPOA (Lisciotto & Morrell, 1993). In this study we were able to measure a comparable (1.5-fold) induction of ER-α mRNA expression in this region after CX (see Figure 4.4.) indicating the effectiveness of the surgery.

Secondly the increase in ER-α mRNA expression that was found in the DG and that of ER-β mRNA in CA1 and the DG indicates a region-specificity of the regulation of ER mRNAs. Moreover, the increased expression in the present study after gonadectomy was observed in females (OVX) but not in (castrated) males (CX), suggesting that the regulation of ER mRNA is sexually dimorphic. Additionally there was also a sex difference in the basal expression of ER-α mRNA in the hippocampus, although this was found only in the 2 week gonadectomy experiment but not in the 72h experiment. This apparent discrepancy might have stemmed from the difference in the endogenous level of sex steroid hormone in the female rats which fluctuates cyclically thus contributing to the difference in the basal expression of the ER-α mRNA from one experiment to the other.

Tight regulation of the expression of steroid hormone receptors will ensure that the appropriate amount of the receptor is available at the appropriate time in the correct cells to achieve the maximal responses of the target tissue.

Numerous previous studies have shown that region-specific, dose-dependent and, to a lesser extent, sex-specific regulation is a common feature of steroid receptors, including ER. For instance, Shughrue *et al.* 1992 have shown that the expression of ER-α mRNA within the hypothalamus is quantitatively different among many nuclei and varies across the 4-day oestrous cycle according to the fluctuating level of E_2. Furthermore, they demonstrated that the expression of ER-α transcript was significantly elevated following OVX (Shughrue, Bushnell, & Dorsa, 1992). Perhaps analogous to the present ISH study, Lauber *et al.* 1991 showed that the expression of ER-α mRNA in the ventromedial and arcuate nuclei in OVX rats was decreased by E_2 treatment in a dose-dependent manner. Moreover, they also showed that there was a sex difference in the basal level of ER-α mRNA between OVX and CX rats in the two nuclei and that the level of ER-α transcript in CX rats was maintained throughout the treatment suggesting that E_2 did not down-regulate the ER-α transcript in CX rats (Lauber *et al.*, 1991). As well as ER-α mRNA, ER-β mRNA has been shown to be modulated in a region specific and dose-dependent manner. In a recent ISH study by Osterlund *et al.* 1998 both transcripts have been shown to respond to E_2 treatment when OVX rats were given high dose of E_2 (170 µg) but a low dose of E_2 (17 µg) was largely ineffective. With a high dose of E_2 the expression of ER-β mRNA was reduced in the medial amygdala, the anterodorsal (MeAD) and posterodorsal (MePD) nuclei while the increase was observed in the arcuate nucleus. With a low dose of E_2, however, the expression of the ER-β transcript was only reduced in the MeAD but not anywhere else (Osterlund *et al.*, 1998).

The molecular mechanism underlying the complex regulation of ER mRNA expression is not fully understood. Several previous studies indicate a tissue-specific alternative promoter usage as a mechanism in the regulation of ER gene expression which might partly explain the differential responses observed in a variety of tissues (see Chapter 1). The region-specific pattern of changes in ER mRNA in response to GDX within various subfields of the hippocampus in our study suggests that the regulation of these transcripts might also be under the influence of different

promoter-usage. Such a mechanism has recently been demonstrated for the GR in the hippocampus (McCormick *et al.*, 2000).

Although this present study does not show that the increase in the receptor mRNA following gonadectomy would necessarily result in an increase in the receptor protein, previous reports have demonstrated that oestrogen receptor protein in certain areas of the brain, including the hippocampus, is regulated by circulating sex steroids. For instance, a previous study has shown that oestradiol benzoate administered to OVX rats led to a decrease in the concentration of ER protein by up to 50% in the hippocampus (Maggi *et al.*, 1989). This indicates that ER protein is physiologically functional and can be regulated in the hippocampus. It also indirectly implies that the increase in ER mRNA observed in our study might precede an increase in the actual receptor protein. These findings reinforce our present study and suggest that oestrogen has the potential to regulate the sensitivity of the hippocampal neurones to hormonal modulation, presumably, via regulation at the receptor level.

It seems an obvious possibility that the up-regulation of ER mRNA observed in this present study was primarily due to the absence of 17-β oestradiol (E_2) as a result of gonadectomy. Autoregulation of receptor mRNAs and/or protein by their cognate ligands, a process known as *homologous regulation*, has been established for many steroid hormone receptors, including ER. Such a mechanism involves partly a regulation of rate of gene transcription or an altered receptor mRNA stability, as well as some other possibilities as previously discussed (see Chapter 1 for more detail). The presence of an ERE in the promoter region of ER-α such as in the rainbow trout suggests a possibility of direct autoregulation of ER by its own ligand (Le Dréan *et al.*, 1995).

However in the study to test this, whereby E_2 was replaced in the OVX rats for 2 weeks, the level of the ER mRNAs did not significantly differ from that in OVX females treated with vehicle. The effectiveness of E_2 replacement was validated by determining the effects on uterine weight (Figure 4.6.). Thus the lack of

response of ER mRNA upon E_2 replacement was likely to be due to factors other than inadequate E_2 treatment. This indicates that E_2 removal was not the only factor responsible for the increase in ER mRNA expression after OVX. This present finding suggests that ovariectomy may have removed additional ovarian factors that modulate ER expression and biosynthesis. One such candidate might be progesterone (P_4). In a breast cancer cell line down-regulation of ER-α expression could be induced by P_4 *in vitro* (Read, Greene, & Katzenellenbogen, 1989). *In vivo*, P_4 has been shown to attenuate ER-α synthesis in rodent brain (Blaustein & Brown, 1984; Kato & Onouchi, 1984). Thus removing the ovaries would inevitably result in the loss of the endogenous source of P_4. However, P_4 is only transiently secreted in the normal rat oestrous cycle, and initial comparisons in the present study were made between OVX and intact rats at random stages of the cycle. For this reason, it was not considered appropriate to test P_4 replacement. Whether P_4 modulates the ER expression in the hippocampus is presently unknown.

It is perhaps possible that a sustained high circulating concentration of E_2, as indicated by the oestrus-state of the uteri post mortem in the present study, has different actions on ER mRNA expression from the intermittent secretion during the oestrous cycle. For ER-α, opposite effects of E_2 on expression are seen between the uterus and liver (Koritnik, Koshy, & Hoversland, 1995; Shupnik, Gordon, & Chin, 1989), reflecting the complex regulation of ER expression. On the other hand, it may be possible that a lower dose of E_2 might have been effective; perhaps the dose-response relationship is U-shaped? This was, however, not explored in the present study. Nonetheless, previous studies have shown that, at the behavioural level, hormone replacement with relatively high E_2 (mimicking pro-oestrus level) were ineffective (Luine & Rodriguez, 1994) while comparatively low E_2 (producing di-oestrus/early pro-oestrus concentration) did enhance cognitive performance in OVX rats (Luine *et al.*, 1998).

Although the notion of sex difference in the CNS has been mainly directed towards structures involved in reproduction, accumulating evidence over the past several years suggests that perhaps the hippocampus is also one of the structures in the brain which shows (subtle) sex differences. Synaptogenesis on excitatory spines in the hippocampal CA1 pyramidal neurones fluctuates cyclically in parallel with the natural ovarian cycle. This effect was only observed in female but not in male rats (Gould *et al.*, 1990a; Lewis, McEwen, & Frankfurt, 1995; Woolley & McEwen, 1992). The sex difference in the regulation of ER-α and -β mRNA in the DG may underlie the sex difference in the reactive outgrowth of the commissural afferent fibers in the DG. Morse *et al.* 1986 have shown that removal of the entorhinal cortex results in less fiber outgrowth in the DG which was observed only in female rats. Upon hormone replacement therapy the outgrowth was brought back to the control levels again only in females, whereas fiber outgrowth in males was unaffected (Morse, Scheff, & DeKosky, 1986). Additionally, male rats have also been shown to have a larger and less lateral (more asymmetric) DG than females, and neonatal testosterone treatment induced the genetically female DG to appear like that of male (Roof & Havens, 1992).

Sexual dimorphism can also be demonstrated at the behavioural level, particularly with regard to spatial ability as well as the strategies used in spatial learning between sexes (Beatty, 1984; Einon, 1980; Williams, Barnett, & Meck, 1990; Williams & Meck, 1991). For instance, Roof and Havens 1992 showed that the performance of adult male and female rats in a Morris water maze task shows a sex difference with male rats learning the task more rapidly than females (Roof & Havens, 1992). In humans it has been repeatedly demonstrated that males score better on certain types of spatial abilities while females excel on others (Caplan, MacPherson, & Tobin, 1985; Postma, Izendoorn, & De Haan, 1998; Postma *et al.*, 1999). All this evidence supports the notion that there is a sexual dimorphism between the sexes regarding learning and memory and that the underlying neural mechanism might perhaps stem from the sex difference in the hippocampal

morphology and physiology which is brought about partly by effects of gonadal hormones.

The underlying mechanism by which sex steroids mediate their effects in the hippocampus is perhaps via morphological alterations in the hippocampal neurones. Numerous studies suggest that E_2 regulates hippocampal dendritic spines and synapses, an action which appears to involve NMDA receptor activation. Removal of endogenous sources of ovarian hormones results in a reduction in the number and density of dendritic spines as well as synapses in the apical dendrites of hippocampal CA1 pyramidal neurones (Gould et al., 1990a; Woolley & McEwen, 1992; Woolley & McEwen, 1993; Woolley et al., 1997). This decrease can be prevented or reversed by treatment with E_2 (Gould et al., 1990a). Moreover, cyclic synaptic turnover in the hippocampus displays a remarkable plasticity as well as a high degree of specificity. Oestrogen-induced synaptogenesis occurs on dendritic spines and not on dendritic shafts and such synaptogenesis fluctuates significantly within a short period of the natural ovarian cycle. Surprisingly, such synaptic plasticity is specific only to CA1 and does not occur on CA3 pyramidal neurones or dentate granule neurones (Woolley et al., 1990a). Perhaps these synapses may be involved in learning and memory processes as reflected by long-term potentiation (LTP), the sensitivity of which correlates with excitatory synapse density and E_2 level (Warren et al., 1995).

Non-conventionally, E_2 has been shown to regulate hippocampal neuronal excitability through a non-genomic mechanism in which it rapidly potentiates glutamate receptor-mediated excitatory post-synaptic potentials (EPSPs) (Wong & Moss, 1992) and excitatory post-synaptic currents (Gu & Moss, 1996) on hippocampal CA1 pyramidal neurones. These rapid effects (occurring within minutes) have been proposed to involve activation of intracellular second-messenger coupling systems (Gu & Moss, 1996; Moss, Gu, & Wong, 1997). These actions, however, may involve ER expressed in the cell membrane.

4.5.2. Regulation of ER mRNAs in the hippocampus by adrenal steroid manipulation

In the study involving adrenal steroid hormone manipulation, the expression of ER-α mRNA remained stable through the different treatments. The only change in ER-β mRNA expression that was found was a decrease in the CA1 subfield after ADX, which was prevented by glucocorticoid replacement. Repeated stress did not affect expression of either transcript in intact rats. Thus basal glucocorticoid secretion maintains ER-β mRNA expression in the CA1 subfield.

Replacement of CORT in the present study was sufficient to fully reverse both the increases in CRF mRNA expression in the dorsomedial parvocellular PVN neurones (Figure 4.9.) and the increase in thymus weight following ADX. Although there was no significant increase in the expression of CRF mRNA following stress exposure, CORT secretion was nonetheless significantly increased in the stressed groups. Several studies have demonstrated that exposure of rats to either repeated (daily but intermittently) or chronic (continuous) stress causes elevations in plasma CORT and changes in CORT-sensitive tissues, such as decreased thymus gland weight (Hauger *et al.*, 1988; Kiss & Aguilera, 1993). All these parameters are indicative of chronic activation of the HPA axis. Numerous studies have demonstrated that chronic stress increases CRF mRNA expression in the parvocellular PVN (Kiss & Aguilera, 1993; Makino, Smith, & Gold, 1995; Mamalaki *et al.*, 1992). However, other studies have failed to find any up-regulation (Harbuz *et al.*, 1992). Thus the sensitivity of CRF expression towards stress exposure appears to be dependent upon the type of stress used in the study.

The hippocampus, including CA1 neurones in particular, express a high level of GR (and MR), so it is possible that CORT positively regulates ER-β expression directly through GR and/or MR in the same neurones. Previously, glucocorticoids with growth hormone, or thyroid hormone have been shown to stimulate ER-α expression in hepatocytes (Stavreus-Evers, Freyschuss, & Eriksson, 1997). There are

other examples of regulation of expression of members of the steroid/thyroid superfamily by one another as previously discussed. Oestrogen has been shown to decrease GR and MR expression in hippocampal neurones (Burgess & Handa, 1993). The role of ER-β in mediating these effects of sex steroids on GR and MR expression is unknown but subtle regulation might be possible. The sensitivity to glucocorticoids of ER-β expression in the CA1 subfield is particularly interesting since this subfield is where oestradiol induces increased dendritic spine density, synapses, and NMDA binding sites on pyramidal neurones, and enhances LTP (Warren *et al.*, 1995; Weiland *et al.*, 1997; Woolley *et al.*, 1997). In rats, E_2 treatment enhances retention in a spatial discrimination task, whether given systemically or into the hippocampus, with effects in both males and females, and evidently involving cholinergic mechanisms (Packard, 1998; Simpkins *et al.*, 1997). It is not yet known whether adrenalectomy affects these actions of E_2.

The present study indicates that effectiveness of ER-β ligands on CA1 neurones may be reduced in the absence of glucocorticoids. However, the results also indicate that in the presence of stress levels of glucocorticoids there may be no change in effectiveness of oestrogens as a result of changes in ER expression. The lack of changes in ER-α or -β mRNA expression in the CA3 subfield indicates that actions of oestrogen here are unaffected by changes in corticosterone exposure, perhaps because GR expression is low; notably, this subfield is where stress and corticosteroids cause atrophy of dendrites (McEwen, 1999). Similarly, in the DG, where E_2 stimulates neurogenesis (Tanapat *et al.*, 1999), the present study indicates that E_2 action will not be affected by glucocorticoid effects on ER-α or -β mRNA expression, as these were not found.

From the present finding it is inferred that actions of E_2 are likely to be enhanced by stimulation of ER-β mRNA expression by basal, non-stress, secretion of glucocorticoids. Such an action of basal secretion of glucocorticoids may be expected to be through MR since they have a higher affinity for corticosterone, and are thus the predominant mediator of glucocorticoid action at basal levels (Reul, van

den Bosch, & de Kloet, 1987). This facilitation of oestrogen action through maintenance of ER-β expression could contribute to the known positive effects of low levels of glucocorticoids on cognitive aspects of hippocampal function (Landfield, Baskin, & Pitler, 1981).

Our present finding further suggests that any modulatory effects of oestrogen upon the HPA axis during stress exposure is unlikely to be mediated via ER in the hippocampus. Recent studies have shown that many of the components involved in the regulation of the HPA activity are modulated by sex steroid hormones. The synthesis of the ACTH secretagogues, CRF and VP, in the PVN is influenced by gonadal steroids (Almeida et al., 1992; Bohler et al., 1990; Greer et al., 1986; Haas & George, 1988; Vamvakopoulos & Chrousos, 1993). Likewise the synthesis and binding properties of corticosteroid hormone receptors in brain regions regulating the HPA axis have been shown to be modulated by sex steroids (Carey et al., 1995; Patchev & Almeida, 1996; Viau & Meaney, 1991). Indeed, the gene encoding CRF has been identified to contain the ERE (Vamvakopoulos & Chrousos, 1993), providing the evidence for direct genomic regulation of CRF by E_2. Our current finding that there is no change in ER-α or -β mRNA expression following stress exposure in the hippocampus perhaps is not surprising but reinstates the notion that E_2 exerts a variety of actions in different ways at distinct loci.

Conclusion

The expression of ER-α mRNA in the DG, and ER-β mRNA in the CA1 and the DG increase over a prolonged period after OVX, but do not change in castrated males; chronic E_2 replacement did not reduce ER expression after OVX. The expression of ER-β mRNA in the CA1 subfield of the hippocampus is positively regulated by basal glucocorticoid secretion, but does not respond to the increased corticosterone secretion of chronic stress. ER-α mRNA expression in the hippocampus was not affected by adrenal corticosteroid manipulation.

The present finding in this chapter shows that ER mRNAs are present in loci not traditionally involved in aspects of reproduction and are regulated in a region-, time- and sexually dimorphic pattern; how these steroid hormone transcripts are regulated is not fully understood, however. Recent studies indicate that E_2 enhances performances of learning and memory tasks both in animal models and in humans (for review see Luine, 1997). These sex steroid hormones appear to exert a physiological influence upon learning and memory since differences in aspects of cognition have been demonstrated over the course of the natural menstrual cycle as well as during the peri- and post-menopausal periods (Hampson, 1990; Phillips & Sherwin, 1992b). However it is not clear which ovarian hormone(s) – E_2 or P_4 or, indeed, both are responsible, although replacement studies in women suggest that E_2 may be perhaps most important in maintaining and restoring cognitive functions (Phillips & Sherwin, 1992a; Sherwin, 1994). The influence of gonadal hormones on memory processes appears to involve actions on brain structures such as the hippocampus and basal forebrain. The hippocampus is known to play an essential role in memory (Morris et al., 1982; O'Keefe & Nadel, 1978; Squire, 1992) and in particular spatial memory (Olton & Papas, 1979; Olton, Walker, & Gage, 1978). Ovarian steroid deprivation resulted in a learning impairment which was reversed upon steroid replacement (Singh et al., 1994). This present study suggests a possibility of direct action of sex steroids upon the hippocampus.

It is not known whether actions of E_2 on the hippocampus are mediated by ER-α or -β or indeed by both (Pettersson *et al.*, 1997). The detection of both ER-α and -β mRNA and of changes with both transcripts in the hippocampus, however, suggests that oestrogens might predominantly confer their activity via both receptor subtypes in a synergistic manner. A consequence of the pattern and dynamics of ER mRNA expression seen in the hippocampus following chronic withdrawal of ovarian hormones, is that, if translated into function receptor protein, the sensitivity of neurones in CA1 or the dentate gyrus to E_2 action will be increased in the absence of the ligand.

The implications for the effects on hippocampal function of continuous oestrogen treatment therapy in post-menopausal women are that ER-α and -β expression will not be reduced, and thus the sensitivity to E_2 will be retained. Indeed, prolonged withdrawal of E_2 may increase expression of both ER-α and -β, increasing sensitivity to E_2 with the replacement therapy. This perhaps explains the mechanism of how benefits of HRT is brought about in post-menopausal women. Indeed, in a recent study by Luine *et al.* 1998 chronic E_2 treatment for 12 d, but not 3 d, facilitated spatial memory, as assessed by choice accuracy on a radial arm maze, in OVX rats, suggesting enhancing effects of E_2 on learning and memory over a relatively prolonged period (Luine *et al.*, 1998).

