REGULATION OF 11-BETA-HYDROXYSTEROID DEHYDROGENASE IN RAT BRAIN AND CLONING OF THE GENE PROMOTER REGION.

by

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A thesis submitted for the degree of Doctor of Philosophy

UNIVERSITY OF EDINBURGH

1992
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DECLARATION

I declare that the studies presented in this thesis are the result of my own independent investigation unless otherwise indicated in the text.

This work has not been and is not being concurrently submitted for any other degree.
ACKNOWLEDGEMENTS

I am indebted to Professor Christopher R. W. Edwards who accepted me in his department and who has always shown support and enthusiasm for my work.

I would like to thank my supervisor, Dr Jonathan Seckl, who played myriad important roles throughout my three years in the department. I am particularly grateful for his encouragement, guidance, advice, and discussion and also for his generosity and his good sense of humour.

I am indebted to Dr Brian Walker who helped me a lot, in the last two years, from very useful discussion on the "shuttle" or general physiology, through computer processing and English grammar, to general encouragement and friendship.

I am very grateful to Dr Paul Teelucksingh for his help and his true sympathy, friendship and encouragement.

I am very grateful to Dr Karen Chapman for her very useful advices on molecular biology techniques and for her criticism on the last manuscript and part of this thesis, as well as being always enthusiastic and available to share with me her vast knowledge on gene transcription.

I wish to thank Drs John Mullins, Bob Hill, Ian Jackson, and Benoit Arveiler for their help on designing some experiments.

I would like to thank the staff of the Biomedical Research Facilities Center for their help.

I wish to thank the staff of Medical photography and Medical artistry for their patience in doing some work for me.

I would like to thank all the staff of the department of Medicine, and I am particularly grateful to Mrs Greta Proven, Mrs Barbara Beattie and Mr Daniel Burt for their great help and sympathy.

I wish to thank Mark, Rafn, Alison, and Brian with who I shared an office, for their help and their friendship.

This work was supported by grants from the Wellcome trust/Royal Society of Edinburgh, the Scottish Hospital Endowments Research Trust and the Sir Stanley and Lady Davidson Research Fund.
À mes parents et à mes deux frères, Jean-Paul et Bruno, pour toute l'affection que je leur porte et pour leur continuel encouragement au cours de mes longues études.

À ma nièce et filleule Joséphine

À mon neveu Patrick, à mes nièces Armelle, Tania et Kay et à leur dévouée maman, Brigitte.

À Pascale Pennarun qui remplace la soeur que je n'ai pas eu, ma meilleure amie depuis 10 ans.

À ce beau pays qu'est l'Écosse....
à Benoît
ABSTRACT

Adrenal corticosteroid hormones play diverse and important roles in development and homeostasis. Their complex effects are predominantly mediated by intracellular receptors, which are of two types; mineralocorticoid (MR, type I) and glucocorticoid (GR, type II). MR bind aldosterone and physiological glucocorticoids (cortisol, corticosterone) with equivalent high affinity in vitro, whereas GR show a low affinity for aldosterone but bind physiological glucocorticoids and the synthetic glucocorticoid dexamethasone with high affinity. In vivo, kidney MR selectively bind aldosterone despite a hundred-fold molar excess of circulating glucocorticoids. This selectivity is due to the high activity of the enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) which catalyses the reversible conversion of physiological glucocorticoids to inactive products, but does not metabolise aldosterone, thus preventing glucocorticoid access to renal MR. This thesis describes firstly the presence and subregional distribution of 11β-OHSD in rat brain using Northern analysis, in situ hybridisation, and an 11β-OHSD activity assay. The enzyme was not only found in aldosterone selective regions, such as periventricular areas of the hypothalamus, but also in hippocampus where corticosterone is the physiological ligand for MR, and, more surprisingly, in cerebellum which contains very low levels of MR. Therefore it was postulated that 11β-OHSD might modulate glucocorticoid access to both types of corticosteroid receptor in brain. Furthermore, brain but not renal, 11β-OHSD was up-regulated by chronic glucocorticoid but not aldosterone administration, suggesting a role for the enzyme in controlling long-term neuronal glucocorticoid exposure. The importance of 11β-OHSD in brain was further substantiated by the finding of high 11β-OHSD mRNA levels and activity in rat brain subregions in the early postnatal period, with regionally specific developmental patterns of activity. The enzyme may play an important role in the developing brain by protecting tissues from (or exposing them to) elevated corticosterone levels. To understand the molecular mechanisms underlying tissue-specific and ontogenic regulation of 11β-OHSD a rat genomic clone was isolated and partially sequenced. Differential promoter usage of the gene was demonstrated in kidney; three transcription start sites were detected.
within one kilobase of 5' flanking region of the gene, using primer extension and ribonuclease protection analyses. Corresponding transcripts were detected on Northern blots of kidney, but not liver RNA, using specific oligonucleotide probes. Sequence analysis revealed a number of putative transcription factor binding sites. These findings might explain, at least in part, tissue-specific differences in the regulation of 11β-OHSD mRNA and activity.
Chapter 1: General introduction
1.1 Adrenocortical steroids

1.1.1 Discovery of the corticosteroids

In the 1930's, various groups of workers began the isolation of pure crystalline compounds from adrenal extracts (Mason et al, 1936; Wintersteiner & Pfiffner, 1936). Advances in steroid chemistry led to the separation of numerous compounds and some were found to possess significant biological activity: corticosterone (compound B of Kendall), cortisol (compound E of Kendall), 11-dehydrocorticosterone (compound A of Wintersteiner & Pfiffner), 11-deoxycortisol (compound S of Reichstein), oestrone, and progesterone. In 1940, Hartman and Spoor prepared a concentrate producing high sodium retaining activity and the active principle was named the sodium factor. Further purification was achieved and its physico-chemical properties together with its hydrophylic nature led Simpson and Tait (1953) to call this compound "electrocortin". The definitive name of aldosterone was given after elucidation of the structure which showed that electrocortin was the 18-aldehyde of corticosterone (Simpson et al, 1954).

1.1.2. Biosynthesis of the adrenal cortical hormones

The adrenal cortex comprises three morphologically distinct regions. The outermost region, the zona glomerulosa, is composed of small clusters of compact cells and secretes aldosterone, the major mineralocorticoid. The cells in the middle layer, the zona fasciculata, are larger and are arranged in radiating cords. The innermost layer, the zona reticularis, is composed of smaller cells arranged in a loosely reticular pattern. Zona fasciculata and reticularis are functionally indistinguishable. They produce the glucocorticoids, cortisol and/or corticosterone and synthesise relatively large amounts of adrenal androgens.

All the adrenal cortical hormones are steroids derived initially from cholesterol, the basic ring structure of which is preserved throughout the various biosynthetic steps (Fig.1.1.).
Some of the steps involved in the steroidogenesis are shown in Fig. 1.2. The end products fall into two main categories, the C21 steroids, which have a two-carbon side-chain attached at C17, and the C19 steroids, which have either a =0 or -OH group at C17. The C19 steroids possess androgenic activity but removal of a further carbon atom (C19) results in C18 compounds with oestrogenic properties. The C21 steroids possess both mineralocorticoid and glucocorticoid activities and the further small differences in structure between them determine which of these predominates. Aldosterone structure is represented as a 11\(\beta\)-hydroxy,18-aldehyde but in solution the molecule harbours an 11,18-hemiacetal bridge (Edwards and Hayman, 1991). Cortisol is the major glucocorticoid in many species including man, but corticosterone is the predominant glucocorticoid in rat and mouse. Recent studies have shown that two distinct 11\(\beta\)-hydroxylase proteins are expressed in the adrenal cortex of rat (Lauber & Muller, 1989; Matsukawa et al, 1990), mouse (Domalik et al, 1991) and human (Curnow et al, 1991). One of these proteins, found only in the zona glomerulosa, performs all steps necessary for the synthesis of aldosterone. The second protein found in all cortical zones, performs 11-\(\beta\) and 18-hydroxylations but lacks the 18-oxidase activity needed for aldosterone biosynthesis. Thus, distinct forms of 11\(\beta\)-hydroxylase catalyse the formation of mineralocorticoids and glucocorticoids.

Adrenal steroid hormones are liberated as soon as they are synthesised and only small amounts can be detected in the gland. In the
Figure 1.2. Synthesis of (a) glucocorticoid hormones, (b) mineralocorticoid hormones, and (c) androgens.

(adapted from R.N. Hardy, 1981)
circulation, both glucocorticoids and mineralocorticoids tend to bind to plasma proteins; cortisol and corticosterone combine mainly (89.5%) with an α-globulin, which is called corticosteroid-binding globulin (CBG) or transcortin. A further 6.6% is bound to albumin and the remaining 4% is free. Only 62.6% of aldosterone is bound (41.6 % to albumin, and 21% to CBG) but the total amounts of aldosterone in the plasma (<400 pmol/l) are minute by comparison with total plasma glucocorticoid concentration (~140-700 nmol/l) (Dunn et al, 1981). The half-life of cortisol is 50-90 min as opposed to 15-25 min for aldosterone.

1.1.3. Actions on target tissues

1.1.3.1 Glucocorticoids

The glucocorticoids exert a multiplicity of actions on a wide range of tissues. They have an important role in the control of carbohydrate, fat, protein and purine metabolism and influence the function of the cardiovascular system, skeletal muscle, the central nervous system, lymphoid, connective and other tissues. They exert an important "permissive" action and thereby influence the responses of many tissues to other hormones e.g. catecholamines. They have a powerful anti-inflammatory effect and also increase the capacity to withstand almost any stress, whether it derives from the internal or external environment. In some species, e.g. sheep but not man, glucocorticoids play a crucial role in the initiation of parturition. The actions of glucocorticoids briefly reviewed thereafter are detailed in (Orth et al, 1991).

**Effects on metabolism:**

Glucocorticoids maintain the reserves of glycogen in the liver by multiple mechanisms. They activate glycogen synthase by dephosphorylation and inactivate the glycogen-mobilising enzyme glycogen phosphorylase. They stimulate breakdown of protein and release of amino acids from skeletal muscle. They also directly activate key hepatic gluconeogenic enzymes, such as glucose-6-phosphate and phosphoenol pyruvate carboxykinase (PEPCK). The PEPCK gene contains two glucocorticoid responsive elements (GRE) located in its 5'-flanking region that mediate activation of the transcription of the PEPCK gene by
interaction with the glucocorticoid-receptor (Type II) complex. Glucocorticoids also exert an anti-insulin effect, inhibiting glucose uptake and utilisation in peripheral tissues, in part by direct inhibition of glucose transport into the cells. Other gluconeogenic hormones such as glucagon and epinephrine, are ineffective without the permissive effect of glucocorticoids. Glucocorticoids enhance the sensitivity of lipolysis to catecholamines in target tissues and the glycerol released during lipolysis provides a substrate for glucose.

Effects on immunological function and inflammatory processes:

Various effects of the glucocorticoids on components of the immunological and inflammatory responses have been described. One of the principal glucocorticoid effects on immune cells is on their traffic to and from the peripheral circulation. The decrease in peripheral lymphocytes number is due to redistribution of lymphocytes from the intravascular compartment to the spleen, lymph nodes, thoracic duct, and bone marrow. Glucocorticoids exert immunosuppressive effects on T cells in vitro, such as inhibition of proliferation and inhibition of transcription of the gene for interleukin 2, a potent T cell growth factor. Glucocorticoids also inhibit the proliferation of B cells and monocytes and inhibit the phagocytic and cytotoxic function of macrophages. Glucocorticoids inhibit the mediators of inflammation such as histamine, a potent vasoactive agent, prostaglandins, and plasminogen activators which produce active kinins that cause the vasodilatation and increased capillary permeability characteristic of inflammation.

Effects on musculoskeletal and connective tissues:

Glucocorticoids exert direct effects on bone by inhibiting osteoblast function, thus decreasing new bone formation, and indirectly by decreasing intestinal calcium absorption.

Their catabolic effect on muscle protein (mentioned earlier) is the basis for profound myopathy that sometimes results from glucocorticoids excess.

Glucocorticoids modulate fibroblasts proliferation and a number of differentiated functions of fibroblasts. They also suppress synthesis of the extracellular matrix components, collagen and hyaluronidate. Therefore
excess glucocorticoids results in impaired wound healing and friable connective tissues.

Effects on fluid and electrolyte homeostasis:

Evidence of glucocorticoids modulation of blood pressure comes from patients with glucocorticoid excess, but the basis for the hypertension in such patients is not clearly understood.

A defect in free water clearance in patients with glucocorticoids deficiency is associated with increased plasma arginine vasopressin (AVP) concentrations. Administration of cortisol, but not aldosterone, restores water diuresis. Glucocorticoid receptors are present in AVP producing cells of the parvicellular division of the paraventricular nucleus and glucocorticoid deficiency increases the AVP mRNA levels in the paraventricular nucleus but not in the supraoptic or suprachiasmatic nucleus of the rat.

Glucocorticoids induce increased plasma atrial natriuretic factor (ANF) levels in intact and adrenalectomised animals. They stimulate increased ANF mRNA content and increase processing of the ANF(1-126) precursor into mature ANF(99-126). These effects explain suppression of natriuresis after adrenal enucleation (=removal of the adrenals, with the capsule and adherent zona glomerulosa cells left in situ)

Gastrointestinal effects:

Glucocorticoids have direct effect on sodium transport in the colon. The use of steroids analogues specific for glucocorticoid receptors and of mineralocorticoid receptors blockers demonstrated that this is a true glucocorticoid receptor-mediated effect. Moreover, it has been shown that in the rat descending colon Na.K-ATPase α1 and β-subunit gene expression is acutely regulated by dexamethasone but not aldosterone (Fuller & Verity, 1990).

Developmental effects:

Excess glucocorticoid inhibit linear skeletal growth in children, presumably due to, at least in part, direct inhibitory effects on bone and connective tissues as reported earlier.

Pulmonary differentiation is dependent on and is accelerated by glucocorticoids. Glucocorticoids induce synthesis and release of surfactant
by type II pneumocytes. The effect on surfactant gene transcription depend on glucocorticoids levels: lower concentrations are stimulatory but higher levels are inhibitory.

Neural crest cells are precursors of a variety of more differentiated cell types, including autonomic ganglion cells and adrenomedullary cells. Under the influence of glucocorticoids, neural crest precursor cells that invade the embryonic adrenal gland cease to express neuron-specific gene products, such as neurofilaments, and acquire the characteristic morphology of adrenomedullary chromaffin cells.

**ACTH secretion:**

The glucocorticoids strongly inhibit the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. This is discussed under "control of secretion", section 1.1.4.

**1.1.3.2 Mineralocorticoids**

Aldosterone is the most potent endogenous mineralocorticoid. It promotes reabsorption of Na\(^+\) and excretion of K\(^+\) from urine, sweat, saliva and gastrointestinal contents. Its most important physiological action is that which it exerts on the renal tubules, since electrolyte excretion is normally controlled by this means. Aldosterone acts on the distal convoluted tubules to promote reabsorption of Na\(^+\) in exchange for K\(^+\) or H\(^+\), and thereby increases extracellular volume and blood pressure. Aldosterone acts on the brain in some species to promote salt appetite and increase blood pressure (McEwen et al, 1986a, De Kloet, 1991).