The presence of both gonadal and adrenal steroid receptors in the hippocampus as well as the susceptibility of this structure to actions of both types of steroid (see Luine, 1997), despite being in rather opposing directions, suggests a possibility of mutual regulation of both molecules. The results imply that maintenance of ER-β mRNA expression in CA1 by basal glucocorticoid secretion may ensure E_2 action here alongside glucocorticoid action, while E_2 replacement after ovarian failure is expected to be able to act on a still E_2-sensitive hippocampus.

By studying the expression of ER in a variety of tissues including the hippocampus it is hoped that one could perhaps devise an oestrogenic molecule that

specifically targets one organ while leaving the others unaffected. The benefits would be paramount if such moiety is available, particularly those counteracting the cognitive decline; the process, inevitably, accompanies human ageing.

Chapter 5

Regulation of oestrogen receptor-β mRNA in the hypothalamic paraventricular and supraoptic nuclei

5.1. Introduction

The hypothalamus is considered important in organising the neural and neuroendocrine defence of homeostasis. The paired SON and PVN nuclei are particularly important in the control of endocrine and autonomic responses. Most of the OXT and VP magnocellular neurones project their axons through the pituitary stalk and terminate on capillaries in the neurohypophysis where the two peptides are released from their nerve terminals into the systemic circulation (for reviews see Leng, Brown, & Russell, 1999; Swanson & Sawchenko, 1983). In the periphery, VP is involved in maintaining osmotic balance by promoting reabsorption of water in the collecting ducts of the renal tubules and in maintaining blood pressure through vasoconstriction (Hatton & Li, 1998). Its synthesis and secretion is, in turn, controlled by plasma osmolarity as well as by osmotic blood pressure and volume (Hatton & Li, 1998). On the other hand, the principal functions of OXT secreted by the neurohypophysis are related to reproduction: it is essential for milk ejection, and important in promoting established parturition (Russell & Leng, 1998). In rats, OXT is also natriuretic, involving a stimulatory action on atrial natriuretic peptide secretion (Jankowski *et al.*, 1998) and thus co-ordinates with VP in maintaining body fluid osmolarity. Its synthesis has also been shown to respond to changes in plasma osmolarity (Hatton, 1990).

The ovarian steroid oestrogen has long been recognised as a modulator of the neurosecretory system influencing the OXT and VP neurones. Oestradiol, 17-β (E_2) has been shown to stimulate the release of both OXT and VP secretion from the

magnocellular neurones (Skowsky, Swan, & Smith, 1979; Yamaguchi, Akaishi, & Negoro, 1979). Systemically administered radiolabelled-oestrogen can be detected in OXT (Rhodes, Morrell, & Pfaff, 1982) as well as VP neurones (Sar & Stumpf, 1980). Administration of E_2 stimulates the peripheral release of OT immunoreactivity (Amico, Seif, & Robinson, 1981). Changes in OXT mRNA in the SON vary across the oestrous cycle with a marked increase in expression during oestrus (Van Tol et al., 1988). At the end of pregnancy, the decline in progesterone secretion, and relative increase in E_2 secretion has been reported to result in increased OXT gene expression in the magnocellular neurones (Van Tol et al., 1988; Zingg & Lefebvre, 1988; Zingg & Lefebvre, 1989). In vitro E_2 has been shown to induce OXT gene transcription (Burbach et al., 1990). Conversely, many previous reports have failed to find changes in OXT or VP gene expression in response to sex steroid hormones manipulation. For instance it has been previously reported that gonadectomy (both ovariectomy (OVX) in females and castration (CX) in males) alters neither the expression of VP/OXT mRNA (Carter, Pardy, & Murphy, 1993; Crowley & Amico, 1993) nor OXT pituitary contents (Crowley & Amico, 1993). Roy et al. 1999 also reported that treatment with E_2 in OVX rhesus monkeys failed to modulate the expression of VP mRNA in the magnocellular neurones (Roy, Reid, & Van Vugt, 1999).

How oestrogen exerts its effects upon neurones in the SON and PVN remains unclear since these neurones lack the classical oestrogen receptor (ER-α, (Corodimas & Morrell, 1990), although these neurones have been shown to concentrate $[^3H]$-E_2 in in vivo binding studies (Corodimas & Morrell, 1990; Rhodes et al., 1982). Recently, Kuiper et al. 1996 cloned a novel oestrogen receptor, designated ER-β, from the rat prostate cDNA library (Kuiper et al., 1996). This receptor shares a considerable degree of homology and shows an overlapping but non-identical tissue distribution with ER-α, particularly within the central nervous system (see Chapter 3). A striking difference is that while the SON and PVN show a complete absence of ER-α, the PVN in particular expresses a high level of ER-β (both mRNA and protein), and a lesser degree of expression is found in the SON. A substantial number

of studies have shown co-localisation of ER-β-like-immunoreactivity (-ir) or mRNA within the OXT- and VP-immunopositive neurones in the SON and PVN (Alves *et al.*, 1998; Hrabovszky *et al.*, 1998; Li, Schwartz, & Rissman, 1997; Shughrue, Komm, & Merchenthaler, 1996). Moreover, the up-stream regulatory elements of the OXT and VP genes contain sequences similar to an oestrogen-response element (ERE) (Adan *et al.*, 1993; Mohr & Schmitz, 1991; Richard & Zingg, 1990 for OXT and Watters, Poulin, & Dorsa, 1998 for VP), suggesting a possible direct genomic action of E_2 upon the OXT and VP genes, perhaps via the ER-β subtype. Collectively these findings indicate a possibility that neurones in the SON and PVN can be regulated by E_2.

5.2. Aims

Much information on the influence of sex steroid hormones upon the hypothalamic SON and PVN is available but the mechanism mediating these effects remains largely unknown. Since the effects of the hormones are in large part mediated by the receptors, any changes in their expression would alter the responsiveness of the cells in target organs, and might help indicate the circumstances in which this factor is important. Because of the prominent expression of ER-β mRNA in the SON and PVN neurones we proposed to investigate *in vivo* regulation of the transcript by sex steroid hormones and its expression under conditions known to alter the activity of the magnocellular neurones. In particular, we also determined the expression of ER-β mRNA in the ventral group of parvocellular, predominantly oxytocin, neurones in the PVN that project to the brainstem and spinal cord, and are involved in autonomic regulation (Sawchenko & Swanson, 1982). These neurones express ER-β mRNA more strongly than other neurones observed in the study.

5.3.   Methods

We employed both steroid hormone manipulations as well as physiological conditions known to stimulate neurones in the SON and PVN to determine whether and how ER-β mRNA might be regulated in these neurones, using quantitative ISH as a measure of expression.

5.3.1. Regulation of ER-β mRNA in the hypothalamic SON and PVN by sex steroid manipulation

As previously reported, steroid hormone receptors, including ER-β, can be regulated by their own ligands. However the relatively recent discovery of ER-β has not as yet led to many detailed studies on the regulation of its mRNA transcript in the magnocellular neurones, especially in *in vivo* models. A recent study by Patisaul *et al.* 1999 has shown that ER-β mRNA expression in the PVN in ovariectomised rats is decreased by E_2 treatment, but increased by phyto-oestrogen ingestion, demonstrating the plasticity of ER-β expression in these neurones (Patisaul, Whitten, & Young, 1999).

5.3.1.1. Effect of gonadectomy

To assess whether ER-β mRNA can be regulated by sex steroids, in the first experiment, the endogenous source of sex hormones from both male and female rats was removed by bilateral gonadectomy and left to recover for 72 h prior to decapitation. These were the same rats as those used in Chapter 4, section 4.3.1.1.

5.3.1.2. <u>Effects of exogenous 17β-oestradiol (E$_2$)</u>

5.3.1.2.a. <u>Effect of acute E$_2$ treatment in intact male rats</u>

One group of adult male Hooded-Lister rats (n=5/group) was injected with E$_2$, 100 μg/kg subcutaneously daily for 72 h. Another group of male and another group of random cycling female rats were injected with vehicle as controls over the same period of time. Rats were left for 72 h prior to decapitation.

5.3.1.2.b. <u>Effect of chronic E$_2$ treatment in ovariectomised rats</u>

Two groups of adult random cycling female Hooded-Lister rats (n=6/group) underwent bilateral ovariectomy (OVX) under halothane anaesthesia. While still anaesthetised, one group of rats had a subcutaneous implant of a silastic capsule containing 15 mg/ml of E$_2$ in oil. The other group had a vehicle-containing implant as a control. These were the same rats as those used in Chapter 4, section 4.3.1.2.

5.3.1.3. <u>Expression of ER-β mRNA in the hypothalamic PVN and SON during pregnancy and parturition</u>

The role of OXT in parturition is well established. In rats, the store of OXT peptide in the posterior pituitary is substantially increased so that by the end of pregnancy *ca.* 30% excess of OXT is accumulated, most of which would be depleted during the ensuing parturition (Leng *et al.*, 1988). Thus the oxytocinergic magnocellular neurones are prepared during pregnancy to switch from basal activity to the maximal activation during the 60-90 min of parturition. Although not all, a number of previous studies indicate that magnocellular OXT mRNA is increased in pregnancy, particularly immediately prepartum (12-24 h) (Crowley *et al.*, 1993; Horwitz *et al.*, 1994; Van Tol *et al.*, 1988; Zingg & Lefebvre, 1988; Zingg & Lefebvre, 1989) suggesting that the OXT gene has been turned on. This increased expression of the OXT gene occurs in association with pronounced changes in the

secretion of E_2 which rises throughout pregnancy and reaches a peak at term while progesterone (P_4) secretion declines precipitously 24-48 h prior to delivery (Bridges, 1984). Exposure of ovariectomised virgin rats to a hormone regime simulating the profile of E_2/P_4 secretion in pregnancy (Crowley *et al.*, 1995) also results in an increase in OXT mRNA expression in the magnocellular neurones. Given the importance of E_2 priming (with P_4 withdrawal) in regulating the magnocellular OXT gene in late pregnancy, it is possible that E_2 might mediate the induction of the OXT gene, perhaps involving altered expression of the ER-β present in the hypothalamic magnocellular neurones.

Adult female Sprague-Dawley rats were obtained from the supplier (Bantin and Kingman) and allowed to acclimatise to a 12 h light:dark cycle for at least 2 weeks prior to use. They were mated overnight with sexually experienced male rats. The day on which the vaginal plug was present in the morning was designated as day 1 of pregnancy. Pro-oestrus virgins (determined by daily vaginal smears), day 15 and day 22 (parturient, 90 min after birth of the second pup) pregnant rats (n=6/group) were killed by decapitation.

5.3.2. Expression of ER-β mRNA in the hypothalamic SON and PVN during physiological stimulation

5.3.2.1. Effect of adrenal steroid manipulation

Disturbance in hydromineral balance following manipulation of adreno-corticosteroid hormones is known to alter the activity of neural inputs to the magnocellular neurones, and hence their activity. Secondary adrenocortical insufficiency mimics the syndrome of inappropriate secretion of antidiuretic hormone whereby plasma VP is elevated in the presence of hyponatraemia (Oelkers, 1989; Share & Travis, 1970). Thus, in these situations it appears that VP secretion is not down-regulated appropriately to plasma sodium. Replacement of glucocorticoids restores the basal plasma VP concentration (Ahmed *et al.*, 1967; Seif *et al.*, 1978).

On the other hand, elevated glucocorticoid levels inhibit VP release in response to hypoxia (Raff *et al.*, 1984), hypotension (Raff, Skelton, & Cowley, 1990), and osmotic stimulation (Papanek & Raff, 1994b). These findings suggest that glucocorticoids might also control the basal and stimulated VP release from the magnocellular neurones. As far as we are aware, there has been no study to date investigating the effects of gonadal steroids in controlling the VP gene under these conditions. The presence of ER-β within the magnocellular neurones and the known involvement of sex steroid hormones in the hypothalamo-neurohypophysial system has prompted us to look at the expression of ER-β mRNA in magnocellular neurones in states with altered adrenocortical activity.

In this study bilateral ADX was performed which will reduce basal plasma glucocorticoid thus mimicking the condition of adrenal insufficiency. In parallel we also subjected the rats to stress exposure, a condition which is expected to increase plasma glucocorticoid concentration. We elected to use male rats because there are no differences between males and females in patterns of ER-β mRNA distribution in the PVN and SON (see Chapter 3), while variations in E_2 exposure through the oestrous cycle are avoided, and testosterone is a precursor for E_2 in the male brain; the SON contains abundant aromatase enzyme (Sanghera *et al.*, 1991).

5.3.2.1.a. Effect of bilateral adrenalectomy and corticosterone replacement

These were the same rats as those in Chapter 4, section 4.3.2.1. and were treated as follows;

1)   Sham-operated plus subcutaneous vehicle (corn oil) injection

2)   Bilaterally adrenalectomised (ADX) plus subcutaneous vehicle injection, given 0.9% NaCl to drink

3)   ADX plus replacement of corticosterone (CORT) at a dose of 10 mg/kg in oil via subcutaneous injection daily (at 1600 h), given 0.9% NaCl to drink

5.3.2.1.b. Effect of stress exposure

One group of adult male Hooded-Lister rats (n=5/group) was exposed to a cycle of stressors over 72h while the other group of male rats was left undisturbed and served as controls. These were the identical rats as previously used in Chapter 4, section 4.3.2.2.

Rats were killed by decapitation 72 h following surgery or after the beginning of stress exposure. The effectiveness of the treatments was confirmed by measurements of HPA axis parameters as described in Chapter 4.

5.3.2.2. Effect of 3-day chronic salt loading

In rats both VP and OXT neurones are activated by increased extracellular fluid osmolarity, especially as a consequence of increased sodium concentration. The consequence of increased osmolarity is that 3 days of maintaining 2% NaCl solution as a sole source of fluid intake results in a large increase in both VP and OXT mRNA content in the magnocellular SON and PVN, and a depletion of the peptide contents in the neurohypophysis (see below). Thus induction of hyperosmolarity using stimuli such as chronic salt loading provides a useful model to study the regulation of gene expression in the magnocellular neurosecretory system. An *in vivo* study by Crowley *et al.* 1993 showed that an increase in osmolarity failed to induce OXT/VP mRNA accumulation in gonadectomised animals, and that the response can be restored upon sex steroid replacement (Crowley & Amico, 1993). This indicates that circulating gonadal steroid is required to drive these responses to osmotic stimulus. The underlying mechanism in mediating these sex steroid responses seems unclear but might act via the nuclear ER-β present in the magnocellular neurones.

One group of adult Hooded-Lister male rats (n=6/group) was given 2% NaCl solution to drink while the other group continued to receive tap water *ad libitum* to

drink. 72 h later the rats were decapitated. Trunk blood was collected for determining concentrations of plasma Na^+ and K^+.

At the end of each experiment quantitative ISH (described in detail in Chapter 2, section 2.2.2.-2.2.4.) was employed to determine the expression of ER-β mRNA in the SON and PVN. Specifically two neuronal subpopulations within the PVN were quantified; one was the posterior magnocellular neurones, in the lateral zone, projecting to the neurohypophysis and the other the medial parvocellular neurones, in the ventral zone, projecting to the brainstem and spinal cord (referred to, herein, as magnocellular division and ventral parvocellular division of the PVN, respectively). The sub-divisions of the paraventricular nucleus were defined after Swanson and Kuypers (Swanson & Kuypers, 1980). In addition some sections through the dorsomedial parvocellular division of the PVN, from rats in the adrenal steroid manipulation study, were hybridised with a ^{35}S-labelled antisense riboprobe for CRF mRNA. Background measurements were made over adjacent tissue with no evident expressing neurones (dorsal to the SON) and subtracted to obtain the net value of silver grain counts. The slides were coded so that the experimenter was unaware of the treatment of the rats at the time of evaluation. Animal means were calculated for each variable, and these values were used to calculate group means.

In each experiment Student's t-test or one-way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls post-hoc comparison (except for the measurement of CRF mRNA and plasma CORT concentration following ADX where Kruskal-Wallis one-way ANOVA on ranks was applied) were used as appropriate to determine the statistical significance of differences between groups. The alpha value was set at $P < 0.05$. Results are expressed as the group means±sem.

5.4.   Results

Specific hybridisation signal from the ER-β antisense riboprobe in the hypothalamic SON and PVN was clearly evident both at the cellular level (see Figure 3.11 & 3.12.) seen in dipped emulsion autoradiographs as well as at the macroscopic level in film autoradiograms (Figure 5.1A.). Sections that were hybridised with a sense probe for ER-β mRNA revealed no hybridisation signal in the SON or PVN indicating the specificity of the probe and methodology used in the present study (see Chapter 3).

Although the experiments conducted in the present study did not differentiate the expression of ER-β mRNA between the OXT-containing and VP-containing neurones, the expression was nonetheless present throughout the rostro-caudal extent and in both the dorsal (predominantly oxytocin-containing) and ventral (predominantly vasopressin-containing) portions of the SON. The most prominent expression was found in the ventral parvocellular group of neurones in the PVN that express OXT and project to central autonomic sites, as also seen in other studies (Alves *et al.*, 1998; Hrabovszky *et al.*, 1998). There was sparse labelling for ER-β mRNA in the parvocellular dorsomedial zone, containing CRF neurones (some of which also contain VP-producing neurones) projecting to the median eminence. The differential cellular distribution between ER-β and CRF mRNA in the PVN is evident in Figure 5.1A. & 5.1B. (Note that sections were taken from the same brain *ca.* 10 μM apart).
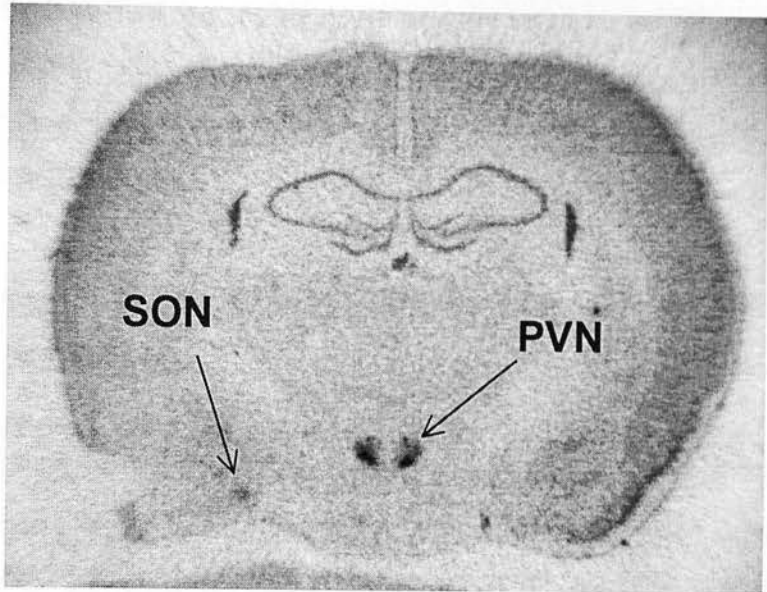
Figure 5.1A. Representative autoradiograph from *in situ* hybridisation for ER-β mRNA antisense riboprobe in the supraoptic (SON) and paraventricular (PVN) nucleus. The strongest hybridisation signal for ER-β mRNA was found in the ventral parvocellular group of neurones in the PVN, projecting to central autonomic sites.
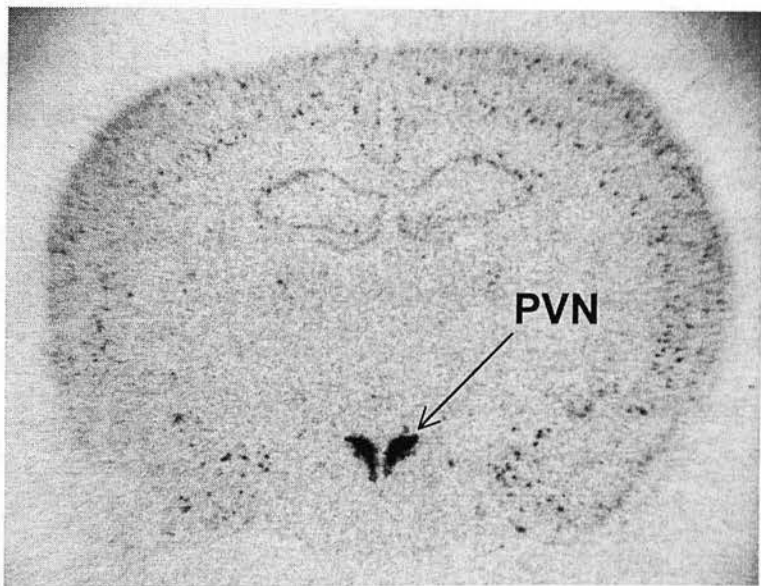
Figure 5.1B. Representative autoradiograph from *in situ* hybridisation for CRF mRNA antisense riboprobe in the paraventricular nucleus (PVN). The expression of the CRF mRNA was confined to the parvocellular dorsomedial portion of the PVN, projecting to the median eminence. (This section is 10 µM caudal to the section in Figure 5.1A.)

Changes in the expression of ER-β mRNA in the SON and PVN occurred following specific hormonal manipulations and physiological stimulation suggesting that oestrogens could potentially influence the activity (and perhaps function?) of neurones within these hypothalamic nuclei, and that this influence may be modified by changes in ER-β expression.

5.4.1. Regulation of ER-β mRNA in the hypothalamic SON and PVN by sex steroid manipulation

In the first experiment the silver grain signal from ER-β mRNA ISH in the magnocellular SON from sham-operated female (SF) rats was found to be significantly greater (*ca.* 1.4-fold) than that from the sham-operated male (SM) rats ($P < 0.05$, ANOVA, $P < 0.05$ Student-Newman-Keuls, SF *vs* SM) (Figure 5.2A.). This sex difference in expression was abolished at 72 h after gonadectomy (GDX), although GDX itself did not significantly alter the expression of ER-β mRNA in either sex.

Within the magnocellular division of the PVN there was a similar trend in the expression of ER-β mRNA transcript, although the difference in the expression between SF and SM was not statistically significant. (Figure 5.2B.) Similarly in the ventral parvocellular division of the PVN there was no sex-difference in the expression of ER-β mRNA. However, 72 h after GDX, the grain density for ER-β mRNA here from OVX rats was significantly increased (*ca.* 25%) ($P < 0.05$, ANOVA, $P < 0.05$ Student-Newman-Keuls) while the expression from CX rats remained relatively unchanged compared to their respective controls (Figure 5.2C.)
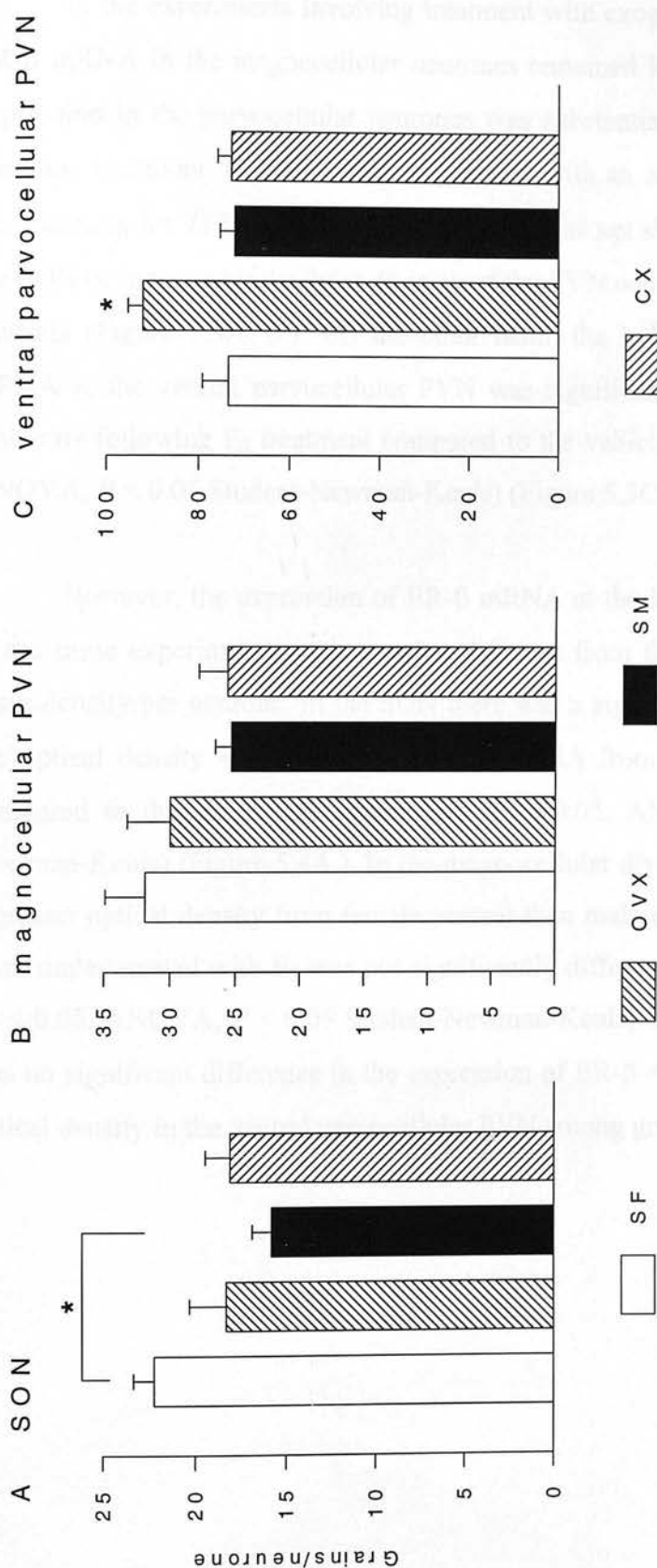
Figure 5.2. Effects of gonadectomy on ER-β mRNA expression in the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei. Measurements of silver grain counts per neurone in autoradiographs from *in situ* hybridisations. The expression of ER-β mRNA in the SON from sham-operated female (SF) rats was significantly greater than in sham-operated male (SM) rats. Gonadectomy (72 h) abolished this difference (A). There was a similar but non-significant trend in the expression of ER-β mRNA transcript in the magnocellular PVN (B). The expression of ER-β mRNA in the ventral parvocellular PVN was significantly increased in the ovariectomised (OVX) but not in the castrated (CX) rats compared to their respective controls (SF/SM) (C). Data were analysed by one-way ANOVA, followed by Student-Newman-Keuls post-hoc comparison ($P < 0.05$). Asterisk indicates statistical significance between groups, n=5/group. (SF = sham-operated female, OVX = ovariectomised female, SM = sham-operated male, CX = castrated male)

In the experiments involving treatment with exogenous E_2, the expression of ER-β mRNA in the magnocellular neurones remained largely unaffected while the expression in the parvocellular neurones was substantially modulated by the same hormone treatment. In intact male rats treated with an acute dose of E_2 (100 µg/kg s.c. injection for 72 h), the ER-β mRNA level was not significantly altered in either the SON or in the magnocellular division of the PVN compared to the vehicle-treated controls (Figure 5.3A./B.). On the other hand, the hybridisation signal for ER-β mRNA in the ventral parvocellular PVN was significantly decreased (*ca.* 30%) in male rats following E_2 treatment compared to the vehicle control groups ($P < 0.05$, ANOVA, $P < 0.05$ Student-Newman-Keuls) (Figure 5.3C.).

However, the expression of ER-β mRNA at the level of film optical density in the same experiment was somewhat different from that obtained at the level of grain density per neurone. In the SON there was a significant increase (*ca.* 15%) in the optical density value indicating ER-β mRNA from male rats treated with E_2 compared to that from vehicle controls ($P < 0.05$, ANOVA, $P < 0.05$ Student-Newman-Keuls) (Figure 5.4A.). In the magnocellular division of the PVN there was a greater optical density from female control than male control rats while the value from males treated with E_2 was not significantly different from either control group ($P < 0.05$, ANOVA, $P < 0.05$ Student-Newman-Keuls) (Figure 5.4B.). Lastly, there was no significant difference in the expression of ER-β mRNA as indicated by film optical density in the ventral parvocellular PVN among groups (Figure 5.4C.).
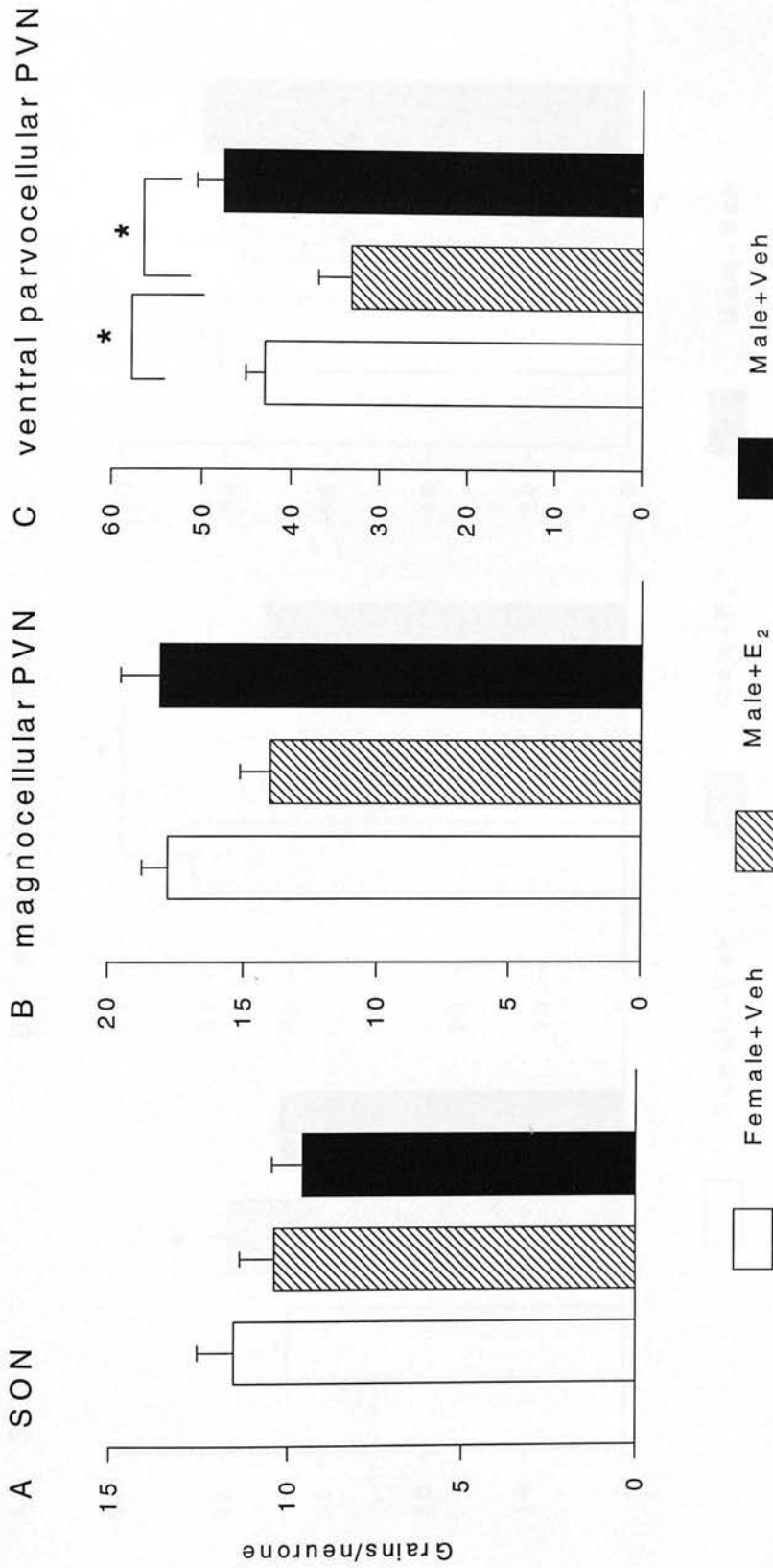
Figure 5.3. Effects of 17-β oestradiol (E$_2$) on ER-β mRNA expression in intact male rats. Treatment with E$_2$ (100 µg/kg s.c. injection daily for 72 h) did not significantly alter the expression of ER-β mRNA in the SON (A) nor in the magnocellular PVN (B). There was no significant difference here in the expression of ER-β mRNA between vehicle-injected female and male rats. On the contrary the expression of ER-β mRNA in the ventral parvocellular PVN from intact male rats following E$_2$ treatment was significantly reduced compared to those in the controls of both sexes (C). Data were analysed by one-way ANOVA, followed by Student-Newman-Keuls post-hoc comparison ($P < 0.05$). Asterisk indicates statistical significance between groups, n=5/group.
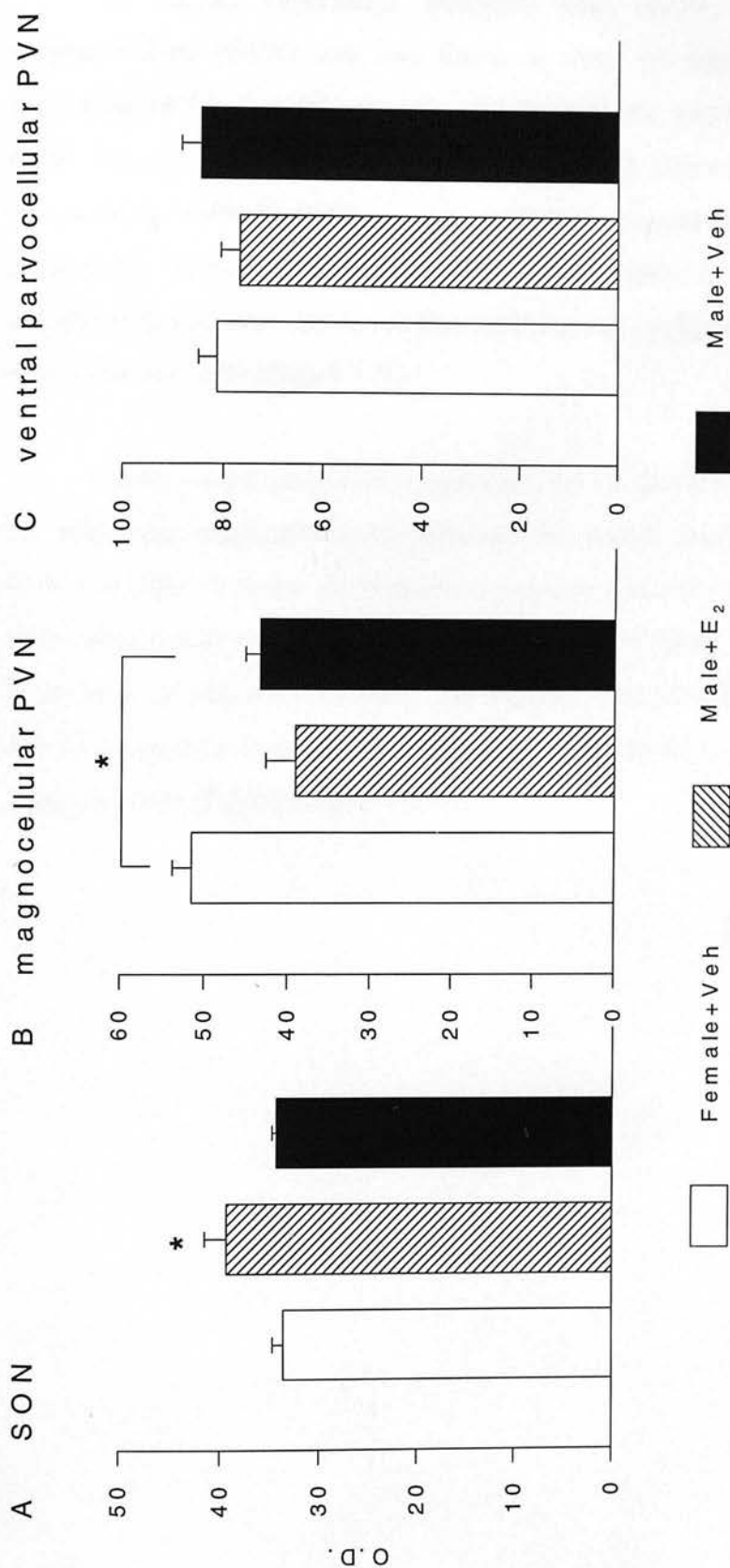
Figure 5.4. Effects of 17-β oestradiol (E$_2$) on ER-β mRNA expression in intact male rats. Measurements of optical density from film autoradiographs from *in situ* hybridisations. The relative optical density represents the overall expression of ER-β mRNA expression from the entire nuclei (units were given arbitrarily). Treatment with E$_2$ (100 μg/kg s.c. injection daily for 72 h) significantly increased the expression of ER-β mRNA in the SON (A, $P = 0.03$) but not in the magnocellular (B) nor the ventral parvocellular PVN (C). The expression of ER-β mRNA from vehicle-injected female was significantly greater than that from male rats although this was found only in the magnocellular PVN (B, $P < 0.05$). Data were analysed by one-way ANOVA, followed by Student-Newman-Keuls post-hoc comparison ($P < 0.05$). Asterisk indicates statistical significance between groups, n=5/group.