Specific actions of glucocorticoids and mineralocorticoids in the central nervous system will be further detailed in Chapter 3 and 4.

**1.1.4. Control of secretion**

**Glucocorticoids**

The structure of the adrenal cortex is maintained by ACTH which also stimulates the synthesis and release of glucocorticoids and androgens.
ACTH interacts with membrane-bound receptors on the adrenocortical cells that activate adenylate cyclase and increase intracellular cyclic AMP (cAMP). This, in turn, activates a cAMP-dependent protein kinase (protein kinase A) that ultimately leads to activation of the first step in adrenal steroidogenesis: the conversion of cholesterol to pregnenolone. ACTH also enhances the later steps in steroidogenesis via cAMP and stimulates the synthesis of i) low-density lipoprotein (LDL) receptor which increases the uptake of cholesterol, ii) adrenodoxin which is needed for transfer of reducing equivalents, and iii) sterol carrier protein 2 which is required for transport of cholesterol from intracellular lipid stores to mitochondria.

ACTH secretion is regulated by multiple hormones. Corticotropin-releasing hormone (CRH-41) and arginine vasopressin (AVP) are physiologically the most important, but catecholamines, angiotensin II, serotonin, oxytocin, ANF, cholecystokinin, vasoactive intestinal peptide, PHI-27 (a gastrointestinal and hypothalamic peptide) and gastrin-releasing peptide have been implicated (reviewed by Jones & Gillham, 1988). CRH-41 is synthesised by neurons of the parvicellular division of the hypothalamic paraventricular nucleus (Swanson et al, 1983). Their axons project to the median eminence, where CRH is secreted in the hypophyseal portal blood and carried to the pituitary. CRH acts on the anterior pituitary corticotrope cells by binding to cell surface receptors and activating adenylate cyclase, thereby increasing intracellular cAMP levels and activating cAMP-dependent protein kinase A. This results within a few seconds in secretion of ACTH and subsequently in increased pro-opiomelanocortin (POMC) gene transcription (Giguère et al, 1982; Lundblad & Roberts, 1988).

In addition, there are three control and response systems which may operate simultaneously (Fig.1.3.): 1) an intrinsic circadian rhythm mediated by the hypothalamus and CRH-41, modified by light and sleep, and resulting in a fluctuating level of plasma cortisol (or corticosterone) with a maximum at about 06.00 h and with a minimum level at 22.00 h in man; 2) a response to mental and physical stress such as hypoglycaemia, hypoxia, asphyxia, hypotension, haemorrhage, anaesthesia and surgery, probably also via CRH-41 and ACTH; 3) a feedback loop with modulation of ACTH secretion by cortisol or corticosterone (and synthetic glucocorticoids) at the level of the pituitary, hypothalamus (PVN) and higher brain areas (hippocampus). It has been proposed that negative
feedback of corticosteroids on stress-induced HPA activity is mediated primarily by GR in the PVN and the anterior pituitary but MR and GR mediate coordinately the corticosteroid control of the hippocampal influence on the HPA axis, which is state-dependent, conditional, and modulatory. In addition, MR may mediate the control of basal activity of the HPA axis, particularly when glucocorticoids levels are low and occupy few GR (morning in rat, evening in man)(reviewed by De Kloet, 1991).

**Figure 1.3.:** Control of glucocorticoid secretion: the hypothalamo-pituitary-adrenocortical axis. —> : stimulation; —X : inhibition.

**Mineralocorticoids**

The secretion of aldosterone is controlled largely by the renin-angiotensin system and by potassium. ACTH and other POMC-derived peptides, sodium ions, and other agents such as AVP, beta-adrenergic
agents, serotonin, dopamine, ANF and somatostatin are minor modulators (Quinn & Williams, 1988).

Renin is an enzyme released from the juxtaglomerular cells in the kidney and cleaves the α2 globulin angiotensinogen, leaving the decapeptide angiotensin I. This in turn loses two further amino acids due to the action of angiotensin converting enzyme (ACE), which occurs mainly in the lungs. The resulting octapeptide, angiotensin II, is responsible for stimulating the synthesis and release of aldosterone. The release of renin is stimulated by many situations but particularly under circumstances such as a decrease in the filtered load of sodium in the renal glomerulus and a reduction in pressure in the afferent glomerular arteriole (Fig.1.4.) (Goodfriend et al, 1984).

![Diagram](Figure 1.4): The renin-angiotensin-aldosterone regulatory system. ACE= angiotensin converting enzyme. A I, A II & A III: angiotensin I, II & III.

The intracellular mechanism of aldosterone secretion has been extensively studied. There is a general agreement on the main role of calcium as mediator of aldosterone secretion. In glomerulosa cells, characterised by a sustained response to angiotensin II, a general two-branch model has been proposed: calmodulin and C-kinase. The calmodulin branch is involved in the initial response and is triggered through the increase of inositol 1,4,5-triphosphate and endoplasmic reticulum Ca++ mobilisation which leads to activation of various kinases, followed by increased aldosterone secretion. The delayed response is
mediated by the C-kinase system: the increase of Ca++ and diacylglycerol convert the C-kinase into its Ca++-sensitive forms with the production of phosphoproteins leading to increased aldosterone secretion. Other mechanisms have been proposed for potassium and ACTH: they increase Ca++ influx or activate the cAMP system without affecting diacylglycerol or inositol 1,4,5 triphosphate (Rasmussen, 1986).

1.2. Steroid receptors

1.2.1. Introduction

In the classic model, steroid hormones elicit their effects on target cells after traversing the cell membrane and activating endogenous intracellular steroid hormones receptors. These steroid hormone receptors function as hormone-dependent nuclear transcription factors that modulate cell function by altering the expression of specific genes or gene networks (Reviewed by Yamamoto, 1985).

In addition to the well-known nuclear actions of intracellular steroid receptors, direct steroid action on membranes may occur via membrane receptors. Rapid behavioral responses to short-term exposure to steroids have been reported, for example short-term exposure of rats to progesterone is associated with rapid changes in behavior (Schumacher et al, 1990).

Recently, Orchinik et al (1991) have identified a corticosterone-specific, high affinity receptor in synaptic membranes from an amphibian brain. Aldosterone and dexamethasone (a synthetic glucocorticoid) are poor ligands for this membrane-bound receptor which appeared to mediate male reproductive behavior.

Wohling et al (1992) described an aldosterone-specific membrane receptor in human mononuclear leukocytes. In contrast to the cytosolic mineralocorticoid receptor which does not distinguish between aldosterone and cortisol or corticosterone in vitro (see section 1.2.3.), aldosterone was found to bind to this aldosterone-membrane receptor with a Kd of 0.15 nM and was not displaced by cortisol or canrenone (a mineralocorticoid antagonist) at concentrations of up to 0.1 μM. This receptor appeared to mediate aldosterone stimulation of the sodium-
1.2.2. Steroid receptor superfamily

Within the past seven years, the human and rat cDNAs of the major nuclear steroid receptors have been cloned (reviewed by Green & Chambon, 1988; Evans, 1988; Berg, 1989; Beato, 1989; O'Malley, 1990; King, 1991). Comparative analysis of steroid receptor sequences with each other and with other nuclear receptors led to the identification of a steroid hormone receptor superfamily which includes the receptors for glucocorticoid hormone (GR), mineralocorticoid (MR), thyroid (TRα, TRβ and TRγ), oestrogens (ER), androgens (AR), progestagens (PR), retinoic acid (RARα, RARβ & RARγ), vitamin D3 (VDR). Other superfamily members have been identified, although their activating ligand is unknown. For this reason, these superfamily members are referred to as "orphans" (reviewed by O'Malley, 1990; King, 1991). Orphans includes two ER-related proteins called ERR1 and ERR2, COUP transcription factor, the nerve growth factor-induced NGFI-B protein, dioxin receptor, the hepatic nuclear factor HNF4, the drosophila gene encoded by E75 locus, and the drosophila gene tailless and certain oncogene products (v-erb A) and c-erb A identified subsequently as a thyroid hormone receptor (Weinberger et al, 1986; Sap et al, 1986).

Steroid hormone mediated transcriptional regulation involves five main reactions requiring different functional domains of the steroid receptor:

1) steroid hormone binding to its receptor and transforming it from an inactive to an active state. This involves dissociation of the receptor from the inhibitory protein Hsp90. 2) dimerisation of the receptor which is essential for efficient DNA-binding activity 3) transfer of the hormone-receptor complex from the cytoplasm to the nucleus (at least for the glucocorticoid receptor) 4) specific binding of the hormone-receptor...
complex to short DNA sequences called steroid response elements (SRE), found usually in the 5'-regulatory region of target genes 5) interaction with the transcription machinery which activates or represses basal transcription of genes containing SREs.

1.2.2.1. Functional domains of steroid receptors

Proteolytic digestion, mutational analysis and construction of chimaeric receptors have revealed a clear-cut domain structure shared by every member of the steroid receptor superfamily (reviewed by Green & Chambon, 1988; Evans, 1988; Berg, 1989; Beato, 1989; O'Malley, 1990; King, 1994). Sequence comparison has revealed a N-terminal region which is highly variable among species with respect to both sequence and length, a highly conserved DNA-binding domain and a C-terminal ligand-binding domain. The non-conserved N-terminal region corresponds to the major immunoreactive epitope of the glucocorticoid receptor (Carlstedt-Duke et al, 1982). The DNA-binding and ligand-binding domains appear to form independent structural and functional entities. Throughout all the steroid receptors these two domains, in particular the DNA-binding domain, share maximal sequence homology (Fig. 1.5).

In response to hormone binding, the ligand-binding domain (or region E) affect all aspects of receptor activity. This includes receptor dimerisation, nuclear localisation, DNA-binding and transcriptional activation.

Overlapping hydrophobic segments within region E promote dimerisation and steroid binding activity by forming a hydrophobic pocket within which one molecule of steroid hormone is proposed to bind. Two nuclear localisation signals have been defined for the GR (Picard and Yamamoto, 1987) one of them is encoded in the ligand binding domain and the other in the hinge region, and both signals would direct the hormone-receptor complex to the nucleus.

Transcriptional activation domains of the steroid receptors are located both in the ligand binding domain and in the N-terminal region of the molecule. Activation of transcription may involve interaction of the DNA-bound hormone-receptor complex with a component of the transcriptional machinery and initiation or stabilisation of the formation of a transcription initiation complex (Hollenberg & Evans, 1988).
Figure 1.5. Schematic amino acid comparison of members of the steroid hormone receptor superfamily. Primary amino acid sequences have been aligned on the basis of the regions of maximum amino acid similarity, with the percentage amino acid identity indicated for each region in relation to the GR. The amino acid position for each domain boundary is shown. Amino acid numbers for all receptors represent the human forms with the exception of v-erb-A and E75.

(adapted from Evans, 1988)
Another possibility is that steroid receptors displace nucleosomes thus allowing transcription factor recognition of previously inaccessible DNA sites (Richard-Foy & Hager, 1987; Cordingley et al, 1987).

The DNA-binding domain (also called region C) is sufficient for specific DNA binding in vitro and in vivo, although high affinity DNA binding requires additional information encoded within N- and C-terminal sequences (Danielsen et al, 1987). Region C is highly basic and contains a number of conserved cysteine residues, eight of which are involved in forming two zinc fingers (Evans & Hollenberg, 1988; Frankel & Pabo, 1988). Zinc fingers were first described in *Xenopus laevis* transcription factor TFIIIA in which a pattern of two conserved cysteines and two histidines residues are coordinated to a zinc atom (Miller et al, 1985; Lee et al, 1989). In the case of the glucocorticoid receptor each zinc finger consists of two pairs of cysteines co-ordinated to a zinc atom (Härd et al, 1990). Two copies of the zinc finger motif are present in the DNA-binding domain and the zinc ion present in each finger is essential for DNA-binding activity (Fig.1.6). The N-terminal zinc finger is involved in sequence-specific contacts; two amino acids, located at the beginning of the α helix which directly interacts with DNA, have been shown to be critical in determining specificity of GR versus ER (Mader et al, 1989; Danielsen et al, 1989). Mutational analysis in the second finger indicates that this region is also required for efficient DNA-binding (Danielsen et al, 1987; Hollenberg et al, 1987). In addition, there are evidence that for TR, which can bind both an ERE and a TRE, the second zinc finger is involved in discriminating between half site spacing of the SRE (Umesono & Evans, 1989).
Figure 1.6: Model of the dimeric complex between the GR DNA-binding domain and the GRE. The residues essential for discrimination between GRE and ERE as well as two AT base pairs in the center of each of the GRE-half site, indicating the differences between this GRE and the consensus ERE sequences, are coloured in red. Residues important for protein-protein interactions are coloured in green. (from Härd et al, 1990)
1.2.2.2. Steroid response elements

Many steroid hormone responsive genes have been cloned. Gene-transfer experiment and comparative analysis of hormone responsive genes has allowed identification of consensus sequences for steroid response elements (Martinez & Wahli, 1991). They can be divided into two groups: a) responses to glucocorticoids, progesterone, androgens and mineralocorticoids are mediated by a single element (AGAACANNNTGTTCT) and, GR, PR, AR, and MR all encode the "Gly-Ser" pair of specificity amino acids; b) responses to oestrogens, thyroid hormones, retinoic acid and vitamin D3 are mediated by ERE-like sequences that have in common the "Glu-Gly" pair of specificity amino acid.

This promiscuous DNA-binding behaviour observed amongst members of the steroid hormone receptor superfamily contrasts with the hormone-specific activation of genes in specialised cell types. This could be explained, in part, by the differential ability of steroid receptors to recognise distinct features of a common response element. For example, PR and GR binding to a GRE is very similar but mutations in the MMTV GRE differentially affect induction by progesterone or glucocorticoid (von der Ahe et al, 1986; Chalepakis et al, 1988). Also, it is becoming clear that steroid receptors contain promoter-specific and cell-type specific transcriptional activation domains that restrict the transcriptional activity of certain steroid receptors to particular promoters and cell types (Tora et al, 1989).

In addition, there is increasing evidence which suggests that the affinity of a particular steroid receptor for its response element can be modulated by accessory proteins (reviewed by Hager & Archer, 1991).

1.2.3. Corticosteroid receptors: how many types?

A second order of promiscuous behaviour has been observed between MR and GR. MR have been shown to bind and be transformed by both aldosterone and glucocorticoids in vitro (Krozowski and Funder, 1983). In vivo, MR bind to aldosterone in tissues such as kidney, parotid and colon, but cortisol (or corticosterone) is the physiological ligand to
MR in hippocampus and heart. Cloning of human GR (Hollenberg et al., 1985) and MR (Arriza et al., 1987) cDNAs has demonstrated that the two receptors are highly homologous; human MR and GR have 57% identity in the steroid-binding domain and 94% sequence identity in the DNA-binding domain (respectively, 59% and 76% for rat MR and GR). Furthermore, isolation of a rat MR cDNA from a brain cDNA library offered the possibility of demonstrating that the renal MR which bind aldosterone in vivo are identical at least at the level of the mRNA, to the brain MR which bind corticosterone in vivo (Arriza et al., 1988; Patel et al., 1989).

The hormone binding specificity of human MR has been investigated by Arriza et al (1988) in CV1 cells which lack endogenous steroid receptors. In these experiments an expression vector encoding the human MR (trans-vector) is co-transfected into cells with a reporter vector (cis-vector) as shown in figure 1.7.

**Figure 1.7.** The co-transfection assay

The reporter gene is a chimaera of two components: a regulatory region from a gene known to be sensitive to glucocorticoids (GRE from Mouse mammary tumour virus = MMTV) linked to the coding region of a gene whose expression can be easily measured (CAT=bacterial chloramphenicol acetyl transferase). MMTV promoter was chosen because a) the DNA-binding domain of the MR and the GR were found to be nearly identical (94% homology) and were therefore likely to have identical DNA binding specificity; and b) to date no mineralocorticoid sensitive gene has clearly been demonstrated. Glucocorticoids (cortisol and corticosterone) were found to be effective in activating both MR and GR, and, surprisingly, activated MR at a ten-fold lower concentration than GR (Kd :
5 nM versus 0.5 nM). The MR was thus found to have little intrinsic ability to discriminate between mineralocorticoids and glucocorticoids. Therefore, the questions posed were: what is the physiological role of MR as a high affinity glucocorticoid receptor in vivo; and what is the nature of the factor(s) conferring aldosterone selectivity upon MR in kidney and other epithelial tissues, despite the 100-fold higher concentrations of competitive circulating glucocorticoids?

In an attempt to answer the first question, Arriza et al (1988), prompted by the finding that GR and MR act through common responsive elements, speculated that MR could provide a high-sensitivity, low response function to glucocorticoids, while the GR would act with low sensitivity but high response. This is particularly relevant to cells in which the MR and the GR are coexpressed such as hippocampal neurons (Evans & Arriza, 1989).

The factor which confers in vivo aldosterone selectivity on renal MR was originally thought to be CBG. CBG sequesters glucocorticoids and, because of its differential tissue distribution, could influence local glucocorticoid concentration. High levels of CBG in kidney would reduce available glucocorticoids from plasma, conferring aldosterone selectivity, whereas levels of CBG in the hippocampus would suggest that glucocorticoids may be the predominant ligand for MR (Funder and Sheppard, 1987). This model was abandoned when it was discovered that 10-day-old rats lack CBG but still show aldosterone selectivity in kidney MR (Sheppard & Funder, 1987).

Relatively recent studies from two groups, our group in Edinburgh and John Funder's team in Melbourne, demonstrated that mineralocorticoid-specific actions of MR are determined, at least in part, by a steroid modifying enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) (Edwards et al, 1988; Funder et al, 1988). 11β-OHSD metabolises glucocorticoids to forms which do not bind to the MR, thus allowing aldosterone, which is not a substrate for the enzyme by virtue of its 11-18 hemiacetal bridge structure (Edwards et al, 1991), to selectively gain access to its receptors.
1.3 11β-hydroxysteroid dehydrogenase

1.3.1. Background and historical aspects

After secretion, glucocorticoids are actively metabolised in the liver and a variety of other tissues. Figure 1.8 depicts the principal pathways of cortisol metabolism in man. One of the transformations cortisol undergoes is its conversion to cortisone (corticosterone to 11-dehydrocorticosterone in rat), by oxidation of the hydroxyl group in position 11, to a keto group. This step is important in that glucocorticoids possessing a hydroxyl group at C11 are active whilst those with a keto group are inactive. The first studies on cortisol-cortisone conversion were done in the 1950s, where labelled cortisol (Hellman et al, 1954; Migeon et al, 1956; Peterson et al, 1955) or cortisone (Burnstein et al, 1953; Burton et al, 1953) was administrated to volunteers and the various metabolites produced were identified. It was found that conversion of cortisol to cortisone was reversible and mediated by the enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD). In 1960, Osinsky described 11β-OHSD activity in homogenates of human placenta and showed that the enzyme required NADP+ or NADPH, depending upon the direction of the reaction.


However it was only in the late 1980s that the crucial role of the enzyme was discovered after observations of congenital and acquired 11β-deficiency in man.
1.3.2. 11β-dehydrogenase deficiency: syndrome of apparent mineralocorticoid excess and liquorice-induced hypertension

The syndrome of apparent mineralocorticoid excess is a rare but often fatal cause of mineralocorticoid hypertension. Worldwide only 21 children and one adult have been reported (Shackleton & Stewart, 1990). The clinical picture is of severe hypertension with suppression of the renin-angiotensin-aldosterone axis and hypokalaemia. It was in the late 1970s that Ulick (1979) first documented a defect in the peripheral metabolism of cortisol to cortisone, as evidenced by marked elevation of the ratio of the urinary steroid metabolites tetrahydrocortisol (5β-THF) + allo-tetrahydrocortisol (5α-THF) to tetrahydrocortisone (THE) (see Fig. 1.8.). This impaired conversion was confirmed by giving 11α-3H-cortisol. When one mole of this steroid is converted to cortisone, one mole of 3H-H2O is produced. Patients with this syndrome produce virtually no 3H-H2O.

For several years the mineralocorticoid responsible for this condition was unknown. Numerous bioassays on plasma and urine from such patients failed to detect increased mineralocorticoid activity and hence the term syndrome of "apparent mineralocorticoid excess" (AME) was coined. In 1983, Oberfield et al documented sodium retention, potassium loss and hypertension, all suggesting marked mineralocorticoid excess, following ACTH and cortisol infusion in their AME patient. This effect of cortisol appeared to be mediated by mineralocorticoid receptors since administration of spironolactone, an aldosterone antagonist, resulted in reduction of blood pressure, natriuresis and antikaliuresis, and replacement of cortisol by the pure glucocorticoid dexamethasone, which bind to GR rather than MR, produced a marked natriuresis, potassium retention and recovery of the renin-angiotensin-aldosterone system. Oberfield et al (1983) postulated an abnormality in the MR, recognising cortisol as a mineralocorticoid. Stewart et al (1988) in Edinburgh suggested that renal 11β-dehydrogenase was an important physiological mechanism, protecting exposure of the kidney MR to cortisol by shuttling it to the inactive steroid cortisone. Failure of this mechanism in AME results in the kidney seeing cortisol as a potent mineralocorticoid. This mechanism is illustrated in Figure 1.9.
Figure 1.8. Major pathways of cortisol metabolism. The broad arrow indicates a shift of metabolism towards cortisol in the syndrome of apparent mineralocorticoid excess. DH= Dihydro; TH=tetrahydro.

(From Stewart and Edwards, 1991)
This mechanism was demonstrated by observing the effects of inhibiting the conversion of cortisol to cortisone in vivo. It had been recognised for many years that ingestion of liquorice can produce a mineralocorticoid excess syndrome. This was thought to be due to the binding of the active component of liquorice (glycyrrhizic acid = GI or its hydrolytic product glycyrrhetinic acid = GE) to the mineralocorticoid receptor. The affinity however is very low, \(10^{-4}\) that of aldosterone) and the circulating levels in patients on liquorice are much lower than would be required to activate the receptor. In addition, liquorice does not produce sodium retention in the absence of functioning adrenal tissue (see Stewart & Edwards, 1991 for review). This led Stewart et al to examine the possibility that liquorice acts by inhibiting 11β-OHSD. Their studies in normal volunteers confirmed that the sodium retention produced by liquorice is indeed associated with a significant change in cortisol metabolism indicating inhibition of 11β-OHSD (Stewart et al, 1987). Animal experiments performed in parallel with these studies in man have
confirmed that GI and GE inhibit renal $11\beta$-OHSD both in vivo and in vitro (Monder et al, 1989). In vitro a $K_i$ of $10^{-9}$ M for GE was observed, indicating potent inhibition. Furthermore, animal studies also show that carbenoxolone (the hemisuccinate derivative of GE) potentiates the mineralocorticoid effect of corticosterone, for example sodium transport in toad bladder (Gaeggeler et al, 1989; Brem et al, 1989) and in rat (Sou.ness & Morris, 1989). However, carbenoxolone may also inhibit $5\beta$-reductase as well as $11\beta$-OHSD (Latif et al, 1990), placing limitations on interpreting the effects of carbenoxolone as being solely related to altered glucocorticoid metabolism (Monder, 1991a).

1.3.3. Purification, production of antibodies and cloning of $11\beta$-OHSD

1.3.3.1. Purification of rat liver $11\beta$-dehydrogenase

A long-running debate about $11\beta$-OHSD enzyme is whether the $11\beta$-dehydrogenase and the $11\beta$-reductase are two separate enzymes, components of an $11\beta$-hydroxysteroid dehydrogenase complex, or a single multifunctional enzyme that contains both dehydrogenase and reductase activities. To determine which, if any, of these hypotheses is correct, Lakshmi and Monder, in New York, undertook the purification of $11\beta$-OHSD. They were successful in purifying the $11\beta$-dehydrogenase component of the complex but the purified enzyme was devoid of $11\beta$-reductase activity (Lakshmi & Monder, 1988). They therefore proposed that the $11\beta$-dehydrogenase and the $11\beta$-reductase were structurally two independent units. This was supported by the finding that AME patients are deficient in $11\beta$-dehydrogenase activity but they convert cortisone to cortisol normally.

Purification of the rat liver $11\beta$-dehydrogenase was achieved in a single step from detergent solubilised extracts of rat liver microsomes, after absorbing the enzyme on a NADP-sepharose affinity column. The molecular weight of monomers of the enzyme is 34 kD and the enzyme is a glycoprotein.
1.3.3.2. Production of antisera

Polyclonal antibodies were generated against the purified enzyme in rabbits. The antibodies were immunoprecipitins but 11β-dehydrogenase activity was not completely suppressed in the antigen-antibody complex, suggesting that the epitopic regions did not include the active site. Two antibody preparations, 56-125 and 56-126, used to detect 11β-dehydrogenase antigen in Western blots, generated different staining patterns for kidney, liver, brain, testis and heart. Using the two antibodies together, the total number of antibody-reacting components in kidney was three, and that in liver was two. In brain a shorter species of 26 kD was detected and in testis a 47 kD was found in addition of the 34 kD species present in every tissues and corresponding to the purified enzyme. These were the first data indicating that the enzyme may occur in several forms, and that the distribution of these forms is to some extent tissue-specific (Monder & Lakshmi, 1990).

Castello et al., (1989), in an independent study, generated a mouse anti-11β-OHSD monoclonal antibody from rat microsomal proteins. They detected a single 35 kD species on immunoblots of kidney. Interestingly, this antibody bound both oxidative and reductive 11β-OHSD enzymatic activities, as detected by capture of the antigen (solubilised renal microsomal proteins) by the anti-11β-OHSD antibody followed by incubation of this complex with $^3$H-corticosterone or $^3$H-11-dehydrocorticosterone and measurement by HPLC of the $^3$H-metabolites formed.

1.3.3.3. Cloning of cDNAs

Agarwal and coworkers (1989), in New York, isolated a rat liver cDNA clone encoding 11β-OHSD by screening a lambda expression library with the antibodies raised against 11β-dehydrogenase. Sequencing of a 1265 bp insert revealed an 861 bp open reading frame predicting a polypeptide of 287 amino acids with a molecular weight of 31.8 kD. The difference between this value and the previously reported 34 kD molecular weight of the enzyme was attributed to oligosaccharide residues, as the enzyme is a glycoprotein, which was confirmed by the finding that
two potential "Asn-X-Ser" N-glycosylation sites were located at residues 158-160 and 203-205. The cDNA was expressed in Chinese hamster ovary cells by transfection of a recombinant plasmid containing the 11β-OHSD cDNA driven by the SV40 promoter. It was found that both 11β-dehydrogenase and 11β-reductase activities were encoded by this cDNA. Nevertheless, these activities were expressed at levels that were too low to perform kinetics or physico-chemical studies, so that it was not clear if the reductase activity of this enzyme was physiologically significant. To address this problem the cDNA was then expressed in osteosarcoma cells infected with recombinant vaccinia virus which permit expression of relatively high levels of the enzyme (Agarwal et al, 1990). This study confirmed that the recombinant enzyme contained both oxidising and reducing capabilities. Its enzymatic properties were similar to those found in rat liver homogenates: molecular weight, sugar content, optimum pH and kinetics constants were comparable; 11β-reductase is quite labile but can be stabilised if accumulation of NADP+ is prevented; GE is a potent inhibitor of 11β-dehydrogenase but inhibits 11β-reductase poorly. Interestingly, 11β-dehydrogenase activity of cells treated with 50 ng/ml of A1-tunicamycin was decreased by 50% compared with that of untreated cells but 11β-reductase was not affected by the treatment. These data suggest that glycosylation is important for 11β-dehydrogenase activity.

This rat liver cDNA was also used as a probe to investigate the distribution of 11β-OHSD mRNA in various tissues (Agarwal et al, 1989; Moisan et al, 1990a; Krozowski et al, 1990; Whorwood et al, 1991). A single species of approximately 1700 nucleotides was found in most tissues but kidney contains at least 3 other species (1900 nt, 1600 nt, 1500 nt) and colon contains a large species of 3.4 kb. These data provided another piece of evidence for the existence of multiple 11β-OHSD isoforms. This will be reviewed in detail in Chapter 5.

Tannin et al (1991) isolated a human 11β-OHSD cDNA clone from a testis library by hybridisation with the rat liver cDNA. The open reading frame of this cDNA is of 876 bp, which predicts a protein of 292 amino acids. The sequence is 77% identical at the amino acid level to the rat cDNA. Surprisingly, only a single mRNA species was detected in human kidney using this cDNA as a probe.

The availability of human cDNA allowed localisation of the 11β-OHSD gene to chromosome 1. This 11β-OHSD gene was then isolated from a
chromosome 1-specific human genomic library using the human cDNA as a probe (Tannin et al, 1991). Human 11β-OHSD gene consists of 6 exons and is at least 9 kb long. Cloning of the rat homologue of this gene is presented in Chapter 5.

Very recently variant cDNA clones were isolated from a rat kidney library. In particular, a 5' truncated cDNA clone present only in kidney was identified. The missing fragment (26 amino acids) encodes for a hydrophobic peptide which could be a signal sequence for membrane insertion (Krozowski et al, 1992). Thus the deletion of the leader sequence from the microsomal 11β-OHSD protein may result in a nuclear localisation of the kidney-specific 11β-OHSD form. Our study on the rat gene shows that these two mRNAs are generated from a single gene by differential promter usage (Chapter 5). In addition they showed the presence of three alternate sites of polyadenylation which contributes to the differences in mRNA sizes detected on Northern blot of kidney.