In another experiment, treatment with chronic E_2 over 2 weeks in ovariectomised (OVX) rats was found to exert no significant effect upon the expression of ER-β mRNA in either the SON or the magnocellular division of the PVN (Figure 5.5A./B.). On the contrary ER-β mRNA level in the ventral parvocellular PVN in OVX rats following E_2 treatment was evidently lower than those in the vehicle control group, although this cannot be demonstrated statistically due to the insufficient number in the control group (n=2; due to sections lost during processing for ISH) (Figure 5.5C.).

Lastly, in the pregnancy experiment, the ER-β mRNA hybridisation signal in the SON, the magnocellular division and the ventral parvocellular division of the PVN from day 15 or day 22 (parturient) pregnant rats were not significantly different from those found in the pro-oestrus virgin controls (Figure 5.6A. & 5.7.). However, at the level of film optical density, the expression of ER-β mRNA in the SON from day 15 pregnant rats was significantly greater compared to parturient or pro-oestrus virgin controls (Figure 5.6B.).
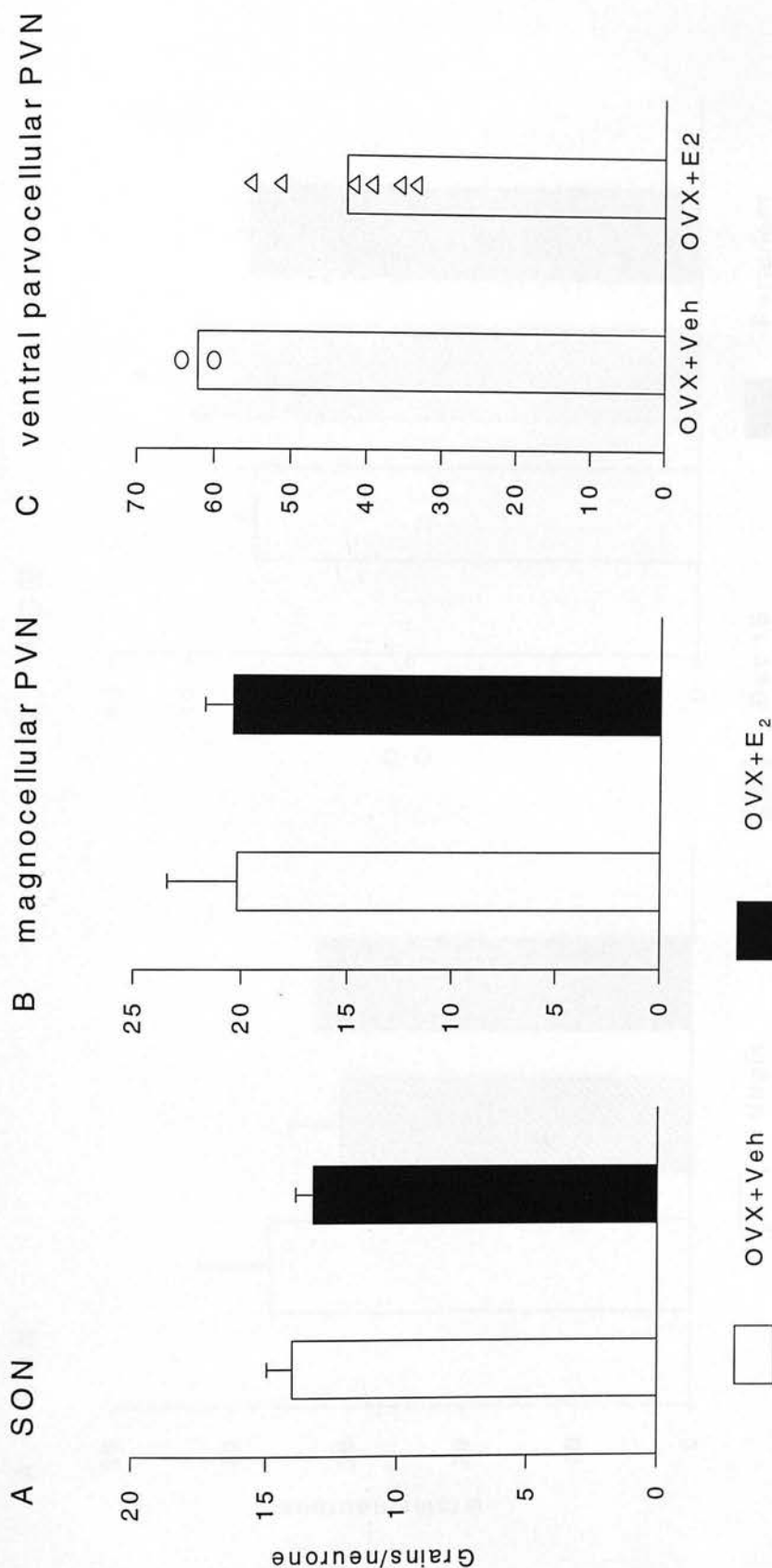
<u>Figure 5.5.</u> Effects of chronic 17-β oestradiol (E₂) treatment on the expression of ER-β mRNA in ovariectomised rats (OVX). Graph shows the expression of ER-β mRNA in the SON (A) and the magnocellular PVN (B) in OVX rats treated with E₂ (15 mg/ml s.c. implant) and those treated with vehicle over a period of 2 weeks. There was no significant difference between groups. Data were analysed by Student's *t*-test, n=6/group. However, within the ventral parvocellular PVN the expression of ER-β mRNA in OVX rats following E₂ treatment was (clearly) reduced from that in the vehicle control group although this can not be demonstrated statistically due to the insufficient number in the control group (C, n=2 for control and n=6 for E₂-treated group).
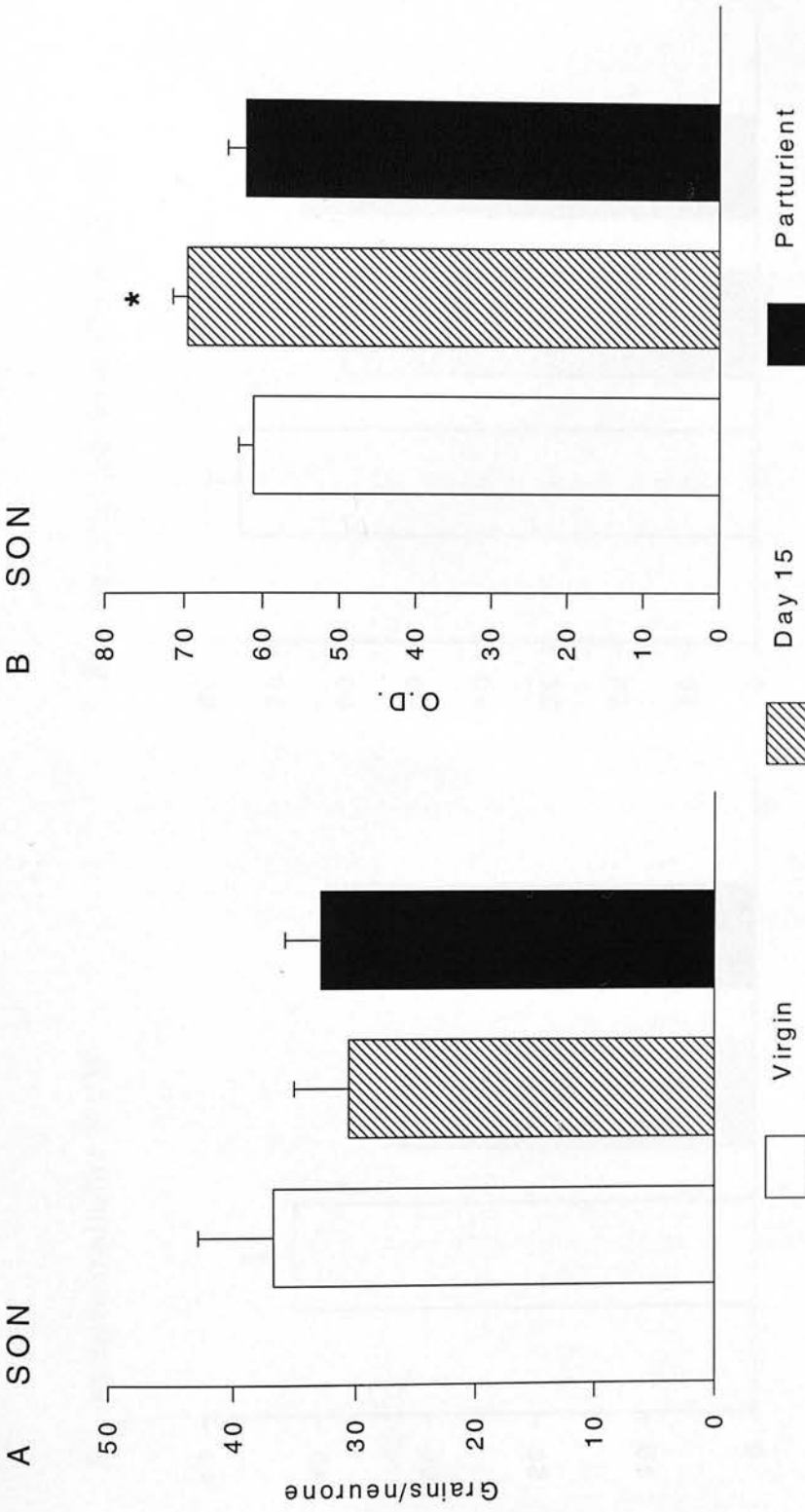
Figure 5.6. Graph shows the expression of ER-β mRNA in the SON at different stages of pregnancy. At the level of grain density per neurone, there was no significant difference in the expression of ER-β mRNA at either day 15 or 22 (parturient) of pregnancy compared to pro-oestrous virgin controls (A). However, at the level of film optical density, the value indicating ER-β mRNA from rats at day 15 of pregnancy was significantly greater compared to parturient or pro-oestrous virgin controls (B). Data were analysed by one-way ANOVA, followed by Student-Newman-Keuls post-hoc comparison (P < 0.05). Asterisk indicates statistical significance between groups, n=6/group.
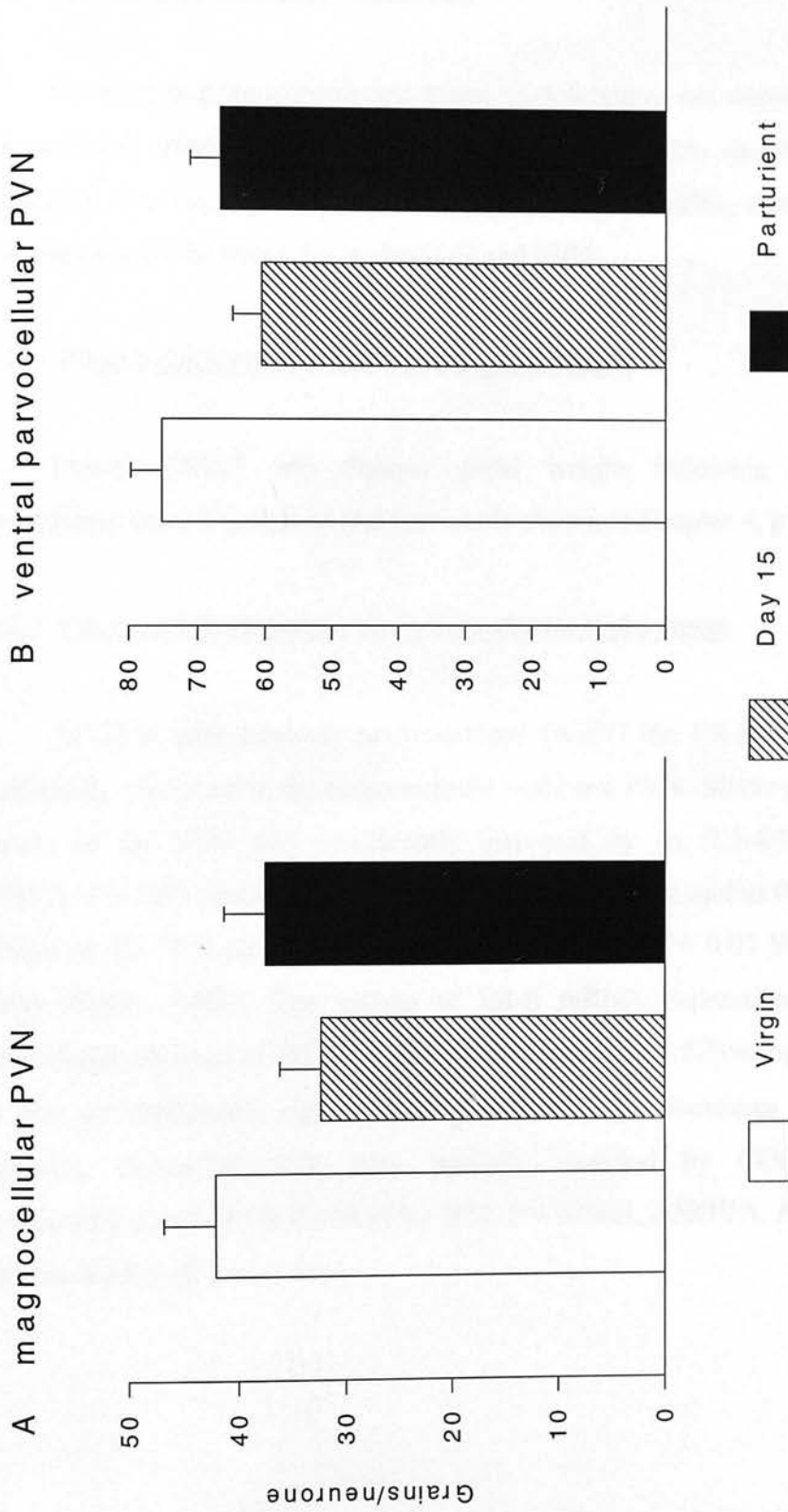
Figure 5.7. Graph shows the expression of ER-β mRNA in the PVN; (A) in the magnocellular subdivision and (B) in the ventral parvocellular subdivision at different stages of pregnancy. There was no significant difference in the expression of ER-β mRNA in both region at either day 15 or 22 (parturient) of pregnancy compared to pro-oestrous virgin controls. Data were analysed by one-way ANOVA, n=6/group.

5.4.2. Regulation of ER-β mRNA in the hypothalamic SON and PVN under specific physiological stimulation

Contrary to prior experiments where modulation of sex steroids was shown to exert minimal effects upon the expression of ER-β mRNA, specific physiological stimulation involving adrenal steroid hormones and salt-loading dramatically altered the expression of the transcript in the SON and PVN.

5.4.2.1. <u>Plasma corticosterone and thymus gland weight</u>

Plasma CORT and thymus gland weight following adrenal steroid manipulation were identical as and previously shown in Chapter 4, p 116.

5.4.2.2. <u>Effect of adrenalectomy and corticosterone replacement</u>

At 72 h after bilateral adrenalectomy (ADX) the ER-β mRNA level was significantly increased in the magnocellular SON and PVN. Silver grain density per neurone in the SON was significantly increased by *ca.* 2.5-fold ($P = 0.0001$, ANOVA, $P < 0.05$ Student-Newman-Keuls) (Figure 5.8A.), and in the magnocellular division of the PVN *ca.* 2.2-fold ($P < 0.005$, ANOVA, $P < 0.05$ Student-Newman-Keuls) (Figure 5.8B.). The pattern of ER-β mRNA expression in the ventral parvocellular division of the PVN showed the same trend following ADX although this was not statistically significant (Figure 5.8C.). The increases in ER-β mRNA expression following ADX were partially reversed by CORT replacement (significantly decreased in the SON by 36%, $P = 0.0001$, ANOVA, $P < 0.05$ Student-Newman-Keuls) (Figure 5.8A.).
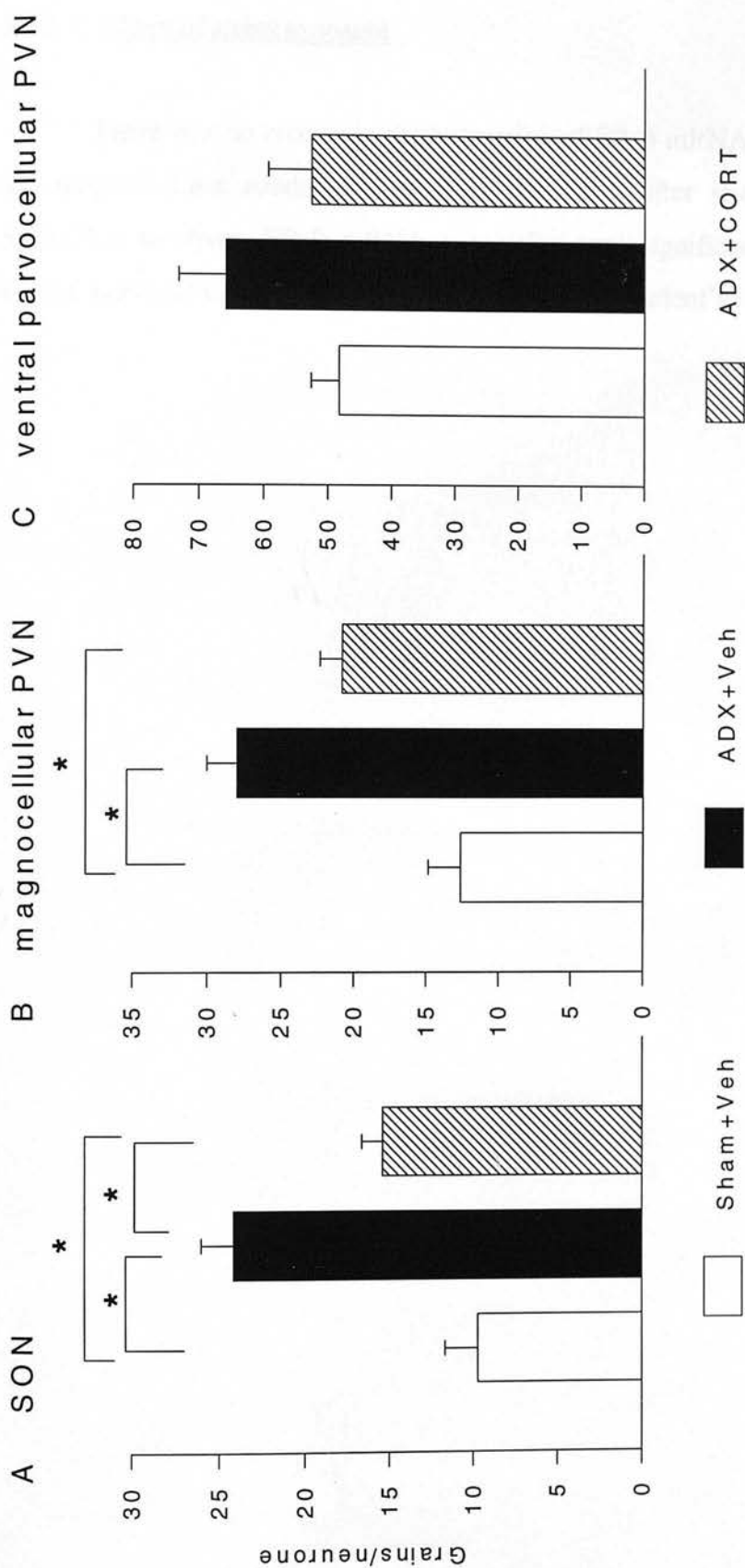
Figure 5.8. Effects of adrenal steroid manipulation on the expression of ER-β mRNA in male rats. Bilateral adrenalectomy (ADX) induced a significant increase in ER-β mRNA expression in the SON (A) as well as in the magnocellular PVN (B) while corticosterone (CORT) replacement (10 mg/kg s.c. injection at 4 p.m. daily for 72 h) partially reversed the effect of ADX on the expression of ER-β mRNA. The apparent increase in the expression of ER-β mRNA in the ventral parvocellular PVN following ADX was not significant (C). Data were analysed by one-way ANOVA, followed by Student-Newman-Keuls post-hoc comparison ($P < 0.05$). Asterisk indicates statistical significance between groups, n=5/group.

5.4.2.3. Effect of stress exposure

There was no change in the expression of ER-β mRNA either in the SON or the magnocellular subdivision of the PVN 72 h after stress exposure (Figure 5.9A./B.); however, ER-β mRNA expression was significantly increased in the ventral parvocellular division of the PVN ($P = 0.02$, Student's t-test). (Figure 5.9C.).
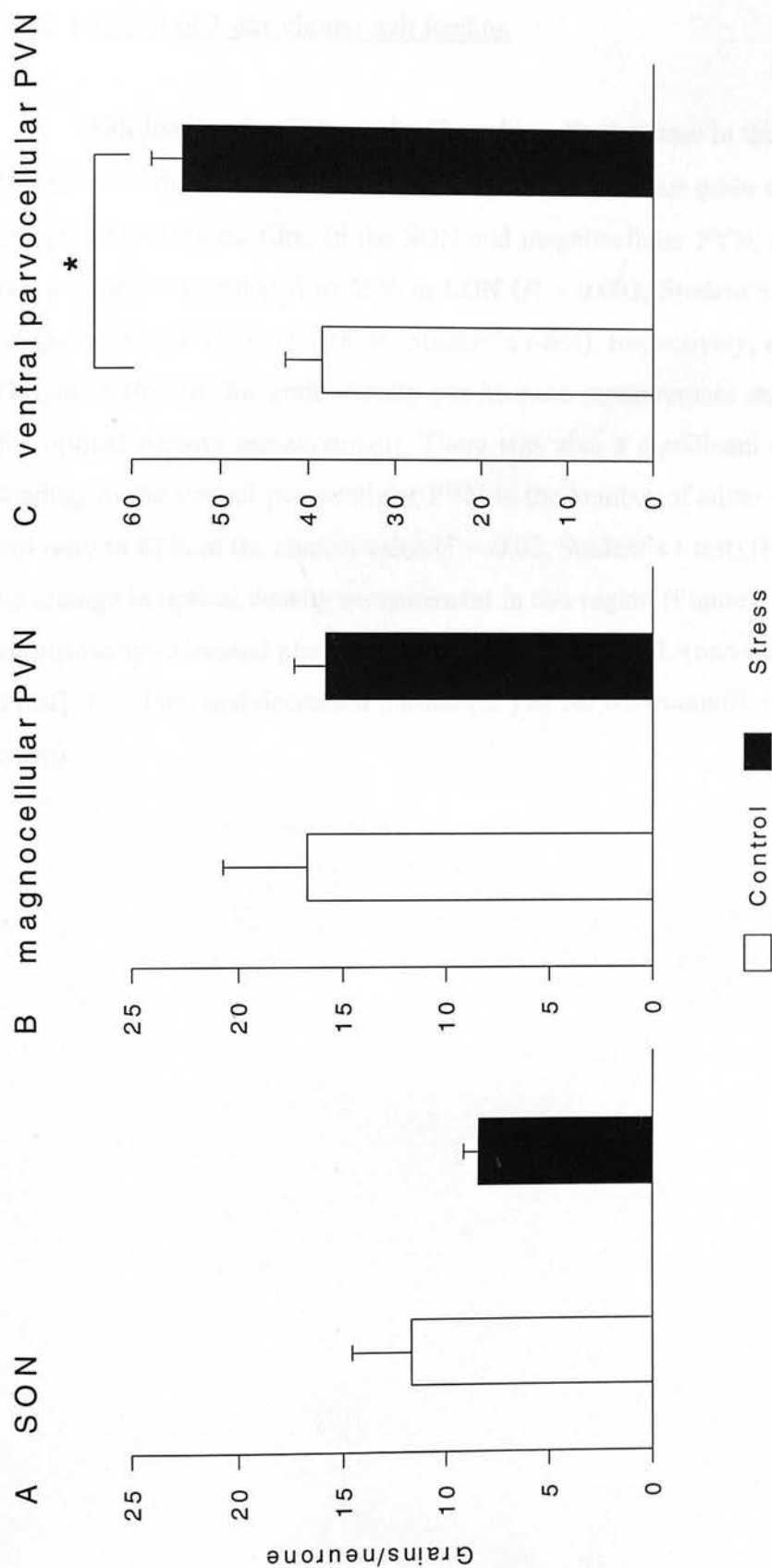
Figure 5.9. Effects of repeated stresses (cold, halothane, and immobilised restraint exposure) on the expression of ER-β mRNA over the period of 72 h. The expression of ER-β mRNA in the SON (A) and the magnocellular PVN (B) from male rats subjected to a cycle of various stress paradigms was not statistically significantly different from that in the controls. On the other hand, the expression of ER-β mRNA in the parvocellular PVN was significantly increased ($P = 0.02$) following 72 h of stress exposure (C). Data were analysed by Student's t-test. Asterisk indicates statistical significance between groups, n=5/group.

5.4.2.4. Effect of 3-day chronic salt loading

Salt loading for 72 h resulted in a dramatic decrease in the expression of ER-β mRNA in the SON and PVN, measured as either silver grain density per neurone or optical density on film. In the SON and magnocellular PVN, silver grain density per neurone was reduced to 39% in SON ($P = 0.003$, Student's t-test) and 38% in magnocellular PVN ($P < 0.0001$, Student's t-test), respectively, of the control value (Figure 5.10A./B. for grain density per neurone measurement and Figure 5.11A./B. for optical density measurement). There was also a significant decrease with salt-loading in the ventral parvocellular PVN in the number of silver grains per neurone, but only to 87% of the control value ($P = 0.02$, Student's t-test) (Figure 5.10C.), with no change in optical density measurement in this region (Figure 5.11C.). Salt loading significantly increased plasma [Na^+] by $ca.$ 13.0 mmol/L (one-tailed t-test [$t = 1.93$, 11 df], $P < 0.05$) and decreased plasma [K^+] by $ca.$ 0.84 mmol/L ($P < 0.05$, Student's t-test).
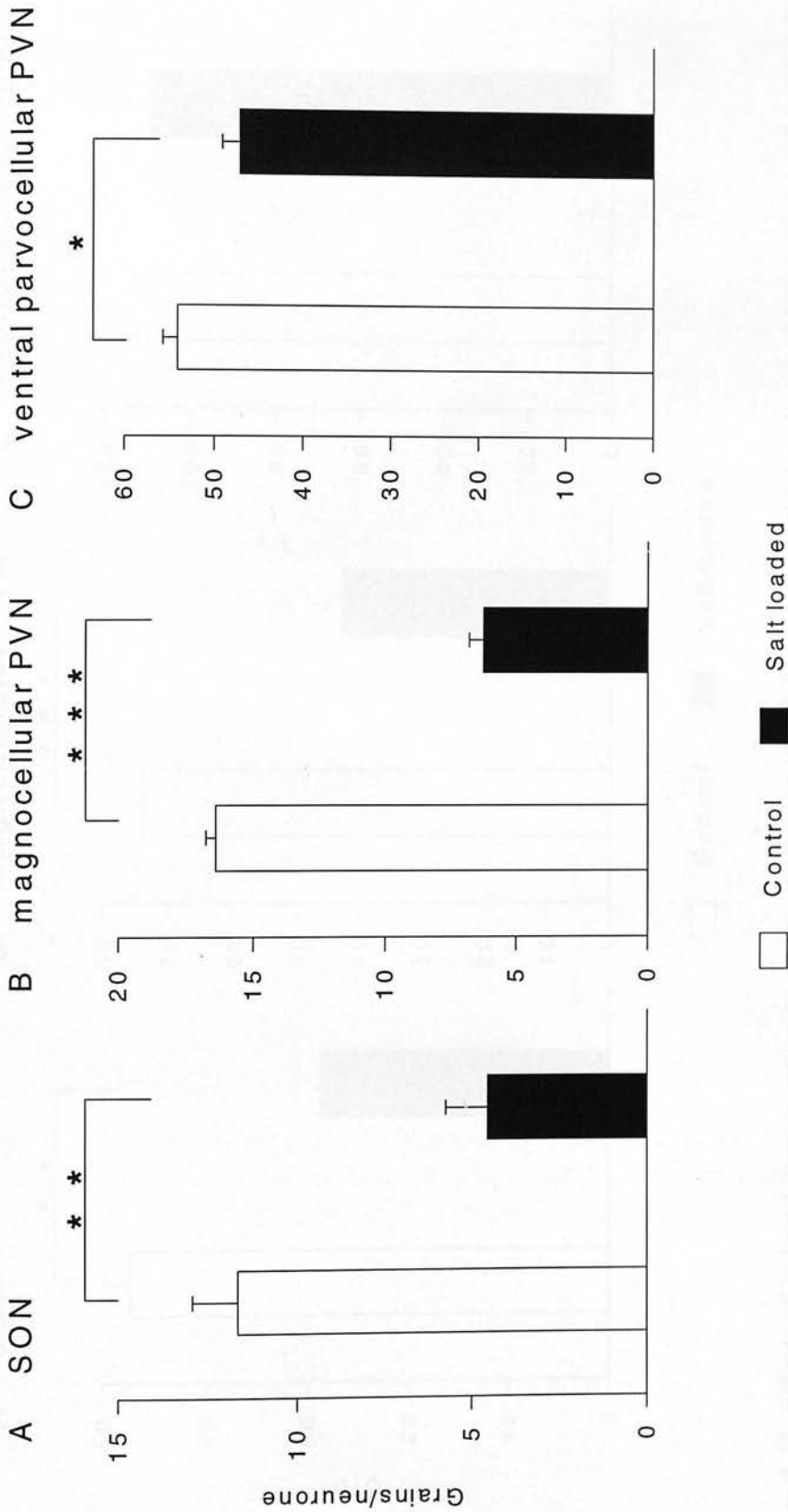
Figure 5.10. Effects of induced hyperosmolarity on the expression of ER-β mRNA in intact male rats. Three day salt loading with 2% NaCl solution to drink resulted in a significant reduction in the expression of ER-β mRNA in the SON (A, $P = 0.003$), the magnocellular PVN (B, $P < 0.0001$) as well as the ventral parvocellular PVN (C, $P = 0.02$). Data were analysed by Student's t-test. Asterisk indicates statistical significance between groups, n=6/group.
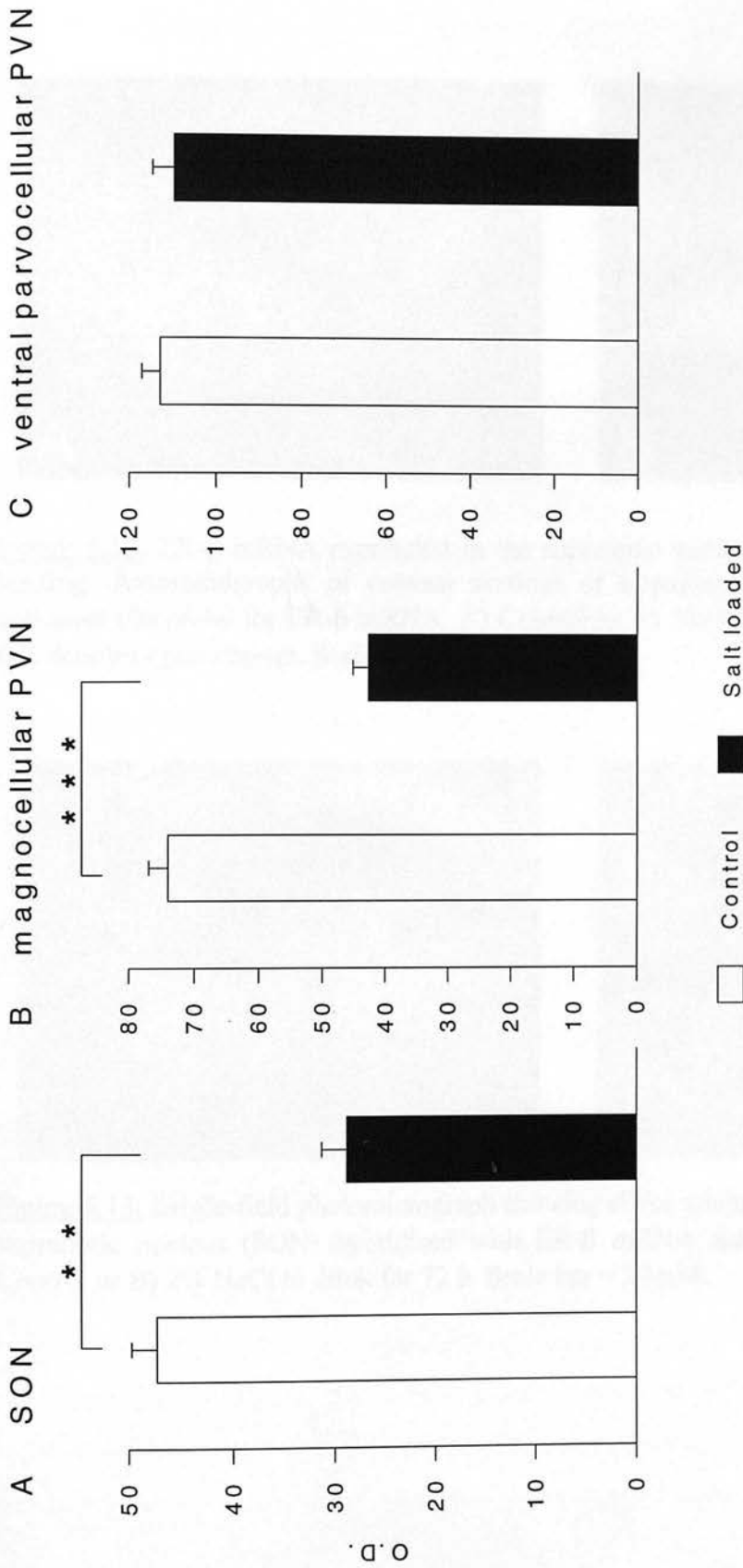
Figure 5.11. Effects of induced hyperosmolarity on the expression of ER-β mRNA in intact male rats. Measurements of optical density from autoradiograms from *in situ* hybridisations. The relative optical density represents the overall expression of ER-β mRNA expression from the entire nuclei (units were given arbitrarily). Three day salt loading with 2% NaCl solution to drink resulted in a significant reduction in the expression of ER-β mRNA in the SON (A, *P* = 0.0005) and the magnocellular PVN (B, *P* < 0.0001). However the effect of chronic salt loading on the expression of ER-β mRNA was not seen in the ventral parvocellular PVN (C). Data were analysed by Student's *t*-test. Asterisk indicates statistical significance between groups, n=6/group.
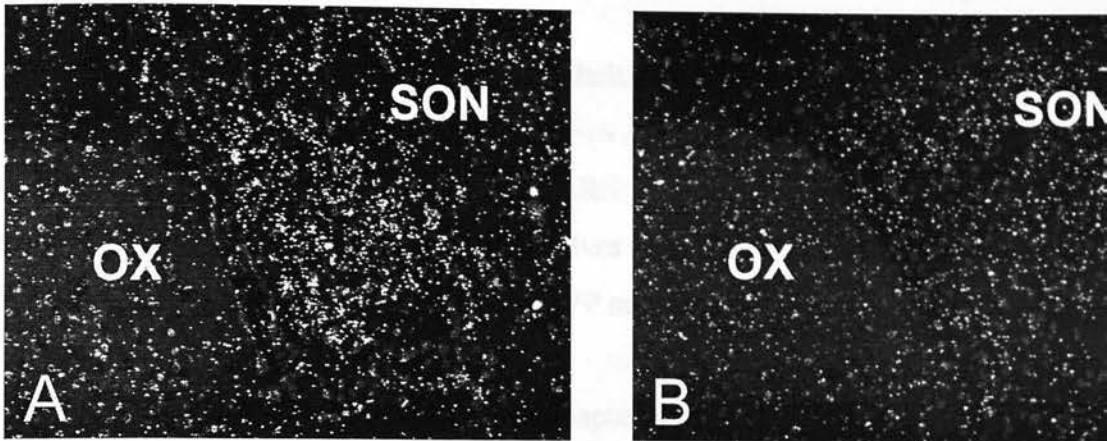
Figure 5.12. ER-β mRNA expression in the supraoptic nucleus (SON) during salt-loading. Autoradiographs of coronal sections of hypothalamus, hybridised with antisense riboprobe for ER-β mRNA. A) Control *vs* B) 2% NaCl to drink for 72 h. OX denotes optic chiasm. Scale bar = 100 μM.
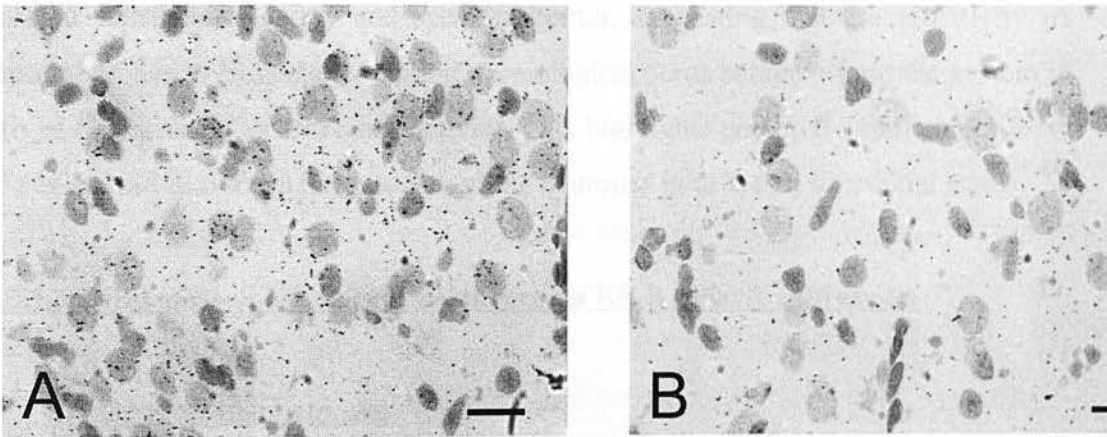
Figure 5.13. Bright-field photomicrograph showing silver grains over neurones in the supraoptic nucleus (SON) hybridised with ER-β mRNA antisense riboprobe. A) Control *vs* B) 2% NaCl to drink for 72 h. Scale bar = 20 μM.