Finally, an 11β-OHSD cDNA was cloned from a sheep liver cDNA library using the rat liver cDNA as a probe. 72% sequence identity was found between this cDNA and the rat liver cDNA (Yang et al, 1991).

1.3.4. 11β-hydroxysteroid dehydrogenase : tissue-specific protector of the mineralocorticoid receptor

1.3.4.1. Original studies

The role of 11β-OHSD as a factor conferring aldosterone specificity was demonstrated experimentally by studies carried on in our laboratory (Edwards et al, 1988) and in Melbourne (Funder et al, 1988). The two studies led to the same conclusions and they will be presented in parallel.

The first step was to show that 11β-OHSD is selectively expressed in aldosterone target tissues but not in other organs containing MR. For this, conversion of 3H-cortisol to 3H-cortisone by rat tissues minces (Funder et al, 1988) or conversion of 3H-corticosterone to 3H-11-dehydrocorticosterone (Edwards et al, 1988) in rat tissue homogenates was analysed. 11β-OHSD activity, estimated as the percentage conversion of 3H-cortisol to 3H-cortisone (or 3H-corticosterone), was highest in kidney, next highest in parotid, and lowest but still significant in colon: all three tissues being classical mineralocorticoid target tissues. Under the
conditions of the study, no discernible activity was found in hippocampus and heart, which both contain high levels of MR but are not selective for aldosterone (Fig. 1.10. A). Enzyme activity was higher in the distal than in the proximal tubules of the kidney. However, localisation of the enzyme by immunohistochemistry, using an antibody raised against a purified hepatic 11β-OHSD (see section 1.3.3.), showed that the enzyme was principally in the proximal tubules. Edwards et al then suggested that the enzyme was acting as a paracrine protector of the MR which are mainly present in distal convoluted tubules and cortical collecting ducts. This is no longer the prevailing model as will be discussed later.

Funder et al looked at the effect of in vivo carbenoxolone treatment on in vitro conversion of 3H-cortisol to 3H-cortisone and found that treatment with carbenoxolone (an 11β-OHSD blocker, similar in structure to GE) was followed by a substantial reduction in enzyme activity in aldosterone target tissues (Fig. 1.10.B).

Finally, both teams compared the binding of aldosterone and corticosterone in the absence or presence of an 11β-OHSD inhibitor (carbenoxolone or GI). Funder et al did receptor binding analysis on cytosolic preparations of kidney, parotid, colon, hippocampus and heart. They found that in the presence of carbenoxolone the level of corticosterone binding markedly increased and approaches if not equals that of aldosterone whereas no differences were observed in hippocampus or heart after treatment (Fig. 1.11). Edwards' et al approach was to compare the uptake of 3H-corticosterone in kidney, with and without GI pretreatment of the animals. The binding of the labelled steroid was detected by autoradiography and is presented in Fig. 1.12. The uptake of 3H-corticosterone in untreated animals is very low and not really different from the non-specific binding control; in contrast, after inhibition of 11β-OHSD by GI, the pattern of 3H-corticosterone binding was greatly changed and was now similar to that of aldosterone.

These latter experiments clearly demonstrated that glucocorticoids gain access to renal MR when the enzyme is inhibited in vivo, strongly supporting that the role of the enzyme in a normal situation is to prevent glucocorticoid occupancy of MR and thereby to confer aldosterone specificity in the tissue where it is expressed. This model fits with the tissue-specific and non-intrinsic specificity of the MR for aldosterone. The enzyme is present and active in 10-day-old rats (Funder et al, 1988)
Figure 1.10. (A) Analysis of *in vitro* conversion of [3H] cortisol (F) to [3H] cortisone (E) by 9-day-old rat tissue minces by H.P.L.C. (B) Analysis of the effect of *in vivo* carbonexolone treatment on *in vitro* conversion of [3H] cortisol (F) to [3H] cortisone (E) by 9-day-old rat tissue minces by H.P.L.C.

Figure 1.11. MR (type I sites) binding of adrenal steroids in vivo in 9-day-old adrenalectomised rats; uptake and retention of [3H] aldosterone ([3H] Aldo) and [3H] corticosterone ([3H] B), and effect of treatment with carbonexolone (CBX).

(adapted from Funder et al, 1988)
Figure 1.12: Autoradiographs of rat kidney sections showing binding of $^3$H-corticosterone. In (a) the label alone was given in vivo. In (b) the rat was given glycyrrhizic acid followed by the labelled steroid. In (c) the same protocol was followed as in (b) with the addition of a large dose of unlabelled aldosterone.

(adapted from Edwards & Hayman, 1991)
Another important result provided by Funder et al, in this study, was to show that 11-dehydrocorticosterone, the product of the reaction catalysed by 11β-OHSD, has very low affinity for MR and therefore does not compete with aldosterone.

1.3.4.2. Further studies of 11β-OHSD in kidney

Kidney has been and is still, by far, the most investigated tissue to define 11β-OHSD function as a factor conferring mineralocorticoid specificity. Localisation of 11β-OHSD activity, immunoreactivity and mRNA have been examined in order to connect the functional relationships with the anatomical locations of 11β-OHSD and MR.

The first approach used to localise the enzyme was by immunohistochemistry. As reported in section 1.3.3.1, Lakshmi and Monder had purified the hepatic 11β-dehydrogenase and raised antibodies against this enzyme. These antisera were used first by Edwards and coworkers (1988) who detected the enzyme mainly in the proximal tubules and not in the distal nephron. Immunostaining was also present either in or immediately adjacent to the vasa recta alongside the papillary collecting tubules. In a separate study, Castello et al (1989) raised a monoclonal antibody against rat renal 11β-OHSD from solubilised microsomal proteins. Immunohistochemistry using this monoclonal antibody showed intense staining in proximal tubule cells and peritubular interstitial cells, confirming Edwards' et al data. Rundle et al (1989) also used the antisera raised by Lakshmi and Monder to explore the anatomical relationship of 11β-OHSD and the MR. Again they found 11β-OHSD in the proximal tubules whereas MR were located in distal convoluted tubules, connected pieces and initial cortical collecting tubules.

All these studies showed that 11β-OHSD and MR are not colocalised and led the authors to the conclusion that the action of 11β-OHSD in the kidney was paracrine rather than autocrine. However, there are problems with this idea since the physical distance between the proximal 11β-OHSD and the distal MR is great, and since 11β-OHSD activity was found both in proximal and distal tubules of the kidney (Edwards et al, 1988).
In 1990, Bonvalet and coworkers, in Paris, reported that the distribution of 11β-OHSD activity along the rabbit nephron corresponds to the renal mineralocorticoid target sites. They examined the conversion of 3H-corticosterone to 3H-11-dehydrocorticosterone in microdissected tubular segments. They found a very high activity of the enzyme in cortical collecting tubules, the cortical part of the thick ascending limb and in the medullary collecting tubules from the outer medulla. Low levels of activity were detected in proximal tubules. In a later study from the same group, this distribution was confirmed in the mouse nephron (Kenouch et al, 1991). These important findings were further substantiated by the study of Náray-Fejes-Tóth and coworkers (1991), who used freshly isolated rabbit cortical collecting duct cells or primary culture of cortical collecting duct cells grown as monolayers on permeable supports, to examine the metabolism of corticosterone. They found that both cell preparations rapidly converted 3H-corticosterone to 3H-11-dehydrocorticosterone, which was the only metabolite detected. However the apparent Km value of 11β-OHSD for corticosterone was ~ 60 nM, 100-fold lower than the Km of 11β-OHSD for corticosterone in the rat liver. Neither freshly isolated nor cultured collecting duct cells converted 3H-11-dehydrocorticosterone back to 3H-corticosterone. Taken together these data provide functional evidence for 11β-OHSD activity in renal aldosterone target cells and suggest an autocrine mechanism for enzyme-mediated receptor protection. These findings also indicate that this enzyme might be a collecting duct-specific isoform of 11β-OHSD given the different Km value; this could also explain the discrepancy between these data and the immunohistochemical studies.

Another method to localise the enzyme in the kidney was the utilisation of the cDNA in in situ hybridisation studies. 32P or 35S labelled antisense cRNA probes were synthesised from 11β-OHSD cDNA (see section 2.2.3.) and hybridised to rat renal section under stringent conditions. Macroscopically, as shown on the in situ hybridisation performed by myself (Fig. 1.13), 11β-OHSD mRNA expression was highest in the juxta medullary cortex-outer medulla with lower expression in the outer cortex and inner medulla, closely resembling the pattern of aldosterone binding in vivo. Microscopic analysis demonstrated 11β-OHSD mRNA expression in all tubular regions, with high expression in proximal and distal tubular epithelia, including the connecting tubule and cortical collecting duct.
(Yau et al, 1991). In a separate study, Stewart et al (1991) reported similar results. These findings reconciled all the previous studies showing the presence of 11β-OHSD in both proximal and distal tubules of the kidney. Clearly several isoforms of the enzyme are present in this organ: they should be closely related since the cDNA hybridises to every form, but sufficiently different for the antibodies to recognise only the isoform.

Figure 1.13: Autoradiogram of a rat kidney section hybridised with a $^{35}$S-labelled cRNA antisense probe.
restricted to the proximal tubules. In parallel with these in situ hybridisation studies, Krozowski et al (1990) have shown that hybridisation of the 11β-OHSD cDNA to Northern blots of kidney total RNA revealed at least four 11β-OHSD mRNA species that are not explained by differences in polyadenylation signal. Recently, the same group reported the isolation of variant 11β-OHSD cDNA clones from a rat kidney library, which will probably provide more information about the 11β-OHSD kidney isoforms (Krozowski et al, 1992). This group also reported that the distal tubular 11β-OHSD isoform is NAD+-dependent, whereas the previously reported hepatic and proximal tubule-specific species is NADP+-dependent (Mercer & Krozowski, 1992). For this, they used a histochemical technique which links steroid metabolism with the production of a colour reaction. 11-hydroxyandrostenedione was used as a substrate and its dehydrogenation is followed in kidney sections by the production of a diformazan derivative of nitroblue tetrazolium following reduction of NAD+ to NADH. Under these conditions activity was seen only in distal tubules and only when NAD+ was the cofactor. However, no activity was detected by this method when physiological glucocorticoids (corticosterone or cortisol) were used as substrates, in either proximal or distal tubules even when cofactor was appropriate. The authors interpretation of this is that the 20 reductase enzyme uses the reduced cofactor at the expense of the color reaction, but no evidence for this was provided.

In conclusion, it is clear that several 11β-OHSD species are present in different part of the kidney. There is undoubtedly a high 11β-dehydrogenase activity in the renal aldosterone target cells, which strongly supports the hypothesis that 11β-OHSD plays a crucial role in aldosterone specificity of renal MR in a autocrine fashion. What remains to be clarified is the structural (and maybe functional) differences between proximal and distal 11β-OHSD. A lot of information on this subject will hopefully be deduced from the cloning of a new renal cDNA (Krozowski et al, 1992) and from our cloning of the rat gene (Chapter 5).
1.3.5. 11β-hydroxysteroid dehydrogenase: modulator of glucocorticoid-glucocorticoid receptor interaction.

Initially it was thought that 11β-OHSD was present only in aldosterone target tissues and that the non-selectivity of the MR in heart and hippocampus could be explained by a lack of this enzyme in these tissues. Indeed 11β-OHSD bioactivity was detected only in aldosterone target tissues in earliest studies (Edwards et al, 1988; Funder et al, 1988). However the enzyme has long been known to be present in tissues which are not thought to be aldosterone targets or to contain MR, many of which have a high concentration of GR. Such tissues include liver, lung, skin, testis, mammary gland, ovary, proximal and distal tubules, pituitary and placenta.

Skin

The association of 11β-OHSD with the GR raises the possibility that 11β-OHSD modulates access of cortisol to this receptor. Teelucksingh and co-workers (1990) from our laboratory, examined this possibility in the skin. Cutaneous vasoconstriction has been shown to be a GR-mediated response (Marks et al, 1982). When hydrocortisone was applied to the forearm of volunteers overnight under an occlusive dressing, there was minimal vasoconstriction. However, when GE (an 11β-OHSD blocker), which alone has no effect, was added to the preparation there was a marked potentiation of the response. Furthermore, these authors showed by immunohistochemistry and by in vitro enzymology that 11β-OHSD is present in the skin. These data have offered an explanation for the anti-inflammatory properties of topical GE (Adamson & Tillman, 1955) and carbenoxolone (Csonka & Murray, 1971).

Vascular smooth muscle and heart

Corticosteroid hormones play an important role in the control of blood pressure but the mechanisms involved are unknown. The presence of MR and GR in vascular smooth muscle (Meyer & Nicholls, 1981; Komel et al, 1982) and in heart (Arriza et al, 1987; Barnett et al, 1988; Funder et al, 1973) provides evidence of direct action of corticosteroids on the arterial
wall. 11β-OHSD activity has been reported in these tissues (Kornel et al., 1982; Kolanowski et al., 1981; Funder et al., 1989) and 11β-OHSD mRNA has been localised in the cytoplasm of vascular and cardiac smooth muscle but not in endothelium (Walker et al., 1991). Thus 11β-OHSD is appropriately sited to modulate access of glucocorticoids to vascular receptors and could influence vascular resistance (as they do in the skin), cardiac output and thereby blood pressure.

**Testis**

High levels of 11β-OHSD were also found in testis. It is known that cortisol inhibits testosterone production, testicular metabolism and sperm formation through GR-mediated processes (Bambino & Hseuh, 1981, Welsh et al., 1982). The inhibitory effects of cortisol on the synthesis of testosterone is determined by the availability of active steroids derived from the circulation. In the rat testis, 11β-OHSD is found only in Leydig cells and increases with the postnatal increases in Leydig cell number and the developmental rise in serum testosterone (Phillips et al., 1990). The concomitant appearance of 11β-OHSD and testosterone at puberty led the authors to postulate that the enzyme mediates corticosteroid-dependent androgen production. Absence of 11β-OHSD prior to 20 days of age permits cortisol (or corticosterone) to bind and activate GR, interfering with testosterone synthesis. Beyond 25 days, 11β-OHSD converts the glucocorticoid to its inactive 11-oxo-form and releases the inhibition, permitting testosterone production to proceed. This was further supported by in vivo data showing that carbenoxolone potentiates corticosterone inhibition of LH-induced testosterone release (Monder et al, 1991b).

**Ovary**

GR are present in the ovary (Schreiber et al, 1982) and may affect ovarian physiology (Inazu et al, 1990; Baldwin & Sawyer, 1974). 11β-OHSD activity and mRNA has been found in human ovaries (Murphy, 1981; Tannin et al, 1991) and in rat where it has been localised by in situ hybridisation and immunohistochemistry in oocytes and luteal bodies (Benedíksson et al, 1992). Thus, 11β-OHSD may modulate glucocorticoid effects on ovarian function.
Glucocorticoids induce milk production in mammary gland via the GR (Jahn et al, 1987; Quirk et al, 1988). High levels of 11β-OHSD activity are present in the adipose cells of the mammary gland but this activity decreases just after parturition (Quirk et al, 1990a). Activity of 11β-OHSD in adipose cells from pregnant rats is 3-fold higher than in lactating rats (Quirk et al, 1990b). Thus 11β-OHSD in mammary gland may constitute one of the physiological mechanisms preventing premature milk production in response to glucocorticoids.