5.5. Discussion

The expression of ER-β in the hypothalamic SON and PVN neurones has been reported in several previous studies (Alves *et al.*, 1998; Shughrue, Scrimo, & Merchenthaler, 1998), with a stronger ER-β mRNA signal in the magnocellular OXT neurones than in VP neurones in the PVN (Alves *et al.*, 1998), and vice versa in the SON, where ER-β protein is more evident in VP neurones (Hrabovszky *et al.*, 1998).

The experiments described in this chapter have shown that modulation of gonadal steroids produced modest changes in the expression of ER-β mRNA in the magnocellular neurones in male and female rats, and in the PVN only in the ventral group of parvocellular neurones projecting centrally to sites involved in autonomic regulation. On the other hand dramatic changes in the expression of ER-β mRNA in the hypothalamic SON and PVN neurones were observed following changes in adrenal steroid availability and specific stimuli, suggesting that the sensitivity of these neurones to E_2 is altered during physiological perturbations where the system is driven to decreased or increased activity. This highlights potential significant effects of sex steroid hormones in responses of the neurones in different functional states.

5.5.1. Influences of sex steroid hormones on ER-β mRNA expression

a) Magnocellular neurones

In the first gonadectomy (GDX) experiment there was a sex difference in the basal expression of ER-β mRNA in the magnocellular SON, with sham-operated female rats exhibiting a greater expression than sham-operated males. This difference was abolished by GDX. The expression of ER-β mRNA in the magnocellular PVN showed a similar trend to that seen in the SON, although this was not statistically significant. The result indicates that female gonadal hormone positively, while male gonadal hormone negatively, regulates the expression of ER-β mRNA in the magnocellular neurones.

In the subsequent studies, however, this small sex difference in the expression of ER-β mRNA was not found consistently. For instance, in the experiment where intact male rats were treated with exogenous E_2 there was no significant sex difference in the expression of ER-β mRNA in the SON, either at the level of silver grain density per neurone or film optical density. Similarly, the difference in grain density per neurone for ER-β mRNA between male and female rats was not seen in the magnocellular PVN neurones and although such a difference became apparent at the level of film optical density, this was rather small (*ca.* 16%).

Merkelbach *et al.*1975 reported that the 24 h urine excretion of VP was higher in men than in women (Merkelbach *et al.*, 1975). In rats a number of reports also indicated a sex difference in basal plasma VP concentration (Crofton *et al.*, 1985; Crofton, Share, & Brooks, 1988; Stone, Crofton, & Share, 1989) as well as VP release in response to intravenous hypertonic saline injection (Crofton & Share, 1989). A recent report by Dai and Yao 1995 also showed a sex difference in hypothalamic VP mRNA and plasma osmolarity in the rats (Dai & Yao, 1995). There is also a sexual dimorphism in the diurnal stress response of OXT secretion in rats (Carter & Lightman, 1986). Conversely, similar amounts of OXT and VP mRNA have been reported in the SON and PVN of male and female rats (Swaab, Pool, & Nijveldt, 1975b). Thus the finding of a sex difference in ER-β mRNA in the magnocellular neurones in this present study might contribute to the sex difference in basal functional activity in the magnocellular neurones indicated by previous studies, although such a difference in itself is not reliably detected.

Although the sex difference in ER-β mRNA expression in the SON in this study was abolished by GDX, the change in expression of ER-β mRNA was not significantly altered by GDX in either sex. This lack of change was also seen in the magnocellular PVN neurones. This finding, again, reflects the small differences in this effect between the sexes. Following acute E_2 treatment for 72 h there was no significant change in the expression of ER-β mRNA in the magnocellular SON and PVN, measured as silver grains per neurone, in intact male rats compared to that in

vehicle-treated male and female controls. Although there was a significant increase in ER-β mRNA expression, measured as film optical density, this was found only in the SON, and was of the order of about 15%. Similarly chronic treatment of OVX rats with E_2 over 2 weeks did not induce any significant change in the expression of ER-β mRNA either in the SON or the magnocellular PVN.

Collectively these findings suggest that the regulation of ER-β mRNA expression in the magnocellular neurones by sex steroid hormones is rather weak, as revealed by small changes in the expression of the transcript following either exposure to or deprivation of E_2 in male or female rats.

The role of ER-β (in the magnocellular neurones) is not known although we assume it is a ligand-regulated transcription factor that mediates effects of E_2. The oxytocinergic and vasopressinergic magnocellular neurones are known to be influenced by gonadal steroids. As previously mentioned, given that the OXT and VP genes have been identified to possess nucleotide sequences similar to an ERE, it is conceivable that oestrogens, presumably via binding to ER-β in these neurones, could directly modulate the transcription and other downstream consequences of these peptide genes. Nonetheless several previous attempts to characterise oestrogen modulation of OXT and VP genes in the magnocellular neurones have resulted in a rather conflicting literature, with different researchers reporting different results. For instance, it has been previously reported that gonadectomy (both ovariectomy (OVX) in females and castration (CX) in males) alters neither the expression of VP/OXT mRNA (Carter et al., 1993; Crowley & Amico, 1993) nor OXT pituitary contents (Crowley & Amico, 1993) yet in some other studies OVX has been shown to result in a reduction in circulating plasma VP concentrations (Peysner & Forsling, 1990; Skowsky et al., 1979). In another study administration of E_2 failed to modulate the expression of VP mRNA in magnocellular neurones in OVX rhesus monkeys (Roy et al., 1999). Additionally, treatment of OVX rats with E_2 has been shown to either influence or leave unchanged the expression of OXT mRNA in the SON and PVN neurones (Caldwell et al., 1989; Chung, McCabe, & Pfaff, 1991; Miller et al., 1989).

The promoter of the rat OXT gene has been shown to contain a direct repeat of the half-site motif (AGGTCA) as well as an imperfect palindromic sequence similar to the ERE (Richard & Zingg, 1990) supporting the notion that E_2 could directly influence the regulation of the OXT gene. The effects of gonadal steroids on the OXT gene have also been previously demonstrated in many *in vitro* studies (Adan *et al.*, 1993; Adan *et al.*, 1991; Richard & Zingg, 1990). However, Stedronsky *et al.* 1999 have recently looked at the interaction between many nuclear receptors, including ER, and the human OXT gene promoter and suggest that, at least in an *in vitro* model, the human OXT gene is not a natural target for ER (both -α and -β forms) (Stedronsky, Walther, & Ivell, 1999). Instead they have shown that orphan-like receptors, such as SF-1 and COUP-TF1, interacted with a much higher affinity to the natural human OXT ERE, compared to ER-α or -β, suggesting that the orphan-like nuclear receptors, and not the ERs, are the likely candidates to mediate the regulation of the OXT gene. However, since these experiments were performed *in vitro*, where many other factors which might interact with the OXT gene *in vivo* were not included, the possibility that the interaction observed in their study might be different under *in vivo* physiological conditions cannot be ruled out.

These conflicting findings are perhaps reflected by this present study where changes in ER-β mRNA expression in the magnocellular neurones by sex steroid modulation were small and hence the responsiveness of these neurones to sex steroid hormones will also be little affected. So the references that have described effects of E_2 on OXT and VP genes in the literature might not be necessarily directly related to actions of gonadal hormones on these genes in the magnocellular neurones themselves but, rather, secondary to other mechanisms, perhaps as an indirect result of altered hydromineral balance found in some cases, but not in others.

b)   Ventral parvocellular neurones

It is interesting that where modest change in ER-β mRNA expression was observed in the magnocellular neurones following gonadal steroid manipulation, the expression of the transcript in the ventral parvocellular neurones was also evidently

modulated following the same steroid treatment. After OVX, the expression of ER-β mRNA was significantly increased by 25%, while treatment of OVX rats with exogenous E_2 over 2 weeks evidently decreased the expression of ER-β mRNA (although this could not be demonstrated statistically due to an insufficient number of OVX control animals). Similarly treatment with E_2 for 72 h induced a 30% decrease in the expression of ER-β mRNA here in intact male rats compared to their control cohorts. This present finding is also supported by a recent report by Patisaul *et al.* 1999 in which a substantial decrease (44.5%) in the expression of ER-β mRNA in the PVN was observed in OVX rats following E_2 exposure. However, the change that was observed in their study was seen at the level of film autoradiographs whereby the entire PVN which comprises both magno- and parvo-cellular neurones was visualised and quantified. This present study was directed to examine the expression at higher resolution by quantifying the ER-β mRNA hybridisation signal in two specific neuronal sub-populations, each of which sends axons to different sites and mediates distinct physiological functions (Swanson and Kuipers 1980). Thus it is possible that the change in the expression of ER-β mRNA observed in the study by Patisaul *et al.* was perhaps due to changes that occurred in the ventral parvocellular neurones rather than a uniform response of the ER-β mRNA signal from the entire nucleus as they implied (Patisaul *et al.*, 1999). The functional significance of this finding is discussed below.

The regulation of steroid hormone receptor expression is complex. Changes in the expression of ER-β mRNA observed in this study may have been a consequence of altered mRNA stability or altered transcription (see Chapter 1). In the magnocellular neurones, loss of ovarian steroids decreased ER-β mRNA expression while castration in males increased it suggesting that E_2 maintains ER-β at a higher level in females than in males. In contrast, in the ventral parvocellular neurones, the findings indicate that E_2 decreases ER-β mRNA expression in both sexes; the effect being in the opposite direction to the magnocellular neurones. This reinforces the neuronally specific direction of ER-β mRNA regulation by E_2 and

further indicates the plasticity of ER-β mRNA expression in these neurones. The mechanism underlying the differential responses of ER-β mRNA in the two neuronal cell groups is not known but might result from the regulation of gene expression by an alternative promoter usage, as previously discussed (see Chapter 1&4).

5.5.2. ER-β mRNA expression in pregnancy and parturition

a)   Pregnancy

The results in this experiment also showed that even in pregnancy, when rats are exposed to pronounced changes in sex steroid hormone levels, the expression of ER-β mRNA at the level of grain density in the magnocellular neurones was little altered, both at mid-pregnancy (day 15) or during parturition (day 22) compared to that of virgin controls. However, in the SON, there was a significantly greater expression of the transcript at the level of film optical density from day 15 pregnant rats which was *ca.* 14% higher than that of the virgin controls. A small increase in ER-β mRNA expression in the SON magnocellular neurones that was found in day 15 pregnant rats which was apparent at the level of film optical density but not at the level of grain density per neurone suggests that there was an increase in the number of neurones expressing ER-β mRNA without a corresponding increase in the degree of expression by each neurone. This increase in expression might be related to an altered hydromineral balance during pregnancy (see below).

Garland *et al.* 1987 have shown that the level of E_2 rises throughout pregnancy and is significantly higher than in virgin controls from around mid-pregnancy (day 16) reaching a peak at term. Similarly, the level of P_4 is higher than in virgin controls at all stages of pregnancy except towards the end when the level declines precipitously 24-48 h prior to delivery (Garland *et al.*, 1987). During pregnancy and parturition changes in the levels of ER expression have been observed in many brain regions. An increase in the levels of ER-α immunoreactivity (ir) was observed in the medial preoptic nucleus (MPN) on day 16 and day 22 of pregnancy compared to day 8 of pregnancy or day 1 postpartum, and such an increase is

implicated in the induction of maternal behaviour around the time of parturition. Equally the mean ER-α ir per cell in the ventromedial nucleus (VMN) was significantly greater on day 22 compared to day 16 of pregnancy or postpartum day 1; this region is known to be involved in the oestrogen induction of postpartum female sexual receptivity (Wagner & Morrell, 1996).

Gonadal steroids have also been implicated in the regulation of the magnocellular neurosecretory neurones in pregnancy, especially towards the end of gestation. Parturition is a time of high demand for OXT and so in preparation the pituitary stores of OXT increase substantially during pregnancy (Douglas *et al.*, 1993; Leng *et al.*, 1988). The accumulation of OXT peptide in the neural lobe is a result of both a restraint of peptide release from the nerve terminal as well as a possible increase in its synthesis in the magnocellular neurones. Although not conclusively (Brooks, 1992; Douglas *et al.*, 1998), a number of previous studies have shown an up-regulation in the expression of OXT mRNA in the magnocellular neurones during pregnancy, especially immediately prepartum (12-24 h) (Horwitz *et al.*, 1994; Luckman & Larsen, 1997; Van Tol *et al.*, 1988; Zingg & Lefebvre, 1988; Zingg & Lefebvre, 1989) indicating a stimulation of the OXT gene. This increase in OXT mRNA expression occurs in association with the marked changes in the sex steroid hormone profile, when rats are exposed to a rise in E_2 (from *ca.* 30 pg/ml during the early stages of gestation to about 60 pg/ml mid-pregnancy to 80 pg/ml before term) and P_4 during pregnancy (from 70 ng/ml on day 4-10 to an average of 100-152 ng/ml from day 12-20 of gestation) and a subsequent steep decline in P_4 prior to term (to *ca.* 10 ng/ml on day 22) (Bridges, 1984). A correlation of increased OXT mRNA levels in the magnocellular neurones with increased circulating E_2 in rats before term suggests the possibility that E_2 might be an important regulator of OXT gene expression. However, the up-regulation of OXT expression observed in those studies occurred specifically immediately prepartum (i.e. *ca.* 12-24 h prior to term) and not during gestation (not on day 12, 15 or 18, (Van Tol *et al.*, 1988) and not on day 7 (Luckman & Larsen, 1997), it is perhaps not surprising that the

expression of ER-β mRNA at day 15 of pregnancy was barely increased, as seen in this study.

The similar sequential hormone regime (i.e. E_2 and P_4 priming with subsequent P_4 withdrawal) is also seen during other physiological conditions such as during days 10-12 of lactation (Crowley et al., 1993; Thomas, Crowley, & Amico, 1995) and interruption of nursing for 48 h during the first week of lactation (Crowley et al., 1993; Thomas et al., 1995), all of which lead to an increase in hypothalamic OXT mRNA. In fact, exposure of ovariectomised virgin rats to the same hormone regime (Crowley et al., 1995), as well as castrated male rats (Thomas & Amico, 1996), also induced an up-regulation of magnocellular neurone OXT mRNA. Failing to imitate this hormone regime (i.e. either omitting E_2 or P_4 priming or sustaining P_4 exposure) resulted in a blunted response of the OXT gene (Crowley et al., 1995). Intriguingly, the increase in hypothalamic OXT mRNA following this sequential hormone regime, mimicking the sex steroid milieu of pregnancy, was not accompanied by a corresponding increase in the peptide content in the pituitary (Amico, Thomas, & Hollingshead, 1997a; Amico et al., 1997b; Crowley et al., 1995; Thomas & Amico, 1996; Thomas et al., 1995). This raises a question as to whether an influence of sex steroid hormones upon the hypothalamic magnocellular neurones significantly contributes to the increase in neural lobe peptide content. It was suggested that perhaps an increase in OXT mRNA might be related to a central role of OXT, since the increase was not only seen in the magnocellular but also in the parvocellular division of the PVN which is known to send some axons to sites within the CNS (Swanson & Sawchenko, 1983). Perhaps the influence of sex steroid hormones upon the OXT gene may relate to the role of OXT in maternal behaviour, since the aforementioned sex steroid hormone regime has been shown to induce maternal behaviour in OVX virgin rats (Bridges, 1984). Sadly, no evidence so far has been produced about whether such an increase in OXT mRNA would precede an increase in central OXT peptide release in the CNS.

The accumulation in the pituitary OXT store during pregnancy might be mediated via a mechanism of restraint of OXT release, rather than an increase in peptide synthesis, perhaps by endogenous opioid acting on both the OXT neurones as well as the neural lobe over the last few days of pregnancy (Douglas *et al.*, 1994; Douglas *et al.*, 1995). Additionally other mechanisms such as post-transcriptional processing and efficiency of splicing of OXT mRNA and cleavage of preprohormone may also contribute to the availability of the OXT pool. Whether the increase in OXT peptide storage during pregnancy arises from an increase in synthesis remains obscure. Whether E_2 has any stimulatory effect upon the production of the peptide is unknown, although it is clear that changes in sex steroid profile do increase OXT mRNA expression. It might be worth looking at the regulation of ER-β mRNA over a different time frame i.e. 24-48 h prepartum for this is the period where OXT gene stimulation was observed.

b)   Parturition

The results from the present study further demonstrated that parturition itself did not change the expression of ER-β mRNA, relative to day 15 pregnant or virgin rats. Thus the activation of OXT neurones at parturition was evidently not associated with any change in ER-β mRNA expression in the magnocellular neurones. This indicates that E_2 probably does not have any significance influence upon the magnocellular neurones during parturition, at least not through changes in the expression of ER-β mRNA as these was not found.

5.5.3. ER-β mRNA expression during neuronal activation

a)   Magnocellular neurones

As far as we are aware the present study was the first to be undertaken to investigate the regulation of ER-β mRNA in the magnocellular neurones in activated states. This is the first report on changes in ER-β mRNA in these neurones by manipulation of adrenal corticosteroid hormones, or in relation to activation of the neurones by hyperosmotic stimulation. There was a striking increase in expression in

both the SON and magnocellular PVN after ADX, and a striking decrease after salt-loading. In both regions there are both OXT and VP neurones, which have characteristic distributions within the nuclei (Swaab, Nijveldt, & Pool, 1975a; Vandesande & Dierickx, 1975). Although we did not distinguish formally between the two neuronal populations, the large changes in expression that we quantified appeared not to be restricted to any particular subset of magnocellular neurones. Similar changes were seen for both measurements of film optical density overlying these regions as well as silver grain density overlying individual neurones. Together, these measurements indicate altered ER-β mRNA expression within individual as well as across the populations of neurones. Notably there was no effect of repeated stress on ER-β mRNA expression in the magnocellular neurones. This is despite the known stimulation of OXT secretion by stress (see Chapter 1), and the enhancement of this secretion by E_2 (Douglas *et al.*, 2000).