Colon

Fuller & Verity (1990), from Melbourne, demonstrated that in the rat descending colon Na.K-ATPase α1, and β- subunit gene expression is acutely regulated by dexamethasone but not aldosterone which suggest again a GR-mediated effect. Treatment with carbenoxolone sodium also induced subunit gene expression in intact but not adrenalectomised rats, suggesting that inhibition of 11β-OHSD by carbenoxolone increases endogenous glucocorticoid occupancy of GR. This effect of glucocorticoid was confirmed when adrenalectomised animals were pre-treated with carbenoxolone prior to corticosterone (Fuller & Verity, 1991).

In summary, 11β-OHSD clearly plays a role in regulating the access of high levels of glucocorticoids not only to the MR but also to the GR in target cells.

1.3.6. 11β-hydroxysteroid dehydrogenase in fetal tissues and accessory organs

1.3.6.1. Placenta

The level of active corticosteroid to which the fetus is exposed at any given point of the gestation is crucial to its development and maturation. Excess glucocorticoids has deleterious effects ranging from growth retardation (MacHover Reinisch & Simon, 1978) to major teratogenesis. High activity of 11β-dehydrogenase in the human placenta provides a barrier to the transfer of active glucocorticoids to the fetus.
In keeping with this role of barrier, activity of the 11β-reductase component of 11β-OHSD is relatively low in placenta (López Bernal et al, 1980; López-Bernal et al, 1982). 11β-OHSD in placenta resembles 11β-OHSD in distal tubules in that its activity is essentially 11β-dehydrogenase and its role is to inactivate circulating glucocorticoids. In baboons, placental 11β-OHSD activity is apparently regulated by estrogens in vivo (Baggia et al, 1990).

1.3.6.2. Fetal lung

As reviewed earlier (section 1.1.3.1.), glucocorticoids are essential for normal pulmonary differentiation. Although 11-oxidation of glucocorticoids is predominant in the fetus (Murphy, 1981), the fetal lung possesses the ability to reduce ("activate") the abundant fetal supplies of cortisone (or 11-dehydrocorticosterone) and this activity increases with gestational age (Smith et al, 1973; Torday, 1980). Abramovitz et al, 1982 explain these variations by changes in proportions of epithelial and fibroblast-like cells, with 11β-reduction restricted to fibroblasts and 11β-dehydrogenation located in epithelial cells, i.e. alveolar type II cells.

The ratio of reduced to oxidised steroid in tissues at critical stages of development may provide important clues to determining key molecular events necessary to complete development. 11β-OHSD may play a dominant role in ensuring adequate exposure to glucocorticoids at critical stages of development of each maturing organ.

1.4. Aims of the thesis

The aim of this thesis was first to investigate the role of 11β-OHSD in the central nervous system. Adrenal steroids have been reported to have a wide range of actions in the central nervous system, from regulation of cell growth and differentiation, through effects on neuronal and glial metabolism, to changes in appetite and behavioral patterns. Both MR and GR are present in the brain. Glucocorticoids are in vivo ligands of both receptors and aldosterone binds specifically to MR in some neurons
despite competitive concentrations of circulating glucocorticoids. The studies described in Chapter 3 show that 11β-OHSD is present in brain in aldosterone-selective tissues but also in areas where MR are not selective or in cells that contain only GR. Therefore 11β-OHSD appears to have various roles in the brain from conferring aldosterone selectivity to modulating access of glucocorticoids to MR and/or GR. 11β-OHSD activity is regulated in the brain by glucocorticoids and during development in a cell-specific manner (Chapter 4), which led us to the cloning of 11β-OHSD gene (Chapter 5) to determine the molecular basis of this tissue-specific and ontogenic regulation.
Chapter 2: Materials and Methods
In general, basic protocols outlined below are detailed in Sambrook et al. (1989).

2.1 DNA analysis techniques

2.1.1. Restriction endonuclease digestion and electrophoresis of DNA

DNA was digested using 1-3 unit of restriction enzyme per 1 μg of DNA, 1x restriction buffer (supplied with the enzyme) and dH2O to the required volume. Digestion reactions were incubated at 37°C (except BssH II, 50°C) for 1-2 h for plasmid or phage DNA and a minimum of 4 h for mammalian genomic DNA. Enzyme activity was terminated by heating digests to 60°C for 10 min or by addition of stop mix/loading buffer (30% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol, 0.5M EDTA).

For investigatory purposes, 0.2-0.4 μg of DNA was routinely digested in a final volume of 10 μl. For cloning purposes or Southern blot analysis of mammalian DNA, 10-15 μg of DNA were digested in a final volume of 50-200 μl.

Digested DNA was visualised after electrophoresis through an agarose gel containing 0.5 μg/ml of the fluorescent dye ethidium bromide. The concentration of agarose was varied depending on the size of DNA fragments to be resolved; for example, 1% gels were routinely used to separate fragments below 2 kb and above 200 bp, whereas gels as low as 0.5% were used to separate very large fragments. Gels were generally electrophoresed at 60-80 mA in TBE buffer (10 x TBE = 1M Tris-HCl, 0.8M Boric acid, 20 mM EDTA, pH 8.3). The size marker routinely used was "1 kilobase DNA ladder" (Gibco BRL ) which gives a range of mol. wt. sizes between 75 bp and 12 kb.

2.1.2. Subcloning of DNA fragments

Subcloning DNA fragments into plasmid was routinely performed as needed for synthesising specific single stranded RNA transcripts or for DNA sequencing. The subclone could then be selected by analysis of minipreparation of plasmid DNA (see section 2.1.3.1.).
2.1.2.1. **Plasmid vector**

**pBluescript II** (stratagene): A 3 kb plasmid vector derived from pUC 19 (Messing,1983), retaining both the capacity for a high copy number and the ampicillin resistance gene (β-lactamase) (Vieira & Messing, 1982). It contains an artificial polylinker inserted into the β-galactosidase gene (lac-Z) providing a convenient test for recombinant plasmid known as the blue/white test: non-recombinant plasmids are able to synthesise the enzyme which breaks down the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) to release a blue product, however in recombinant plasmid the lac-Z gene is interrupted by foreign DNA and the colonies remain white. This vector also contains the phage T3 and T7 promoter sequences flanking the polylinker thus enabling in vitro synthesis of specific single stranded RNA transcripts, both sense and antisense.

2.1.2.2. **Bacterial strains**

All strains were E. coli K12

**JM83**: Δ ara, (lac-pro AB), rps L, F80, lac Z M15, (rk+, mk+) (Vieira & Messing, 1982). This was the host for pBluescript based plasmids. The lac Z M15 gene is integrated into the host chromosome.

**PLK17**:hsd (rk-, mk+) supE44, lac Z, gal K2, gal T22, met B1, trp R55, hsd R2. This was the host strain for screening the λ DASH II genomic library (section 2.1.4.).

**LE392**: hsd R514 (rk-, mk+) supE44, sup F58, lac Y1 or Δ(lac 1ZY), gal K2, gal T22, met B1, trp R55. This was the host for λ DASH phage based vectors. It was used for large scale λ DNA preparation (section 2.1.3.3.).
2.1.2.3. Bacterial media

LB (Luria-Bertoni) broth and agar; Per litre: 10 g bacto-tryptone (Difco), 5g bacto-yeast extract (Difco), 10 g NaCl. pH 7.5. LB agar contained 15 g agar (Difco) per litre.

NZY broth, NZY agar and Top-agar; Per litre: 5g NaCl, 2g MgSO4.H2O, 5g yeast extract, 10 g NZ amine (casein hydrolysate). In addition, NZY agar contained 15 g agar per litre and Top agar contained 7g agarose (Sigma) per litre.

Terrific broth (Tartof and Hobbs, 1987): per litre; 15 g bacto-tryptone (Difco), 30g bacto-yeast extract (Difco), 5 ml glycerol, 1/10 volumes of 1M K2HP04 added before inoculation. A richer medium than LB broth, it was used for large scale cultures for plasmid preparation since it gave a higher density culture and a better yield of DNA.

Media additives: The antibiotic ampicillin (Sigma) was used to select for plasmid maintenance at a concentration of 100 µg/ml. Stocks were prepared at 1000x concentration and stored at -20°C.

The chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) of the blue/white test was added to the LB agar medium at a concentration of 0.02%. Stocks were prepared at 1000x concentration in dimethylformamide and stored at -20°C.

All media were sterilised by autoclaving. Additives were filter sterilised. Bacteria were grown at 37°C with good aeration for liquid cultures. Bacterial stocks were routinely made in 20% glycerol and frozen at -70°C.

2.1.2.4. Constructing subclones

DNA fragments of interest were purified following fractionation on a low melting point (LMP) agarose gel. The appropriate band was cut out of the gel and DNA was purified from agarose by spinning the gel slice for 30 min in a microcentrifuge filter (Sigma). The latter consist of a 2.5 ml
microcentrifuge tube containing a removable filter insert. The filter is a sterile cellulose acetate membrane of 0.22µm pores. The DNA solution was then cleaned by phenol/chloroform extraction (1:1 volume) and concentrated by ethanol precipitation (1/10 volumes of 3M NaOAc and 2.5 volumes of cold (-20°C) ethanol added, left at -70°C for at least 15 min or at -20°C for a minimum of 30 min). The DNA was then pelleted by centrifugation for at least 10 min and the pellet rinsed in 70% ethanol, dried under vacuum and resuspended in an appropriate volume of TE (10mM Tris-HCl, 1 mM EDTA, pH 7.5). The purified DNA fragment was then inserted into the plasmid vector using the enzyme DNA ligase prepared from phage T4. Ratios of vector to insert DNA were varied around a standard of 1:3 on a molar basis. Ligations were carried out with 25 ng of linear vector in a 10 µl reaction volume (10× ligation buffer: 0.5 M Tris-HCl pH7.4, 0.1 M MgCl₂, 10 mM Spermidine, 1mg/ml BSA, 10 mM ATP, 0.1 M DTT) with 1 µl (10 units) of T4 DNA ligase at 16°C overnight.

2.1.2.5. Bacterial transformation

E. coli JM83 cells were made competent for DNA uptake by the CaCl₂ method (Sambrook et al, 1989). Cells were grown in 100 ml of LB medium to mid log phase (A₅₅₀=0.4-0.5). After harvesting, the cells were resuspended in 10 ml of ice-cold 0.1 M CaCl₂. The cells were then repelleted and resuspended in 2 ml of ice-cold 0.1 M CaCl₂ and stored at 4°C for up to 4 days. For transformation, the ligated plasmid DNA (< 10 µl) was added to 200 µl of cell suspension and left to adsorb for 30 min at 0°C. After a 90 second heatshock at 42°C, the cells were chilled on ice for 1 min, 0.8 ml of LB added and the cells allowed to develop antibiotic resistance at 37°C for 1 hour. Suitable aliquots of the transformation were then plated on selective media and grown overnight at 37°C.

2.1.3. DNA preparation

2.1.3.1. Plasmid DNA minipreparation

This method is a shortened protocol of the alkaline-lysis minipreparation method, in which the volumes and incubation times have been altered (Stephen et al, 1990). 10 ml of LB broth with appropriate
antibiotic added, was inoculated with a selected single colony. This was
grown overnight at 37°C in a sterile tube. 1.5 ml of the culture was pelleted
by centrifugation at 13 krpm for 1 min in a microcentrifuge tube and
resuspended in 150 μl of GTE solution (50 mM glucose, 25 mM Tris-HCl
pH 8.0, 10 mM EDTA). Cells were lysed by adding 300 μl of a freshly prepared
solution of 0.2 M NaOH/1% SDS and placing the tube on ice for 5 min. Cell
debris and chromosomal DNA was precipitated by addition of 225 μl of ice-
cold 3M potassium acetate (pH 4.8) and incubation on ice for 5 min. After
centrifugation, supernatant was removed, and the plasmid DNA precipitated
with one volume of absolute ethanol. After spinning for 5 min, the plasmid pellet was rinsed with 70% ethanol, dried and
resuspended in 40 μl of TE. The resulting DNA was sufficiently pure for
restriction enzyme digestion or DNA sequencing.

2.1.3.2. Large scale plasmid preparation

This protocol is very similar to the minipreparation method and
same solutions are used. 500 ml of LB broth or terrific broth plus
appropriate antibiotic, was inoculated with a selected bacterial colony and
shaken at 37°C overnight. The culture was decanted into two 250 ml
centrifuge bottles and spun at 6 krpm for 5 min. The pellets were
resuspended in 18 ml of cold GTE solution plus 10 mg/ml lysozyme. Then 40
ml 0.2 M NaOH/1% SDS was stirred in and incubated for 10 min at room
temperature. 20 ml of 3M potassium acetate (pH 4.8) was added and the
bottle stored on ice until a white precipitate formed. This was spun out at 5
krpm for 15 min at 4°C. The supernatant was filtered through gauze to
remove coarse precipitate, and 0.6 volumes of isopropanol added to
precipitate plasmid DNA. Samples were left for 10 min at room temperature
and centrifuged at 10 krpm for 20 min to pellet the plasmid DNA. Pellets
were washed with 70% ethanol, lyophilised until almost dried and
redissolved in 2.7 ml of TE. The plasmid DNA was then purified by CsCl
gradient centrifugation. 2.7 g CsCl and 240 μl ethidium bromide were added
and the resulting solution was decanted in Beckman 3.5 ml
ultracentrifugation tubes. Samples were spun for at least 4 h, routinely
overnight, at 100 krpm in a Beckman Ti 100.3 rotor at 20°C. Plasmid bands
were removed with a 18 gauge needle mounted on a 2 ml syringe. The
ethidium bromide was removed by serial extractions with butanol until the
samples were clear. CsCl was removed by precipitating the DNA with 3 volumes of 70% ethanol for 30 min at -20°C. CsCl was redissolved by warming the tube at 37 °C before centrifugation at 10 krpm, for 15 min, to pellet the DNA. The plasmid DNA pellet was then rinsed with 70% ethanol, dried and resuspended in 1ml of TE.

2.1.3.3. Large scale bacteriophage DNA preparation

First, a stock of high titer phage was prepared. Appropriate host cells (LE392) were grown overnight in LB broth containing 10mM MgCl₂ and 0.4% maltose as the sugar induces the maltose operon, which contains the gene (λamb) coding for the λ bacteriophage receptor. These cells were spun down at 3 krpm and resuspended in 0.5 x culture volume of 10 mM MgSO₄, 10⁵ phages in 100 µl of SM buffer (SM= per litre: 5.8 g NaCl, 2.0 g MgSO₄, 50 ml 1M Tris-HCl pH7.5, 5 ml 2% gelatine) were inoculated to 100 µl of the cell culture and the mixture was incubated 20 min at 37°C to allow the bacteriophage particles to adsorb. Then 3 ml of top-agar at 46°C were added, swirled to mix, and immediately plated onto pre-poured NZY-agar 85 mm diameter petri dishes. When the top-agar had hardened, plates were inverted and incubated overnight at 37°C. The plate was inspected for complete lysis of bacteria and confluent plaques. 5 ml of SM was added onto the plate, which was then agitated at room temperature for at least 4 hours. This SM solution containing a high titer of phage was then pipetted in a sterile tube and kept at 4°C.