A key question in interpreting these findings is whether the changes seen in ER-β mRNA expression are a direct result of changes in corticosteroid secretion or availability, or are a consequence of stimuli to the neurones arising from changes in hydromineral balance. First, following ADX, lack of CORT and aldosterone leads to increased sodium excretion, tending to hyponatraemia, but accompanied by water loss, with consequent reduced blood volume and pressure, leading to hypovolaemia and hypotension. In general, hyponatraemia inhibits synthesis (Iványi, Dohanics, & Verbalis, 1995; Robinson *et al.*, 1990) and secretion of VP and OXT (Verbalis, 1993) while hypovolaemia and hypotension stimulate VP secretion (and/or synthesis?), in particular (see Robertson, 1995). The net outcome of adrenocortical insufficiency is the ***stimulation*** *of both VP mRNA* ***synthesis*** in the magnocellular neurones (Carter *et al.*, 1993) and VP secretion (Ahmed *et al.*, 1967; Share & Travis, 1970) leading to more water retention and hyponatraemia; although PVN and not SON VP neurones are evidently stimulated (Suemaru *et al.*, 1990). Thus the increase in ER-β mRNA expression in the magnocellular neurones after ADX is associated with hyponatraemia.

Alternatively, removing adrenal steroids by ADX increased ER-β mRNA expression, and this was only partially prevented by CORT replacement that was nonetheless sufficient to fully reverse both the increase in CRF mRNA expression in parvocellular PVN neurones and the increase in thymus gland weight following ADX. So it seems that perhaps the expression of ER-β mRNA is under the regulation of CORT. However, the identification of glucocorticoid receptor (GR), which CORT could act through, in the magnocellular neurones is controversial. Previous immunocytochemical studies have either failed to detect GR in magnocellular neurones (Fuxe *et al.*, 1985; Uht *et al.*, 1988) or identified GR-immunoreactivity in uncharacterised neurones in the SON and scattered neurones in the PVN (Kiss *et al.*, 1988). Additionally, most previous *in situ* hybridisation studies have not been able to identify GR mRNA in the magnocellular neurones (Aronsson *et al.*, 1988; Herman, 1993). However, up-regulation of GR expression has been demonstrated in the magnocellular neurones in induced hyponatraemia (Berghorn *et al.*, 1995; Iványi *et al.*, 1995). These results suggest that perhaps CORT could be acting in the magnocellular neurones via GR under hyponatraemic conditions to regulate the expression of ER-β mRNA. However, although there was a significant increase in ER-β mRNA following ADX, CORT replacement in ADX animals did not fully normalise the expression of ER-β mRNA raising the question whether another factor from the adrenals (e.g. perhaps aldosterone?) regulates the expression of ER-β mRNA in these neurones. Moreover, in the experiment involving stress exposure, a significant rise in the plasma CORT concentration following repeated stress did not affect the expression of ER-β mRNA in the magnocellular neurones, comparing the control and the stressed groups. Collectively the findings point in the direction that suggests an association between the expression of ER-β mRNA in the magnocellular neurosecretory neurones with changes in hydromineral balance (and hence the activity of these neurones, although see below too), rather than direct regulation by gluco- or mineralo-corticoids.

Indeed, in the subsequent studies a dramatic reduction in the expression of ER-β mRNA in the magnocellular neurones was observed following increased osmolarity induced by 2% NaCl ingestion. Increased hyperosmolarity following chronic salt-loading initially causes intracellular dehydration, and is a strong stimulus to sustained activation of magnocellular OXT and VP neurones in rats (see Leng *et al.*, 1999) leading to an increase in the production of mRNA for these peptides (Carter *et al.*, 1993; Lightman & Young, 1987; Majzoub *et al.*, 1983; Rehbein *et al.*, 1986; Van Tol, Voorhuis, & Burbach, 1987), depletion in neural lobe peptide content (Crowley & Amico, 1993; Jones & Pickering, 1969; O'Keefe *et al.*, 1995) as well as increase in plasma concentration of OXT and VP (Brimble, Dyball, & Forsling, 1978; Burbach *et al.*, 1984; Wade *et al.*, 1982). The present finding that ER-β mRNA expression in the magnocellular neurones is reduced following salt-loading suggests that the sensitivity of these neurones to E_2 would also be decreased under this condition.

The roles of sex steroid hormone in the regulation of OXT and VP genes during perturbation of osmotic balance, particularly by salt loading, has been extensively explored both *in vivo* and *in vitro*. An initial *in vivo* study by Crowley *et al.* 1993 has shown that the response of the OXT and VP genes (i.e. their mRNA accumulation) to increased osmolarity induced by salt loading in the SON and PVN is absent in gonadectomised (GDX) rats (both male and female), and that the response can be restored, at least in male rats, upon testosterone (T) replacement (Crowley & Amico, 1993). This suggests that the gonadal hormones and/or their receptors are required to *permit* the effects of osmotic stimulation upon OXT and VP gene expression. Absence of the response in the VP and OXT genes to hyperosmolarity was observed following GDX in both male and female rats, while T can be further aromatised into E_2 (Lieberburg & McEwen, 1977), and there is ER-β, but not AR (although see below too), in the magnocellular neurones. This suggests that the *permissive* (or *stimulatory*) effect of E_2 and T upon osmotic stimulation on OXT and VP gene expression could conceivably be mediated through ER-β. Yet in the present study, osmotic stimulation induced a dramatic decrease in ER-β mRNA

expression in the magnocellular neurones indicating a possible reduction in the sensitivity of these neurones to E_2 under this condition. In other words, the response of the OXT and VP genes to increased osmolarity evidently only occurs in the presence of E_2, but the expression of the receptor (ER-β) mediating such a task is decreased. This raises the question of whether any stimulatory effect of E_2 is mediated by ER-β.

Intriguingly it has been further demonstrated in a subsequent study that T and its more potent metabolite 5α-dihydrotestosterone (DHT), *but not E_2*, restored the osmotic response of VP mRNA in male rat SON neurones (O'Keefe *et al.*, 1995). The DHT binds to androgen receptor (AR) with a higher affinity than testosterone and is androgen-specific as it cannot be further aromatised to E_2 (Kovacs *et al.*, 1984). Thus it is conceivable that effects of DHT upon VP gene expression is mediated via the AR. Despite such speculation, there has been no report to date describing the localisation of AR within either the SON or PVN (Zhou, Blaustein, & De Vries, 1994), except in one study in which AR mRNA has been identified in neurones in the SON, albeit with low degree of expression (Simerly *et al.*, 1990). So DHT (and T) might be acting upon other AR-expressing neurones which in turn project to and influence the VP neurones in the SON. Alternatively, androgens may be modulating the VP neurones through other non-genomic pathways. It is interesting though that the follow-up study of O'Keefe *et al.*, 1995 was carried out only in male rats, yet absence of a response of VP mRNA accumulation to increased osmolarity following GDX was evident in both sexes.

The effects of sex steroids upon the regulation of VP during increased osmolarity have also been studied *in vitro* using the perifused hypothalamo-neurohypophyseal system (HNS) explants. This approach is particularly useful since these neural explant models contain only the magnocellular neurones in the SON together with their axons terminating in the neural lobe and neurones in the organum vasculosum of the lamina terminalis (OVLT) region. This enables the determination of the direct effects of sex steroid hormones upon the magnocellular neurones, as

opposed to their actions on other loci that might secondarily influence the neurosecretory system. These perifused explants show a characteristic response of VP and OXT with both an increase in their peptide release and mRNA content in response to increased osmolarity (Sladek & Knigge, 1977; Yagil & Sladek, 1990). By using these neural explants, Swenson and Sladek 1997 demonstrated that there was no significant difference in VP release stimulated by increased osmolarity between the explants obtained from sham-operated intact *vs.* gonadectomised male rats (Swenson & Sladek, 1997). Furthermore they have shown that exogenous T and its metabolites, DHT, and E_2 at dose which mimics the physiological plasma concentration, inhibited osmotically stimulated VP (and OXT) release from these explants while *only T and E_2*, but *not DHT*, also *inhibited* an increase in VP mRNA content following induced osmolarity. Moreover this inhibition in VP mRNA content was still present using BSA-conjugated E_2 suggesting that non-genomic action of steroids are involved. Thus it seems that the effects of steroids E_2, T, and DHT upon magnocellular VP mRNA accumulation in response to osmotic stimuli observed in *in vivo* and *in vitro* studies are in opposite directions.

Since the *in vitro* model lacks many of the afferent inputs that are present *in vivo*, it remains possible that effects of sex steroid hormones *in vivo* might be acting at sites other than or in addition to the SON, which in turn influence the activity of these magnocellular neurones. These loci include the SFO, MnPO, OVLT, NTS and VML, some of which have also been shown to contain ER and are known to be influenced by sex steroids (Akaishi & Homma, 1996a; Akaishi & Homma, 1996b; Blache, Batailler, & Fabre-Nys, 1994; Pfaff & Keiner, 1973; Rosas-Arellano, Solano-Flores, & Ciriello, 1999; Simerly, 1990). It would be interesting to look at the regulation of gonadal hormones as well as the receptors in these structures using various hormone and endocrine manipulations. This perhaps might enhance understanding of how sex steroids could affect the neurohypophysial system observed in this study.

Despite the rather conflicting results of previous reports both *in vivo* and *in vitro*, it is clear that sex steroid hormones do influence the function of the magnocellular neurosecretory system. Collectively these findings suggest a modulatory role for sex steroid hormones in hypothalamic OXT and VP gene expression. Whether E_2 and/or ER-β has a stimulatory or inhibitory effect, and whether such an action is direct or indirect it is not at all clear at present. Perhaps the role of E_2 and/or ER-β in the magnocellular neurones, on the other hand, might not be involved in the regulation of the OXT or VP genes, but in the regulation of some other genes. Notably, CRF mRNA in the magnocellular neurones is massively up-regulated following salt-loading (see Chapter 1). Moreover, the gene encoding CRF has been identified to contain the ERE (Vamvakopoulos & Chrousos, 1993), providing the evidence for direct genomic regulation of CRF by E_2, perhaps via ER-β present in these neurones. The exact role of ER-β remains to be determined.

b)   Ventral parvocellular neurones

The paraventricular nucleus, in addition to its magnocellular peptidergic projections to the posterior pituitary, contains parvocellular oxytocin- or vasopressin-containing neurones projecting to the locus coeruleus, nucleus tractus solitarii (NTS), dorsal vagal complex, and the intermediolateral cell column in thoracic levels of the spinal cord (Saper *et al.*, 1976). Some of these neurones project directly to sympathetic preganglionic neurones, and are involved in autonomic regulation by the PVN, particularly in defence against plasma volume expansion (Coote *et al.*, 1998; Gilbey *et al.*, 1982). This current study has shown that ER-β mRNA expression in these neurones markedly responded to changes in circulating E_2 as well as during physiological perturbations. OXT evokes hypertensive responses via the dorsal vagal complex, while the responses of dorsal vagal complex neurones to OXT are altered by E_2 exposure (Tian & Ingram, 1997; Tolchard & Ingram, 1993). These neurones showed a significant increase of ER-β mRNA expression after repeated stress, but not following ADX and CORT replacement (although this followed the same direction as the magnocellular neurones, it was not statistically significant). It is likely that these neurones are activated during repeated stress to co-ordinate the

autonomic responses. They showed a modest decrease in ER-β mRNA expression after salt-loading (*ca.* 12% reduction, but nonetheless significant), a condition in which autonomic drive to the cardiovascular system and input from baroreceptors will change. Thus, it seems that for these neurones, the expression of ER-β mRNA may be related to their activity, and sex steroid influences.

Interestingly a report by Estacio *et al.* 1996 showed that following immobilisation for 1 h in the rats, ER-immunoreactivity (ir) was increased within the PVN (Estacio *et al.*, 1996). Although the study did not indicate specifically which subdivision within the PVN the increase in ER-ir occurred and while the antibody employed in their study was a polyclonal antibody generated against the rat ER-α, this also may have detected the immunoreactivity for ER-β, given that the two rat ER peptides share a considerable degree of homology (see Chapter 1). So the increase in ER-ir observed in their studies conceivably was due to an increase in the ER-β protein within the ventral parvocellular neurones following an immobilisation parallel (similar) to the increase in ER-β mRNA observed in the present study.

5.5.4. Mechanisms regulating ER-β mRNA expression: Relationship with hydromineral balance, states of activation or hormone signal?

Since ER-β is capable of binding to oestrogenic compounds and can stimulate the transcription of oestrogen-responsive genes via an ERE this suggests that it functions as a ligand-regulated transcription factor. The presence of both ER-β mRNA as well as protein has been previously characterised in the magnocellular neurones. The present finding which demonstrated that the ER-β mRNA transcript is indeed regulated in these neurones suggests that ER-β is a functional transcription factor mediating the effects of E_2 via genomic mechanism. Nonetheless, the effect of BSA conjugated-steroid hormone, including that of E_2, in regulating the VP gene in the magnocellular neurones described by Swenson and Sladek 1997 also indicates the potential involvement of a non-genomic mechanism (also see Sladek *et al.*, 2000). It is not at all obvious how ER-β mRNA is regulated in the magnocellular

neurones under these circumstances. Whether one or more mechanism is involved is not clear.

a)   Genomic mechanism

At first glance, the dramatic decrease in the expression of ER-β mRNA in the magnocellular neurones that occurred following salt loading offers two obvious possibilities;

1) The expression of ER-β mRNA is related to *hydromineral status* (or Na$^+$?)

2) The expression of the transcript is related to magnocellular *neuronal activity,* a consequence of altered plasma osmolarity

An increase in plasma osmolarity (or hypernatraemia) following salt loading is associated with a down-regulation in the expression of ER-β mRNA in the magnocellular neurones. Conversely, there is a slight increase in the expression of the transcript (observed at the level of film optical density but not by grain density per neurone) during mid-pregnancy when there is an alteration in plasma osmolarity (and volume) with a tendency towards hyponatraemia (see Chapter 1). Similarly, adrenocortical insufficiency, such as following bilateral ADX, is associated with hyponatraemia and results in an increase in ER-β mRNA expression. So the expression of ER-β mRNA seems to vary in relation to alteration in plasma Na$^+$ (mineral) concentration. The problem is how the *actual solute Na$^+$* could influence the expression of ER- β mRNA? Does it somehow *directly* influence the transcription of the gene encoding ER-β or perhaps alter the stability of the transcript? This seems unlikely. So the effect of changes in Na$^+$ content upon the expression of ER-β mRNA would probably be related to an alteration of the activity of neurones consequent on changes in plasma osmolarity instead. Moreover, it is not clear if the reduction in the ER-β mRNA expression is a *consequence* of increased

osmolarity (suggested by the present study), or whether this reduction in the expression of the transcript *allows* the effect of increased osmolarity upon the accumulation of the VP and OXT mRNA to occur (suggested indirectly by Crowley & Amico, 1993). What is the causal relationship between the two?

Looking from the neuronal activity perspective, the expression of ER-β mRNA seems to be associated with an alteration in the activity of the magnocellular neurones. An increase in plasma osmolarity following salt loading excites magnocellular neurones leading to an increase in the firing rates and hence the increased activity of both OXT and VP neurones. This is associated with a decrease in ER-β mRNA expression in the magnocellular neurosecretory cells. Similarly, adrenal insufficiency is associated with elevated plasma VP release and its mRNA synthesis, despite the presence of hyponatraemia, indicating the activation of the magnocellular neurones. Thus, the finding that bilateral ADX led to an increase in the expression of ER-β mRNA, opposite to that observed during salt loading, suggests that either lack of corticosteroids or the expected hyponatraemia (as a result of lack of aldosterone), rather than increased neuronal activity, was the dominant factor. Moreover, in spite of intense excitation of, in particular, the OXT neurones during parturition, albeit over a short time span, there was no change in the expression of ER-β mRNA in the magnocellular neurones.

Some possible explanations to reconcile the discrepancy might be that;

1)   It is not known whether ADX for 72 h led to an activation of VP/OXT neurone secretory activity; this was not explored. Apparently the expression of the mRNA for OXT and VP genes in the magnocellular neurones was found to increase following ADX in some studies (Carter *et al.*, 1993) (and Suemaru *et al.*, 1990, but only in the PVN neurones) but not in others (Davis *et al.*, 1986b; Swanson & Simmons, 1989; Young, Mezey, & Siegel, 1986b). Moreover, an increase in the VP mRNA content in the magnocellular neurones following ADX, indicated in some studies, is not necessarily a result of an increase in the mRNA synthesis. Rather this

could also be due to a *decreased degradation* of the transcript. Thus an increase in ER-β mRNA expression following ADX might not be related to the activation of neurones.

2)   In response to osmotic challenge, there is a continuous release of OXT and VP with an increase in 'non-burst' background firing activity of the magnocellular neurones. On the other hand, parturition evokes an intermittent intense 'burst-like' activity of OXT neurones. Moreover, a decrease in ER-β mRNA expression following salt loading was observed over a relatively prolonged period (72 h) while parturition in rats normally only lasts for 60-90 min. Thus the expression of ER-β mRNA might be dependent on a specific state of neuronal activation and/or perhaps require a longer period of activation. Sadly, the experiment to test this hypothesis seems without the bounds of possibility since normal parturition cannot be prolonged to 72 h, but it would be interesting to look at the expression of ER-β mRNA following shorter periods of osmotic stimulation (e.g. perhaps via acute intraperitoneal administration of NaCl). Another option would be to explore the regulation of ER-β mRNA during lactation since suckling induces a pulsatile 'burst-like' increase in the OXT neurone activity similar to that occurred during parturition but which continues over a period of 12-14 days before pups are weaned. This would be particularly interesting since changes in the sex steroid hormone profile during the first week of lactation are similar to those occurring around the end of pregnancy when OXT mRNA up-regulation in the magnocellular neurones has been observed (see above).

3)   Various inputs are involved in regulating magnocellular neurone activity in each of the conditions examined in the present study. For example, salt loading influences the activity of neurones in the circumventricular organs (e.g. SFO/OVLT/AP) which are indispensable for activation of the VP and OXT neurones (Hamamura *et al.*, 1992; Leng *et al.*, 1989). Thus changes in hydromineral balance following salt-loading will involve these structures, as well as inputs from the brainstem mediating information from baroreceptors (Hochstenbach & Ciriello,

1995). During pregnancy, relaxin acts to sustain an increase in electrical activity of the VP and OXT neurones by activating inputs from these rostral circumventricular structures (McKinley *et al.*, 1997). Thus it is possible that the changes in expression of ER-β mRNA that were observed under the different physiological conditions was a result of the pattern of changes in activity in these inputs to the magnocellular neurones contributing to the overall expression of the ER-β transcript. The underlying mechanism(s) remains to be further investigated.

b)   Non-genomic pathway

Although ER-β mRNA was detected and present in the perikarya, this does not necessarily mean that the protein would only be confined to the cytoplasmic compartment. Instead, this protein might be transported to the cell membrane and act as a membrane-bound receptor to mediate a non-genomic action of E_2. However, this perhaps requires further investigation since

1)   No such membrane-bound ER in the SON or PVN has been reported to date. Moreover, most of the previous immunocytochemical studies detected the presence of ER-β ir in the nuclear compartment and not in the perikarya (except in the hippocampus and lateral septum (Li *et al.*, 1997).