For large scale preparation of phage DNA, 50 µl of high titer phage stock (corresponding to approximately 5x10⁷ pfu) was inoculated to 10¹⁰ LE392 cells harvested as above and diluted in SM buffer. This was incubated 20 min at 37°C and then added to 500 ml of NZY broth, prewarmed at 37°C, in a 2 litre conical flask. Lysis of bacteria started after 12 h incubation after which 10 ml of chloroform was added. Lysed cultures were then treated with DNase I and RNAase, each to a final concentration of 1 µg/ml for 30 min at room temperature. An equal volume of 20% PEG 6000 (Polyethylene glycol, MW 6000, Sigma), 2M NaCl was added to precipitate phage particles and incubated on ice for 1-2 h. Loose precipitates were decanted into 250 ml bottles and spun at 10 krpm for 20 min to pellet the phages. The pellet was resuspend in 3 ml of SM and extracted three times with chloroform to remove all traces of PEG. 0.75
g/ml CsCl was added to the aqueous phase and this was spun in Beckmann 3.5 ml tubes (Ti 100.3 rotor) at 100k rpm for 3 h at 20°C. Phages formed an opaque band in the CsCl gradient, which was extracted and dialysed against 100xTE at room temperature for 1 h to remove the CsCl. Phage particles were then disrupted by the addition of SDS to 0.1% and EDTA to 10 mM and incubated at 68°C for 15 min. 100μg/ml proteinase K was added and incubated at 50°C for 30 min, followed by phenol/chloroform extraction. Phage DNA was then precipitated by addition of NaCl to 0.2 M and two volumes of ethanol. The DNA pellet was resuspended in TE. This bacteriophage DNA was sufficiently pure for enzymatic manipulations.

2.1.4. Phage library screening

The library used was a Sprague Dawley rat genomic library in the lambda DASH II vector (Stratagene, cat n° 945501) and was obtained from Dr John Mullins, Centre for Genome Research, Edinburgh. DNA was prepared from testis and partial digests performed with Sau3A I. Inserts (9-22 kb) were cloned into the BamHI site of λ DASH II.

The library was plated on NZY medium in 20 x 20 cm plates at a maximum density of 2 x 10⁵ plaques per plate to obtain almost confluent lysis. 5 of these plates were prepared so that 10⁶ plaques were screened. To achieve this, PLK17 cells, previously grown to stationary phase in LB broth plus 10 mM MgCl₂ and 0.4% maltose, were spun down and resuspended in 0.5 x volumes of 10 mM MgSO₄. 1 ml of this bacterial suspension was inoculated with an appropriate volume of phage stock (previously titrated) in a 50 ml sterile tube and incubated 20 min at 37°C. The cells were then diluted with 40 ml of molten top-agar (46°C), swirled to mix, and immediately plated onto pre-poured dry NZY agar plate and incubated at 37°C overnight. The phages plaques on the resulting plates were overlaid with dry Nitrocellulose (Schleicher & Schuell) filters which were keyed in place with a needle containing waterproof ink. Transfers were performed in duplicate to cut down false positives. The first filter was left for 2 min and the second 7 min. After lifting, filters were submerged, plaques facing up, in dishes containing the following solutions:

1. 1.5 M NaCl, 0.5 M NaOH. 2 min to denature DNA
2. 1.5 M NaCl, 0.5 M Tris-HCl pH8.0. 5 min to neutralise the pH
(3) 0.2 M Tris-HCl pH 7.5, 2 x SSC. 30 sec. to rinse filters

The phage DNA was fixed to the dry filters by baking 2 h at 80°C. The filters were hybridised and washed as described for Southern blots (section 2.1.5.) and were exposed to autoradiographic film for 1-3 days at -70°C. Duplicate filters were screened with the same probe, and plaques on the original agar plate which corresponded to duplicate spots on the film were selected as potentially positive clones. This was done by cutting out the relevant area of top agar and placing it in 1 ml of SM buffer in a microcentrifuge tube. This stock, which contained a mixture of positive and negative clones, could be stored at 4°C indefinitely while secondary and tertiary screens were carried out. This was done by repeating the procedure above, plating from the selected stock on 85 mm petri dishes at a lower density until a pure stock of the positive phage clones was selected. DNA was then prepared from the clone as described in section 2.1.3.3.

2.1.5 Southern blot analysis

2.1.5.1. Capillary transfer

This method of transferring DNA from an agarose gel to a nitrocellulose or a nylon membrane broadly follows the method of Southern (1975). Following photography of the gel stained with ethidium bromide, the DNA was denatured by immersion in 3 volumes of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 40 min. This was followed by 30 min in neutralising solution (0.5 M Tris-HCl, 1.5 M NaCl pH 5.5). The gel was then laid on a transfer apparatus; a wick of Whatman 3MM filter paper with a reservoir of 20 x SSC (20 x SSC = 3M NaCl, 0.3 M Na Citrate, pH 7.4). A nitrocellulose (Schleicher & Schuell) or a nylon (Hybond-N, Amersham) membrane was carefully placed in direct contact with the entire gel. Two sheets of Whatman 3MM filter paper were placed over the membrane followed by paper towels to a depth of 5 cm. An even weight (1 kg) was placed on the assembly and transfer carried out overnight. The membrane was then baked for 2 h at 80°C (nitrocellulose) or UV irradiated (Hybond N).
2.1.5.2. DNA hybridisation

Pre-hybridisation and hybridisation were carried out in 50% formamide, 5x SSPE, 5x Denhardt's solution (0.1 % BSA, 0.1 % Ficoll, 0.1 % polyvinylpyrrolidone), 200μg/ml denatured herring testis DNA, 0.1% SDS and 1mM EDTA for cDNA hybridisation and in 6x SSC, 4x Denhardt's solution, 0.5% SDS for oligonucleotide hybridisation. These solutions were stable for several months at room temperature. Filters were sealed in plastic bags with about 10 ml of hybridisation mix per 20 cm² of filter. After prehybridisation (42°C for cDNA, 50 °C for oligonucleotide) for at least 20 min, the radioactive probe was carefully added to the bag and hybridised overnight. The bag was then carefully open, the probe disposed of, and the filters were washed in solution of the appropriate stringency (standard wash was 2xSSC, 0.1% SDS for 15-30 min ) until the signal reached an acceptable level. Filters were then wrapped in clingfilm and autoradiographed at -70°C with Kodak XAR-5 film. Films were developed in Kodak D19 developer solution (Ilford) for 2 min and fixed in 1/5 Amfix fixer (Champion).

2.1.6. DNA sequencing

DNA sequences of supercoiled plasmid templates were determined by the dideoxy nucleotide chain termination method using a Sequenase (version 1) kit (United States Biochemical) according to the manufacturer's protocol.

2.1.6.1 Preparation of double stranded DNA templates for sequencing

Prior to sequencing reaction, the DNA template must be denatured to allow access of the sequencing primer. This was achieved by alkaline denaturation which also hydrolyses contaminating RNA. 2 to 5 μg of CsCl purified (section 2.1.3.2.) or minipreparation purified (section 2.1.3.1) supercoiled plasmid DNA were added to 25 μl of denaturation solution (0.2 M NaOH, 2mM EDTA) and incubated at 37°C for 15 min. 2.5 μl of neutralising solution (0.4 M NaOAc pH4.5) was then added, followed by 80 μl of ethanol to
precipitate the DNA. After 15 min at -70°C and centrifugation for 5 min at 13 k rpm, the pellet was rinsed with 70% ethanol and resuspended in sterile dH2O.

2.1.6.2 Sequencing protocol

The first step of the sequencing reaction involved annealing the primer (either the universal or reverse M13 primers, or oligonucleotides specific to internal regions of the insert) to the template, followed by elongation from this primer by the enzyme T7 DNA polymerase. The second step involved chain termination. For each template four separate sequencing reactions were performed. All contained primer, template and all four dNTPs (dATP was radiolabelled) but each contained a different dideoxynucleotide (ddNTP). Incorporation of a ddNTP into the newly synthesised DNA strand resulted in chain termination, as no 3'-OH group was available to form the next phosphodiester bond. A series of DNA fragments was therefore generated, all originating at the primer, and terminating at the sites of incorporation of the appropriate ddNTP.

The annealing reaction contained 2-5 μg of denatured template and 1 pmole of primer in annealing buffer (40 mM Tris-HCl pH7.5, 20 mM MgCl2, 50 mM NaCl) in a volume of 10 μl. This was mixed and centrifuged briefly before incubation for 15-30 min at 37°C. Elongation was achieved by addition of 1 μl DTT 0.1 M, 2 μl nucleotide mix (dGTP, dCTP, and dTTP 7.5 μM each in solution), 5 μCi [α-35S] dATP (Amersham) and 3 units of T7 DNA polymerase for 5 min at room temperature. Four tubes labelled G, A, T, and C containing 2.5 μl of the corresponding termination mix (80 μM of each dNTP and 8 μM of one ddNTP) were pre-incubated for 1 min before addition of 4.5 μl of the elongation reaction to each tube and incubation for a further 5 min at 37°C. Reactions were then stopped by the addition of 5 μl of stop solution (95% formamide, 20 mM EDTA 0.05% bromophenol blue, 0.05 % xylene cyanol FF). The sequencing reactions could be analysed immediately or stored at -20°C. The radiolabelled fragments produced by the sequencing reaction can be resolved on polyacrylamide gels (Sanger & Coulson, 1975) and detected by autoradiography.
2.1.6.3. Polyacrylamide urea gel electrophoresis of sequencing reactions.

Sequencing gels were run between a 37.5 x 45 cm and a 32 x 42.5 cm glass plates in 1 x TBE buffer (section 2.1.1.). The smaller glass plate was siliconised by applying dimethyldichlorosilane in trichloroethane (BDH) to ensure that the gel would not adhere to it at the dismanteling step. The 2 plates were separated by wedge spacer (IBI) creating a gradient gel effect, and held together with bulldog clips. The gel mix contained per 100ml; 42 g urea, 15 ml 40% acrylamide stock (19% acrylamide, 2% bisacrylamide), 10 ml 10 x TBE, 600 µl of 10% ammonium persulfate (crosslinking agent), brought to 100 ml with dH2O and filtered through Whatman #1 filter paper. 40 µl of polymerisation catalyst TEMED (N’-tetramethylethylenediamine) was added immediately before pouring to catalyse the polymerisation.

The gel was allowed to set for at least two hours and was then attached to a perspex vertical gel apparatus (IBI, Model STS-45). The wells were flushed out with electrophoresis buffer before loading the samples to remove unpolymerised acrylamide/urea solution, and the gel was pre-electrophoresed at 70 watts for at least an hour. The samples were heated at 80°C for 5 mins and 2.5 µl of each sequencing reaction loaded per well. Samples were electrophoresed at 70 watts for 2 h for a standard run. The glass plates were then separated from each other and the outer plate carrying the gel was immersed in 10% methanol, 10% acetic acid for 10 min in order to fix the gel. Finally, the gel was peeled away from the plate onto a Whatman 3MM filter paper, covered with clingfilm and dried under vacuum at 80°C on a slab dryer (Bio-Rad, Model 583). Overnight exposure to Kodak XAR-5 film was usually sufficient to clearly read 200-300 nucleotides of sequence.

2.2 Preparation of radiolabelled nucleic acid probe

2.2.1. Random priming of DNA

The technique is based on the method of Feinberg & Vogelstein (1983) where random hexanucleotides act as primers for polymerase chain
extension in the presence of radiolabelled nucleotides. A BCL random primed labelling kit was used. This was a convenient technique since the reaction could be carried out in the presence of minimal amounts of DNA (10 ng), and did not require purification of DNA restriction fragment from agarose, which was only melted and diluted (3 ml dH2O/ g of gel). Routinely, 25 ng of DNA was denatured for 10 min by heating to 100°C. The sample was cooled to room temperature and 5 μl of 4 x random prime buffer/dNTPs was added, followed by 5 μl [32P] dCTP (3000 Ci/ mmol, Amersham) and 1 μl of Klenow fragment of DNA polymerase I. Random prime buffer consists of a mixture of all possible hexanucleotides in 4 x Klenow buffer and 25 μM dATP, dGTP, dTTP. The reaction was incubated at 37°C for 1-2 hours and terminated by adding 2 μl of 0.25M EDTA. In order to reduce the amount of non-incorporated [32P] dCTP, the probe was precipitated by addition of 5 μg of yeast tRNA (acting as a DNA carrier), NaCl to 0.2 M, and 2.5 volumes of ethanol. After 10 min centrifugation, the pellet was rinsed in 70% ethanol, dried under vacuum and resuspended in 100 μl of TE. The probe was denaturated by boiling 5 min immediately before addition to the hybridisation bag.

2.2.2.1 5' end labelling

Oligonucleotides were radiolabelled by T4 polynucleotide kinase. This enzyme transfers the γ-phosphate from [32P] γ-ATP to the 5' OH group of a dephosphorylated oligonucleotide. 30 ng of single stranded oligonucleotide was mixed with 30 μCi [32P] γ-ATP (3000 Ci/ mmol, Amersham) and 10 units T4 polynucleotide kinase (BCL) in a total volume of 20 μl kinase buffer (100 mM Tris-HCl pH 8.0, 0.01 mM MgCl2, 0.005 mM DTT) and incubated for 45 min at 37°C. The reaction mixture was terminated by heating 10 min at 68°C. The labelled oligonucleotide was either directly used (for Southern blot hybridisation) or precipitated in presence of 50 μg yeast tRNA, 2M ammonium acetate and 3 volumes of ethanol at -70°C and resuspended in TE (for primer extension analysis). Specific activity of the labelled oligonucleotide was usually of 0.5- 1 x10^8 cpm/μg of oligonucleotide, as estimated by counting the radioactivity of an
aliquot of the probe purified from free nucleotides and checked on a small polyacrylamide gel.

2.2.2.2. 3' end "tailing" labelling

This method was used when very high specific activity was required as for hybridisation of oligonucleotide onto Northern blot of total RNA.

Oligonucleotides were radiolabelled by the addition of a homopolymeric tail of $[^{32}\text{P}]\alpha$-CTP to the 3' end using the enzyme terminal deoxynucleotidyl transferase. 20 ng of single stranded oligonucleotide was mixed with 100 μCi $[^{32}\text{P}]\alpha$-CTP (3000 Ci/ mmol, Amersham) and 30 units terminal deoxynucleotidyl transferase (BCL) in a total volume of 20 μl reaction buffer (50 mM Tris-HCl, 200 mM potassium cacodylate, 2.5 mM CoCl₂, 0.5 mg/ml BSA pH 6.6) and incubated for 1-2 h at 37°C. The reaction mixture was terminated by heating 10 min at 68°C and the labelled oligonucleotide was purified by ethanol precipitation as described for 5' end labelling. Specific activity of the labelled oligonucleotide was of 2-5 x 10⁹ cpm/μg of oligonucleotide, as estimated by counting the radioactivity of an aliquot of the probe purified from free nucleotides.

2.2.3. Synthesis of radioactive RNA probe

RNA probes were produced in vitro using T3 or T7 phage polymerase systems and were used for in situ hybridisation or RNase protection assays.

A plasmid containing the insert of interest and a flanking T3 or T7 promoter sequence (eg in pBluescript vector) was linearised with a restriction enzyme distal to the insert. After phenol/chloroform extraction and ethanol precipitation, 0.5-1 μg linear DNA template was incubated with a ribonucleotide mix (ATP, CTP, GTP, UTP), $^{32}\text{P}$ or $^{35}\text{S}$ UTP, 10 mM DTT, RNase inhibitor (Promega) and the appropriate polymerase in a total volume of 10 μl in transcription buffer (5x transcription buffer = 200 mM Tris-HCl, pH 7.5; 30 mM MgCl₂; 10 mM spermidine; 50 mM NaCl) for 1 h at 37°C. The proportions of labeled and cold UTP were adjusted to yield probes of the specific activity required. DNA templates were then degraded using RQ1 DNase (Promega). The probe was purified by
phenol/chloroform extraction and ethanol precipitation in presence of carrier. The pellet was resuspended in 100 μl DEP-treated dH2O (0.1% diethylpyrocarbonate in double distilled water, autoclaved) and 1 μl was run on a 20 x 20 cm 4% polyacrylamide urea gel to check the integrity of the probe. The probe was diluted at the concentration required in the appropriate hybridisation mix (see section 2.3.) and kept at -20°C. RNA probes were usually kept no more than 3-4 days since degradation occur rapidly.

2.3 RNA analysis techniques

2.3.1. Tissue dissection

Male rats were killed and the organs of interest rapidly removed and dissected on ice. Tissues were immediately frozen in liquid nitrogen. The rat brain was dissected as follow: The brain was taken out of the skull and turned upside down; the cerebellum was separated from the underlying brainstem by severing the cerebellar peduncles; the hypothalamus was removed as a block lying between the optic chiasm and the mammillary body and extending 1 mm from each side of the midline and 2mm deep. The remainder of the brain was split into halves along the midsaggital plane, then, on each side, the fornix was severed and the hippocampus removed by peeling it away from the wall of the lateral ventricle; a sample of parietal cortex was also dissected.

2.3.2. RNA isolation from rat tissues

Total RNA was extracted following the method of (Chomczynski, 1987). Tissues were homogenised in a denaturing solution (4M guanidinium thiocyanate, 0.025M sodium citrate, 0.5% sarcosyl and 0.1 M β-mercaptoethanol) at a concentration of 5 to 10 ml per g of tissue, using a Dounce tissue grinder. DNA was precipitated by addition of sodium acetate to 0.2M, pH4, and proteins were removed by phenol/chloroform extraction. The aqueous phase containing RNA was precipitated twice with isopropanol and finally resuspended in DEP-treated dH2O. RNA concentration and purity was assayed spectrophotometrically and aliquots stored at -70°C prior to use.
2.3.3. RNA electrophoresis and Northern blot analysis

RNA was fractionated on 1% or 1.2% agarose gels under denaturing conditions to minimise secondary structure which alters mobility. For 100 ml gel mix, the agarose was melt into 88 ml of DEP-treated dH2O and after cooling to 55°C, 10 ml of 10 x MOPS buffer (0.4 M Morpholinopropanesulphonic acid pH7, 100 mM NaOAc, 10 mM EDTA pH8) and 2 ml of formaldehyde solution was added and the gel poured quickly. RNA samples (up to 25 µg) in 5 µl were mixed with 2.25 µl of 10 x MOPS buffer, 4 µl formaldehyde and 11.25 µl formamide, before denaturation for 10 min at 65°C. Then, 1 µl of 1mg/ml ethidium bromide and 2.5 x loading buffer (section 2.1.1) was added and samples were loaded and electrophoresed at 80 mA for 4-5 hours. Then gels were soaked in 20 x SSC to remove formaldehyde and blotted onto nitrocellulose (Hybond C extra) or nylon (Hybond N) (Amersham International, UK) by capillary transfer overnight as for Southern blot (section 2.1.5.) in 20 x SSC. Filters were processed as for Southern blots.

Hybridisation to cDNA probe was as for Southern blot, however a different protocol had to be used for hybridisation of oligonucleotides on Northern blot. This protocol followed the method of Henderson et al (1991). Hybridisation buffer contained 1x SSC, 2x Denhardt's solution, 1% nonfat dry milk, 10% dextran sulfate, 2% SDS, 200µg/ml herring testis DNA, and 200µg/ml polyadenylic acid. Hybridisation temperature were 5°-10°C below the Tm50, the temperature at which 50% of the DNA probe/mRNA hybrids melt. The Tm50 was calculated using the following equation:

\[ T_{m50} = 16.6 \log([Na^+]) + 81.5 + 0.41(\% G+C) - 675/ \text{number of bases in the probe} \]

where [Na+] is the molar concentration of Na+ in the hybridisation mix, and % G+C is the percentage of guanine plus cytosine residues in the probe. The length of the labelled nucleotide tail (section 2.2.2.2.) was not considered in the total probe length. Post-hybridisation washes and autoradiography were as for Southern blot analysis although less stringent.
In situ hybridisation on rat tissue sections

Adult or juvenile male Wistar rats (180-220g) were decapitated and the organs of interest rapidly removed on ice. Tissues were dissected and immediately frozen on dry ice. Tissue was stored at -85°C prior to sectioning in a cryostat at -20°C. 10 μm sections were mounted onto gelatin and poly-L-lysine-coated slides, and stored at -85°C until hybridisation was performed.

Before hybridisation, tissue sections were fixed in 4% paraformaldehyde/0.1M phosphate buffer and washed in 2xSSC made up in DEP-treated water. Filter sterilised prehybridization buffer containing 50% formamide, 0.6M NaCl, 10mM Tris-HCl pH7.5, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.5 mg/ml denatured salmon sperm DNA, 0.5 mg/ml total yeast RNA and 0.05 mg/ml yeast tRNA was pipetted onto the sections (200 μl/slide) and the slides were incubated at 37-42°C for 2 h in sealed boxes.

The RNA probe was denatured and added to hybridisation buffer (50% formamide, 0.6M NaCl, 10mM Tris-HCl pH7.5, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.1 mg/ml denatured salmon sperm DNA, 0.05 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 0.1g/ml Dextran sulphate, and 10 mM DTT) to a final concentration of 10 x 10^6 cpm/ml and applied to the slides (200 μl) after the prehybridisation buffer had been drain off. The slides were incubated overnight at 37-42°C in sealed boxes. Following hybridisation the slides were rinsed twice in 2x SSC for 30 min at room temperature prior to RNase A digestion (30μg/ml for 30 min at 37°C). The slides were washed in reducing salt concentrations to a maximum stringency of 0.1x SSC at 60°C for 60 min. After dehydration in increasing concentrations of ethanol (50%,70% & 90%) containing 0.3M sodium acetate, sections were air dried and exposed to autoradiographic film (Hyperfilm β-max, Amersham International, UK). Similar sections were dipped in photographic emulsion (Ilford, UK) and stored at 4°C for 20-30 days before being developed and counterstained with haematoxylin and eosin.

Controls, carried out to test both the specificity of the hybridisation method and the probe included pretreatment of sections with RNase A (100 μg/ml for 1 h at 37°C) prior to hybridisation with an antisense probe and
hybridisation using labelled-RNA "sense" probe (not complementary to mRNA under test, and applied at 10x10^6 cpm/ml).

2.3.5. Primer extension analysis

This method was used to map 5' termini of 11β-OHSD mRNA. (Moisan et al, 1992b) following the protocol described in Bonthron et al (1988)

The test RNA is hybridised with an excess of a single stranded DNA primer (a synthetic oligonucleotide), complementary to the mRNA and radiolabelled at its 5' terminus. Reverse transcriptase is then used to extend this primer to produce cDNA complementary to the RNA template. The length of the resulting end-labelled cDNA, as measured by electrophoresis through a polyacrylamide gel under denaturing conditions, followed by autoradiography, reflects the distance between the end-labelled nucleotide of the primer and the 5' terminus of the RNA. Best results were obtained when the primer was a 30-40 mer and when it was located 100-200 bp from the 5' terminus.

Oligonucleotides were labelled with ^32P-γ-[ATP] and T4 polynucleotide kinase (section 2.2.2.1.). 10^6 cpm were hybridised in solution to total RNA samples (50 μg) at 60°C for 1 hour in annealing buffer (100 mM KCl, 10 mM MgCl₂, 25 mM Tris-HCl pH 8.5) in a total volume of 12 μl. Primer extension reactions were carried out in 40 μl of 30 mM KCl, 8 mM MgCl₂, 50 mM Tris-HCl, 500 μM each dNTP, 50 μg/ml actinomycin D, 20 units of RNasin (Promega, UK) and 50 units of AMV reverse transcriptase (Life Science) at 42°C for 30 min. After phenol-chloroform extraction and ethanol precipitation, the size of the reaction product was analysed on a 6% polyacrylamide gel by comparison with a sequencing reaction used as a size ladder (section 2.1.6.3.).

2.3.6. RNase protection assay

This method was also used to map 5' termini of 11β-OHSD mRNA and to locate the 5' and 3' splice junctions in relation to sites of cleavage with restriction enzymes in the cloned rat 11β-OHSD gene (Moisan et al, 1992b).

For this an excess of radiolabelled single stranded RNA, complementary to appropriate segments of the template DNA, is hybridised in solution
with the RNA being tested so that all complementary sequences are driven into radiolabelled RNA:RNA hybrids. The unhybridised material (non-complementary RNA and tails of the RNA probe that do not hybridise) is then removed by digestion with RNase, which specifically degrades single stranded RNA. The size of the protected radiolabelled RNA:RNA hybrid fragment is then detected by electrophoresis through a polyacrylamide gel under denaturing conditions, followed by autoradiography.

5x10^5 cpm of 32P[UTP]-labeled antisense RNA probes was hybridised to RNA samples (25-50 µg) overnight at 46°C in 80% formamide, 0.4 M NaCl, 40 mM Pipes pH 6.7 and 1 mM EDTA. Non-hybridised RNA was degraded by the addition of RNAses A (40 µg/ml) and T1 (2 µg/ml) (Boehringer Mannheim) for 1 hour at 30°C. Following proteinase K treatment and phenol/chloroform extraction, the samples were ethanol precipitated and analysed on a 6% polyacrylamide gel in parallel with a sequencing reaction (section 2.1.6.3.).

2.4 11β-OHSD enzyme assay

Adult male Wistar rats (180-220 g) were decapitated and the organs of interest rapidly removed and dissected on ice as described for RNA isolation (section 2.3.1.). Tissues were homogenised in 5 ml of Krebs-Ringer bicarbonate buffer (118 mM NaCl, 3.8 mM KCl, 1.19 mM KH2PO4, 2.54 mM CaCl2.2H2O, 1.19 mM MgSO4.7H2O, 25 mM NaHCO3 and 0.2% glucose, gassed with 95% O2/5% CO2 to pH7.4) using a dounce tissue grinder. The total protein content of the homogenate was estimated colorimetrically (Bio-Rad protein assay kit).

0.5 mg/ml of total protein was then incubated with a final concentration of 12nM 1,2,6,7,^3H-corticosterone (specific activity:84Ci/mmol, Amersham) in Krebs-Ringer buffer (+0.2% BSA) in a total volume of 250 to 1 ml, depending on the amount of tissue available, for 60 min at 37°C. In some experiments, the enzyme co-substrate NADP+ was added before incubation, at a final concentration of 200 µM. After incubation, steroids were extracted with 1 volume of ethyl acetate, dried, and resuspended in ethanol containing 5 mg/ml of unlabelled corticosterone and 11-dehydrocorticosterone. Steroids were separated by thin-layer
chromatography on silica gel plates using as solvent a mixture of 95% chloroform / 5% ethanol (92:8). Areas corresponding to steroids were identified under UV light and scraped into vials before elution with liquid scintillant (Cocktail T, BDH). Radioactivity was quantitated with a β counter. 11β-OHSD activity was determined as the percentage conversion of $^3$H-corticosterone to $^3$H 11-dehydrocorticosterone, calculated from the radioactivity of each fraction.

For inhibition studies, the 11β-OHSD inhibitor β-glycyrrhetinic acid (GE) was diluted in ethanol at a concentration of $10^{-2}$ M and subsequently diluted in water. GE was added to tissue homogenates at $10^{-4}$ M, $10^{-6}$ M, $10^{-8}$ M final concentration and 11β-OHSD activity assayed as above.

2.5. In vivo studies

Male rats (Wistar, 200g) were maintained under conditions of controlled lighting (lights-on from 0.500 to 19.00 h) and temperature (22°C) and water and food ad libitum. Groups of animals (n=3-6 per group) were anesthetised with intraperitoneal fentanyl citrate (0.3 mg/kg, Janssen), fluanisone (10 mg/kg, Janssen) and midazolam (5 mg/kg, Roche) and adrenalectomised or sham-operated (surgery was done by June Noble, Centre for Biomedical Research, W.G.H.). Adrenalectomised animals were injected with dexamethasone at a dose of 0.2 mg/kg body weight/day, subcutaneously, or aldosterone (20μg/kg body weight/day, sub-cutaneously), the remainder received vehicle (5% ethanol-saline). A further group of adrenally-intact rats was injected with the antiglucocorticoid RU38486 (Roussel-UCLAF) (10 mg/kg body weight/day, sub-cutaneously). Rats were sacrificed one or ten days after surgery.

Statistics

Data were assessed by ANOVA followed by Scheffe's test or Student's t-test.. Significance was set at p<0.05. Values are expressed as means±SEM.
Chapter 3: Distribution of 11β-OHSD in brain
3.1 Introduction

Brain contains two distinct types of intracellular corticosteroid receptors, mineralocorticoid (or type I) and glucocorticoid (or type II) (Reul & De Kloet, 1985; Evans & Arriza, 1989). Glucocorticoid receptors (GR) are widely distributed in neurons and glial cells throughout the brain, as judged by immunocytochemical (Fuxe et al, 1985; van Eekelen et al, 1987) and cRNA/mRNA hybridisation procedures (Aronsson et al, 1988; van Eekelen et al, 1988; Chao et al, 1989; Herman et al, 1989; Sousa et al, 1989) which confirmed previous autoradiography using $[^{3}H]$ RU 28362 (a pure glucocorticoid) (Reul & De Kloet, 1986). High levels of GR are found in hippocampus and septum, parvocellular neurons of the paraventricular nucleus where glucocorticoids control the synthesis and release of CRH and vasopressin (Swanson & Simmonds, 1989), ascending monoaminergic neurons of the brain stem, where glucocorticoids control the synthesis of neuropeptide Y, POMC, enkephalin and dynorphin (Fuxe et al, 1985), supraoptic nucleus and cerebellum. Moderate GR concentrations are also found throughout the cortical hemispheres and in many thalamic nuclei, in a patch-like distribution in the striatal areas and in the central amygdaloid nucleus.