2)   If ER-β protein was to be transported to the cell membrane, this perhaps requires assistance from a transporting molecule. No such molecule is known for ER-β thus far.

However, the apparent non-genomically mediated action of E_2 (and T) upon inhibiting the accumulation of OXT and VP mRNA in the neurohypophysial explant substantiates the existence of a non-transcriptional pathway. Moreover, E_2 has also been shown to act rapidly to increase release of OXT and VP from the dendrites of SON neurones (Wang, Ward, & Morris, 1995). There are also rapid effects of E_2 on the electrical activity of the magnocellular neurones (Israel & Poulain, 1999).

Conclusion

It is not known whether ER-β when expressed alone, in sites such as magnocellular SON and PVN neurones, is functional *in vivo*, although *in vitro* ER-β homodimers bind to DNA, and ER-β appears to contain the AF1 and AF2 elements to regulate transcription (Labrie *et al.*, 1999; Tremblay *et al.*, 1999). If ER-β is active alone *in situ*, then it could explain actions of E_2 on OXT and VP neurones that are indicated by other previous studies.

It seems that the effects of sex steroid hormone on the neurohypophysial system and the expression of the ER-β mRNA are not particularly robust. However, under conditions such as pregnancy, ADX or those related to alterations in plasma osmolarity there may be altered effects of E_2 as a consequence of changes in the expression of ER-β mRNA observed in this study. The study leaves open the question of the physiological significance of the influence of sex steroids and/or ER-β in regulating the neurohypophysial system and whether such an effect is stimulatory or inhibitory to the magnocellular neurosecretory system is not at present known. It is clear, however, that the ER-β, at least at the level of mRNA, can and is indeed regulated in these neurones, and this is expected to contribute to the sensitivity of the magnocellular neurosecretory system to E_2. Thus, the influence of sex steroids may decrease in conditions where the roles of OXT and VP in regulating hydromineral homeostasis are paramount, and conversely may increase when corticosteriod secretion (and/or action?), is minimal, then evoking expression of characteristics in these neurones appropriate for their adaptive roles. In the medial ventral parvocellular PVN neurones, the increased expression of ER-β mRNA following repeated stress, but evidently not as a consequence of regulation by glucocorticoids, may enhance regulation of these neurones by E_2 to modulate the autonomic responses.

Undoubtedly the significance of the recent discovery of ER-β and its expression in various structures of the brain including the hypothalamic PVN and SON gives rise to other possible mechanisms of how sex steroids could influence the brain. This is not to say that the mechanism of E_2 action would be only via a genomic pathway.

Chapter 6

General discussion

Although E_2 is generally regarded as a sex steroid hormone, a growing body of evidence in recent years suggests roles of E_2 beyond those associated directly with reproduction. This is also reflected by the extensive expression of both ERs in structures that are not typically involved in reproductive physiology including brain areas that are important for learning and memory such as the hippocampus, as well as autonomic function and homeostasis in a number of the hypothalamic nuclei .

6.1.   Distribution of ER-α and -β mRNA in rat brain

The present study revealed a distinct pattern of expression for both ER-α and -β mRNAs within the rat brain. This suggests the significance of each receptor subtype in mediating specific biological effects in a cell type-specific manner. The degree of expression of each transcript varies considerably from one region to another. Moreover, each transcript is also differentially expressed in a number of individual brain nuclei, as clearly seen in the PVN in the case of ER-β mRNA and in the amygdala for both ER transcripts. Such heterogeneity in the degree and pattern of expression of both ER mRNAs, if translated into receptor protein, could allow E_2 to elicit a pattern of actions in the correct cell types at the right level appropriate for its biological roles. As further demonstrated in Chapter 4 and 5, the expression of both ER transcripts changes with the diverse functional states examined. Such findings indicate the dynamic nature of ER mRNA expression, and suggest shifts in the influence of E_2, and/or other endogenous ER-β ligands, upon specific physiological processes under different conditions.

Ultimately, the impact of the ER-α/-β receptor genes will only be exerted when the transcript is translated into the receptor protein. Nevertheless, the distribution in the brain of both ER mRNAs observed in this study is generally in good agreement with a number of previous reports, both at the level of the mRNA and protein, suggesting that the pattern of expression of ER mRNAs seen here is likely to reflect the subsequent expression of the receptor protein.

6.2.   ER-α and -β mRNAs and the hippocampus

Both ER-α and -β mRNAs are evidently expressed in the pyramidal neurones of the hippocampus and in the granule cell layer of the dentate gyrus. We have shown that the expression of the two ER mRNAs is differentially regulated by sex steroid manipulation in a region- and sex-specific and time-dependent manner. On the other hand, regulation by another hormone signal, specifically from glucocorticoid, was only evident for ER-β and occurred in the hippocampus only in the CA1 subfield where adrenalectomy resulted in a decrease in ER-β mRNA expression, an effect which was reversed upon corticosterone replacement. In contrast stress, which increased corticosterone secretion, did not induce any significant change in the expression of either transcript in any of the subfields examined.

Changes in the expression of ER mRNAs in the hippocampus observed in the present study suggest functional importance of ER. The ER expressed here might orchestrate the morphological changes in the hippocampal neurones that follow changes in sex steroid hormone exposure. Experimentally, exogenous E_2 treatment as well as an increase in E_2 secretion during the pro-oestrus period of the natural oestrous cycle results in increased spine density and *de novo* synapse formation on the pyramidal neurones. Such increases promote synaptic connectivity of the hippocampal neurones contributing perhaps to an enhancement of long-term potentiation, the long-lasting change in excitatory synaptic transmission that seems to be involved in the process of learning and memory.

6.2.1. Physiological implications

With an increasing life span women are more likely to spend a large part of their lives in an oestrogen-deficiency state. Many physiological changes including a decline in cognitive performance as well as an increased susceptibility to neurodegenerative disorders such as dementia accompany the loss of ovarian hormones in postmenopausal women. Oestrogen replacement therapy in ageing women has been shown to improve cognitive function and delay the onset of Alzheimer's disease (Fillit *et al.*, 1986; Tang *et al.*, 1996) as well as to benefit cognition in non-demented women (Jacobs *et al.*, 1998; Resnick, Metter, & Zonderman, 1997).

Thus the role of ER in the hippocampus is perhaps to mediate actions of E_2 in maintaining aspects of cognition that is dependent on the hippocampal formation. It might also be involved in modulating activity of the HPA axis, though paradoxically E_2 enhances responsiveness of the HPA axis, and the increased glucocorticoid potentially damages hippocampal neurones; however, the parallel protective action of E_2 may offset this.

6.3. ER-β mRNA in the hypothalamic SON and PVN

The magnocellular neurosecretory system is modulated by sex steroid hormones. Previous studies have failed to demonstrate expression of ER-α in the magnocellular neurones, so the discovery of ER-β here may explain the enigma of how E_2 could act on them. In this study we have shown that there is a weak regulation of ER-β mRNA expression in the magnocellular neurones by sex steroid hormones. On the other hand, there was a striking increase in the expression of ER-β mRNA here following ADX while a large decrease in expression was observed following activation of these neurones by a chronic hyperosmotic stimulus. Thus ER-β mRNA expression may be negatively regulated by basal secretion of adrenal corticosteroid, and negatively regulated by hyperosmotic cue. The more than 6-fold variation in ER-β mRNA expression in the magnocellular neurones from salt-loading

to adrenalectomy could profoundly alter the sensitivity of these neurones to E_2 under these conditions. In contrast, the expression of the ER-β transcript in the ventral parvocellular PVN neurones involved in autonomic regulation was evidently modulated by the sex steroid hormone treatment while salt loading and ADX produced only modest changes in expression of such transcript. Repeated stress enhanced ER-β mRNA expression only in the ventral parvocellular PVN neurones while leaving the expression of this transcript unchanged in the magnocellular neurones. The expression of ER-β mRNA in both groups of neurones hardly changed during pregnancy and parturition, despite the large sustained increase in E_2 secretion in pregnancy.

It is not clear at present what the physiological role of ER-β in the hypothalamic SON and PVN neurones is. The genes encoding peptides synthesised by magnocellular neurones, OXT and VP, are the obvious candidates to be regulated by E_2 (or other endogenous ligand). However, there are also many others expressed in these two nuclei that could be regulated via ER-β (see Levin & Sawchenko, 1993). Such regulation could be direct, via an ERE in the target gene, or indirect, via an ERE in another gene whose product could then regulated the OXT/VP genes. The upstream regulatory sequences of the OXT gene constitute a composite hormone response element, similar to an ERE which allows binding of many nuclear transcription factors including thyroid hormone, retinoic acid and numerous orphan nuclear receptors (see Burbach et al., 1998). This suggests that these transcription factors, in addition to ER-β, could perhaps interact and potentially control the expression of the OXT gene. For instance, both thyroid hormones and E_2 can activate the rat and human OXT gene promoter in vitro (Adan & Burbach, 1992). Treatment with tri-iodothyronine (T_3) in vivo increased hypothalamic OXT mRNA content, pituitary OXT peptide and plasma OXT concentration (Adan et al., 1992). Indeed, as pointed out in a recent study, thyroid hormone interferes with E_2 in regulating expression of the rat OXT gene. Dellovade et al. 1999 have shown that treatment of ovariectomised (OVX) rats with oestradiol-benzoate (EB) alone did not affect OXT mRNA expression, but in OVX plus thyroidectomised (TX) rats the same hormone

treatment significantly increased OXT gene expression. Moreover exogenous administration of T_3 inhibited an EB-induced increase in OXT mRNA in OVX/TX rats (Dellovade, Zhu, & Pfaff, 1999).

Thus thyroid hormone normally inhibits, and conceals, a stimulatory action of E_2 on OXT gene expression. The present and many previous studies were conducted on rats in a, presumably, euthyroid-state. It thus remains possible that the different responses of the genes in the magnocellular system, at least for OXT, to E_2, whether being stimulatory, inhibitory, gradual or even a complete absence of response, could be due to the lack of control over thyroid hormone status. That said, it does not follow that actions of E_2 upon OXT gene expression are entirely secondary to (or dependent on) the effects of thyroid hormone, but rather this consideration demonstrates the complex interaction of a given set of genes capable of modulating one another to influence the expression of a target gene.

6.3.1. Physiological implications

The ER-β expressed in the magnocellular neurones might be involved in modulating the physiological activity of these neurones. Since VP plays a pivotal role in the control of fluid and electrolyte balance it is likely that E_2, or other ligands, could potentially modulate such a physiological process by changes in the responsiveness of the neurones. It is possible that E_2 might perhaps be involved in altering the sensitivity of the osmotic response of VP in the face of changes in blood volume or pressure as found, for example, during pregnancy.

6.4.   Future studies

6.4.1. Behavioural studies: a knock-out model?

One of the approaches that has been commonly used in assessing the function of any particular gene is via interpretation of the consequences of disruption of the gene in studies on knockout mice. However, this is not as straightforward in the case of the ER-β since the wild-type mice themselves do not normally express ER-β, at least not at the level of mRNA, in the magnocellular SON neurones *(although expression is present in the PVN)* (Shughrue *et al.*, 1997). This suggests that perhaps in mice ER-β may not play any major role in magnocellular neurone physiology. Indeed, although no direct study has been reported on the progression of parturition itself, a study of βERKO mice has shown that these homozygous knockout mice have successful pregnancy, albeit with fewer and smaller litter sizes. Moreover, they have normal breast development and can lactate normally (Krege *et al.*, 1998) suggesting that, at least during lactation, OXT production is not compromised by the lack of ER-β. Whether these findings indicate that ER-β does not mediate any physiological function upon the magnocellular neurones in preparation for parturition, as also indirectly suggested by the lack of changes in the expression of ER-β mRNA during pregnancy and parturition observed in the rats in the present study, or simply reflects a species difference in the expression and function of this receptor between rats and mice is not clear.

Incidentally, shortly following the production of αERKO mice in 1994, transgenic mice over-expressing the wild-type mouse oestrogen receptor-α were also generated. Intriguingly, these transgenic mice demonstrated several aberrant reproductive phenotypes including, in particular, delayed parturition (prolonged up to 4 days beyond normal gestation of 19 days), prolonged labour (up to 3 days to deliver all pups) as well as an increased incidence of still born litters (Davis *et al.*, 1994). Moreover, administration of OXT was not able to rescue females with difficult labour. The finding suggests that the progress of parturition is disrupted in the presence of ER-α over-expression although this might reflect ER functions in the

reproductive tract rather than in the magnocellular neurones since OXT cannot rescue difficult labour while ER-α is not found to be expressed in the magnocellular neurones.

6.4.2. Differential ER-β expression in magnocellular neurones

Although both OXT and VP neurones share the same anatomical location, the SON and the PVN, both peptides are rarely expressed in the same neurones (Kiyama & Emson, 1990; Vandesande & Dierickx, 1979). Moreover, the two peptides generally mediate distinct biological effects. Despite the ubiquitous distribution of ER-β mRNA in the SON which does not appear to be restricted to any particular portion of the nucleus, the basal expression as well as the expression during physiological perturbations of the ER-β mRNA in the OXT-containing *vs* VP-containing neurones might be different. The use of double-labelling histochemistry would allow the detection of OXT or VP peptide and of ER-β mRNA simultaneously thus enabling the determination of any differences in the expression of the transcript between the two populations of neurone.

As discussed in Chapter 5, the expression of ER-β mRNA following salt loading appears to be inversely related to the activity of the neurones. However, the discrepancy in the response of ER-β mRNA expression between salt loading and the lack of changes of the transcript during parturition suggests that the expression may also be governed by other factor(s). The difference might lie in the large time lag discrepancy (72 h salt-loading *vs* 2 h parturition) or the pattern of neuronal activity between the two physiological processes. The advantage of the prolonged time scale during lactation (several days) plus the similar pattern of neuronal activity to that in parturition (intermittent bursting discharge) is thus of key importance to pursue.

Moreover, lactation is probably the only known physiological process where there is a selective release of OXT without VP from the neurohypophysis, at least in a pulsatile pattern during suckling (hence the OXT-knockout mouse cannot transfer milk to its young). Nonetheless VP neurones are also evidently activated in lactation,

probably consequent on the loss of water accompanying milk transfer. Thus by studying ER-β mRNA expression in lactation, one could perhaps begin to partially dissect the response of ER-β mRNA between the two different groups of neurones in a physiological setting.

Another approach in evaluating roles mediated by ER-β (as well as ER-α) is perhaps by using an antagonist, sub-type selective, to specifically inhibit the actions of E_2, or other ligand, upon the target tissues (Sun et al., 1999) including the SON/PVN and the hippocampus.

6.5. Criticism of the experiments

The experiments conducted in the present study used the ISH approach to determine ER mRNA expression. However, the crucial step from the point of view of function is when these transcripts are translated into the receptor protein to mediate the biological effects. In some instances, it has been shown that the mRNA levels do not correlate with receptor protein levels (Uhl, Zingg, & Habener, 1985). The use of immunocytochemistry (ICC), particularly when combined with ISH on the same tissue section, allows the detection of the receptor protein and mRNA simultaneously thus providing the crucial information about translation. However, although ICC does detect the receptor protein, these molecules can exist in inactive forms that either cannot bind to hormones (cryptic receptors), fail to trigger a transduction system (desensitised receptors), or cannot bind to DNA (untransformed receptor). Mono- and poly-clonal antibodies employed in ICC may not be able to distinguish among these different forms (Bolander, 1994b). Moreover ICC, in theory, does not allow an accurate determination of the receptor protein level, except when coupled with fluorescence. On the other hand ISH, when employed within a linear range, gives a hybridisation signal which is quantitatively related to the level of the receptor mRNA. Another approach would be perhaps by western blot analysis which detects the receptor protein, although this technique does not permit the precise anatomical localisation of the protein. This could be particularly problematic especially when

dealing with tissue of high heterogeneity like the brain. Nonetheless this technique is quantifiable.

The OXT and VP genes are the most obvious targets for ER-β in the magnocellular neurones. However, the present study did not study whether there was any change in the activity of the OXT and VP genes in the neurosecretory neurones following stimuli such as salt loading, ADX or parturition. Nonetheless previous studies have shown that activation of OXT and VP gene expression (as indicated by an increase in their hypothalamic mRNA content as well as a depletion of the neural lobe peptide) in the magnocellular neurones via 2% NaCl ingestion occurs within 24 h of initiation of the treatment (Jones & Pickering, 1969; Lightman & Young, 1987) with the effect persisting by for at least 12 days (Lightman & Young, 1987). Similarly, parturition has been repeatedly shown to induce activation of the magnocellular, in particular the OXT neurones (Douglas *et al.*, 1998; Lin *et al.*, 1995; Summerlee, 1981). Despite all that, it is clear that the expression of ER-β mRNA is indeed regulated in the magnocellular neurones under the conditions examined. Whether these changes in the expression of ER-β mRNA is associated with, regulates or is regulated by, the expression of OXT and/or VP genes is subject to further investigation.

Conclusion

In the brain, the gonadal steroid E_2, and hence its precursor T, (and maybe other products?), profoundly affects the activity and the connectivity of certain neuronal populations. Its actions are principally mediated by genomic mechanisms that rely on the expression of intracellular ER. The recent discovery of ER-β, in addition to ER-α, expands ways by which E_2 could modulate the responsive genes, thereby increasing the plethora of functions E_2 could influence in the CNS.

Although the molecular mechanism underlying the changes in ER mRNA expression are far from being understood, the preliminary data obtained from the present study provide an initial step in elucidating the influence of E_2 upon two specific neuronal structures. It is clearly demonstrated, however, that the expression of both ER transcripts is subject to multiple mechanisms of regulation as indicated by the temporal-, regional-, as well as sex-dependency in the pattern of expression of both ER mRNAs. Moreover, the regulation of the ER-β transcript is also influenced by physiological cues such as by osmotic stimulation in functionally relevant neurones.

The functional significance of these findings is the implication for changing the effectiveness of E_2 on neurones in the hippocampus and the SON and PVN as their expression of ER-β mRNA increases or decreases. Future studies on the genes that regulate as well as those that are regulated by ER in these and other relevant loci should yield valuable data contributing to a better understanding of the roles of E_2 and their receptors in neurobiology.

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