MR and their mRNAs show a more selective distribution (Krozowski & Funder, 1983; Arriza et al, 1987 & 1988, van Eckelen et al, 1988; Herman et al, 1989), with high levels in the limbic system (hippocampus, lateral septum and medial and central amygdala), olfactory nucleus, layer II of the cortex and brain stem sensory and motor neurons. Labelled ligand binding studies revealed that most limbic regions retain $[^{3}H]$aldosterone and $[^{3}H]$corticosterone equally well, whereas preferential labelling by $[^{3}H]$aldosterone was observed in the induseum griseum, the anterior hypothalamus, circumventricular areas, such as choroid plexus, and brain stem regions (Birmingham et al, 1984; McEwen et al, 1986b). MR mRNAs have been reported in anterior hypothalamus, subfornical organs, and choroid plexus (van Eckelen, 1989) and in hypothalamic regions after glucocorticoid treatment (Swanson & Simmonds, 1989).

Brain MR and GR were first characterised by their different affinities for natural and synthetic ligands in vitro (Table 3.1).
Table 3.1: Binding affinities of rat hippocampal corticosteroid receptors (from De Kloet, 1991).

GR show the highest affinity for potent synthetic glucocorticoids such as dexamethasone and RU 28362 and the antiglucocorticoid RU38486, a lesser affinity for corticosterone (B) and cortisol (F), and a much lower affinity for aldosterone and the antimineralocorticoid spironolactone. In contrast, MR show negligible affinity for the above RU compounds, moderate affinity for dexamethasone, and equivalent high affinity for aldosterone and corticosterone, which, moreover, is about ten times higher than that shown by GR for corticosterone. In species where cortisol is the major circulating glucocorticoid, both MR and GR show a higher affinity for cortisol. Because corticosterone circulates in distinctly higher amount than aldosterone hippocampal MR are occupied by corticosterone and hippocampal MR could be considered as glucocorticoid receptors. Accordingly, the nomenclature type I/ type II was adopted by some authors. Binding kinetics studies, in the light of the observed higher affinity of MR compared to GR for corticosterone, showed that corticosterone at low dose (down to 2.5 nM) extensively occupies MR in the hippocampus, whereas at the circadian peak and after stress, corticosterone progressively occupies GR. This observation led to the concept of a binary glucocorticoid response in which MR could provide a high-sensitivity, low response function to glucocorticoids, while the GR

<table>
<thead>
<tr>
<th>$K_D$ (nM)</th>
<th>Mineralocorticoid receptor</th>
<th>Glucocorticoid receptor</th>
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<tbody>
<tr>
<td>B</td>
<td>0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>F</td>
<td>1.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Aldo</td>
<td>0.4</td>
<td>16.3</td>
</tr>
<tr>
<td>Dex.</td>
<td>4.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>2.0</td>
<td>29.3</td>
</tr>
<tr>
<td>RU 28362</td>
<td>$&gt;420$</td>
<td>0.7</td>
</tr>
<tr>
<td>RU 38486</td>
<td>$&gt;1000$</td>
<td>1.8</td>
</tr>
</tbody>
</table>
would act with low sensitivity but high response (reviewed in Evans & Arriza, 1989; De Kloet, 1991).

MR are apparently structurally identical in all tissues investigated including kidney and brain, and are translated from a single mRNA species (Arriza et al, 1987 & 1988, Patel, 1989). As discussed in the general introduction (section 1.2.3.), the recombinant human MR expressed in CV1 cells show the same binding affinities that those found by radioligand binding studies in vitro described above. The concept of a MR as a receptor with no inherent specificity for mineralocorticoids over glucocorticoids remains controversial since MR in kidney and in certain areas of the brain listed above, are, in vivo, relatively insensitive to glucocorticoids. Thus, MR in circumventricular organs, medial amygdala and anterior hypothalamus, selectively bind aldosterone whereas corticosterone and dexamethasone are ineffective (McEwen et al, 1986b). Aldosterone at these sites affects salt appetite and blood pressure regulation. Salt hunger developing in adrenalectomised animals can be reduced by low dose of aldosterone (0.1μg/h) whereas corticosterone infused at a dose corresponding to stress levels (50 μg/h), neither mimics nor blocks the effect of aldosterone. However this effect is blocked by the antimineralocorticoid RU 28318 which also almost totally inhibits [3H] aldosterone uptake in rat forebrain (McEwen et al, 1986a). Furthermore, lesions of the anteroventral third ventricle, AV3V (which includes the medial preoptic area and OVLT) attenuate salt appetite and affect blood pressure (Berecek et al, 1982), but in contrast hippocampectomy (which removes limbic system MR) does not alter the actions of adrenal steroids on salt appetite (Magarinos et al, 1986). Aldosterone may act synergistically with the central renin-angiotensin-system (Fluharty & Epstein, 1983, Sakai et al, 1986), atrial natriuretic peptide and ACTH to satisfy salt appetite.

Chronic adrenal insufficiency or adrenalectomy result in reduced blood pressure that can be restored by corticosterone replacement or aldosterone during salt loading. Aldosterone replacement requires increased participation of the renin-angiotensin system, vasopressin, and norepinephrine to maintain blood pressure (Yagil et al, 1986, 1988). Furthermore, intracerebroventricular (icv) infusion of aldosterone (10 ng/h) in rats or dogs produces significant increases in resting blood pressure. In rats this effect is blocked by the concomitant icv infusion of
prorenone, an aldosterone antagonist, and enhanced by salt intake. RU 28318, a selective mineralocorticoid antagonist, infused icv, inhibits the development of aldosterone-induced hypertension. Corticosterone, at a dose of 10 ng/h icv for up to 4 weeks is ineffective. In addition, concomitant infusion of corticosterone antagonises the effect of icv infused aldosterone, suggesting competitive inhibition at the MR site involved in central cardiovascular control (reviewed by Gómez-Sánchez, 1991; De Kloet, 1991).

In contrast, in the hippocampus and septum MR preferentially bind corticosterone in vivo, and their function may even be antagonised by aldosterone. For example, aldosterone blocks the serotonin response to corticosterone in the hippocampus. Bilateral adrenalectomy in rats leads to a reduction in serotonin turnover in dorsal hippocampus. A low dose of corticosterone given subcutaneously immediately after adrenalectomy restores the serotonin turnover to normal values but pretreatment of adrenalectomised rats with aldosterone prevents the uptake of corticosterone to these receptors and thus blocks the response (De Kloet et al, 1983)

GR may also show regional differences in sensitivity to corticosteroids, despite a structural homogeneity. Thus, low doses of corticosterone can prevent the post-adrenalectomy rise of GR levels in hypothalamus and hippocampus, but not in cortex and cerebellum (Brinton & McEwen, 1987; Luttge et al, 1989), whereas low-dose dexamethasone prevents the rise in all brain regions. The mechanism(s) responsible for these site-specific differences in corticosterone sensitivity of cerebral MR and GR in vivo are unknown, although a possible explanation, at least in cerebral cortex where MR and GR are colocalised, is that regulation of both MR and GR levels is mediated by corticosterone binding to MR (Luttge et al, 1989).

The role of 11β-OHSD described in kidney provided a convenient starting point for studies on brain, as 11β-OHSD became the obvious candidate for mediating corticosteroid specificity within the central nervous system, particularly since CBG has been shown to be not expressed in the brain (Hammond, 1990) and its transport from the blood is prevented by the blood brain barrier. Previous recent studies had found 11β-OHSD activity absent in hippocampal extracts in vitro (Edwards et al, 1988; Funder et al, 1988). However, 11β-OHSD activity in brain had been
reported in earlier studies in rat (Mahesh & Ulrich, 1960; Grosser & Bliss, 1963; Peterson et al, 1965), in mouse (Grosser, 1966), in dog (Eik-Nes & Bratteod, 1965) and in baboons (Grosser, 1968). In addition, it was found that dehydrogenase activity was enhanced by adding NADP+ in the incubation buffer (Peterson et al, 1965; Grosser & Bliss, 1963).

The aim of this study was thus to investigate 11β-OHSD distribution in the brain as a first step to the study of its possible role in the localised differences in corticosterone binding to MR and/or GR in the brain.

3.2 Results

3.2.1. NADP+-dependent 11β-OHSD in vitro activity in brain

In tissue homogenates, 11β-OHSD activity expressed as the percentage conversion of 3H-corticosterone to 3H-11-dehydrocorticosterone in 60 min (see section 2.4), showed very low levels of activity in the absence of NADP+ in every brain region tested (under 10% conversion), except in cerebellum where 18% conversion was found. This was significantly more than heart which showed 0.8% conversion (indistinguishable from the assay blank values) but modest level compared to kidney in which 63.4% conversion was found under these conditions. As NADP+ is a co-substrate for 11β-OHSD, we looked at the effects of various concentrations of NADP+ on 11β-OHSD activity by adding it to the incubation buffer (Fig. 3.1.). Addition of 200 μM NADP+ led to a modest increase in activity in kidney (by 15.6%) and heart (by 14.2%) but a marked increase in 11β-OHSD activity in cerebellum (by 46%) (all p<0.001; ANOVA followed by unpaired students t test). Because the brain does not store high levels of NADP+ (Glock & McLean, 1955), we, thereafter, systematically added it to the incubation buffer at a final concentration of 200 μM.

In these conditions we found 11β-OHSD activity in every brain subregion (Fig. 3.2). Highest levels of activity, similar to those found in kidney under the same experimental conditions, were found in cerebellum, hippocampus, cortex and pituitary.
Figure 3.1: Effect of NADP⁺ on 11β-OHSD activity in homogenates of cerebellum compared to kidney and heart. n=5 for 0 and 200 μM NADP⁺, otherwise n=2.

Figure 3.2: 11β-OHSD activity in homogenates of rat brain subregion. n=5
The co-substrate NADP⁺ was added at a final concentration of 200 μM
Northern blots (20μg total RNA) (see section 2.3.2.) hybridised with
32P-labelled 11β-OHSD cDNA probe under stringent conditions (last post-
hybridisation wash was in 0.2 xSSC, 0.1 % SDS at 60°C and for 30 min.)
showed 11β-OHSD mRNA expression in every region of the brain, although
at levels lower than in liver and kidney (Fig 3.3). No expression was
detected in heart under this condition. Within the brain, cerebellum,
cortex and hippocampus showed high 11β-OHSD mRNA expression with
lower expression in brain stem, spinal cord, hypothalamus and pituitary.
The signals detected in testis, pituitary and hypothalamus were weaker
than expected since a high 11β-OHSD activity was found in these tissues.
Loading of RNA on the gel was judged only by O.D. and by visualisation of
the RNA under UV after staining the gel with ethidium bromide. Testis
RNA was obviously underloaded but not pituitary or hypothalamus. Thus,
discrepancy between mRNA and in vitro activity in these two tissues
might be due to dissection artefacts or to the presence of a different 11β-
OHSD species not recognised by the cDNA employed.

Figure 3.3: Northern blot (20 μg total RNA per lane), hybridised with
32P-labelled 11β-OHSD cDNA probe. Li= liver, K=kidney, He=
Heart, T=Testis, Lu= lung, Ce= Cerebellum, Hi= Hippocampus, Co=
parietal cortex, Hy= Hypothalamus, St= brain stem, P= pituitary,
Sc= Spinal cord.
In situ hybridisation using $^{35}$S-labeled cRNA antisense probes confirmed localised cerebral 11β-OHSD mRNA expression. Data from microscopy of emulsion-coated sections, counterstained with haematoxylin and eosin, are summarised in Table 3.2. Highest levels of 11β-OHSD mRNA were found in parietal cortex neurons (layer IV), modest expression was found in the caudal part of the frontal cortex (layer IV only) and piriform cortex. Other cortical neurons did not express 11β-OHSD mRNA. Most, if not all, hippocampal neurons showed high expression of 11β-OHSD mRNA (Fig. 3.4a & b), with the highest levels in CA3 pyramidal cells. Hypothalamus generally showed low 11β-OHSD mRNA expression but localised expression was found in the anterior part of the medial preoptic area (including the organ vasculosum of the lamina terminalis; OVLT) and the posterior part of the arcuate nucleus around the posterior margin of the third ventricle (not shown). In a later in situ hybridisation study in the hypothalamus (Yau et al., 1991b) high levels of 11β-OHSD mRNA were found in paraventricular nucleus. There was also high mRNA expression in anterior lobe of pituitary, vasculature in the meninges and choroid plexus. 11β-OHSD mRNA is very abundantly expressed in the cerebellar cortex (Fig. 3.6) with highest expression in the Purkinje and granule cell layers.
hypothalamus:
anterior medial preoptic area ++
lateral preoptic area ±
suprachiasmatic nucleus -
supraoptic nucleus +
periventricular nucleus -
posterior hypothalamus -
posterior arcuate nucleus ++(+)
striatum ±
lateral septum +
caudate putamen ±
medial habenula ++
lateral habenula ±
amygdala +
olfactory nucleus +++
olfactory tubercle +++
taenia tecta +

hippocampus:
dentate gyrus (granule) ++
CA1 (pyramidal) ++
CA2 (pyramidal) ++
CA3 (pyramidal) +++
molecular layer -
fimbria +
cortex:
frontal (layer IV) ++(+) 
frontal (other layers) ±
piriform +++
parietal (layers II and III) ++
parietal (layer IV) +++(+) 
parietal (layer VI) +
white matter -
meninges +++
choroid plexus +++
pituitary (anterior) +++
pituitary (neurointermediate lobe) ±

Table 3.2: Distribution of 11β-OHSD mRNA expression by in situ hybridisation histochemistry in selected forebrain regions and pituitary.

Key: - absent, ± very weak (just above background), + weak, ++ moderate, +++ strong. (+) denotes higher expression in some areas of the structure indicated.
Figure 3.4: In situ hybridisation of rat brain sections hybridised with $^{35}$S-labelled 11$\beta$-OHSD cRNA antisense probe.

(a) autoradiograph of a coronal section of forebrain.
(b) high power view of CA3 hippocampus
(c) No hybridisation was detected when the sense probe was used in the same conditions.
Figure 3.6. (a) Autoradiograph of a coronal section of rat cerebellum and brain stem hybridised with $^{35}$S-labelled 11β-OHSD cRNA antisense probe. No hybridisation was detected when the sense probe was used in the same conditions.
3.3 Discussion

We found 11β-OHSD bioactivity and mRNA expression in rat brain. In NADP⁺ unsupplemented brain subregions homogenates, enzyme activity is low but is markedly increased, to levels similar to those found in kidney, when the co-substrate NADP⁺ is added to the incubation medium. Although brain levels of NADP⁺ and other adenine dinucleotides are very low compared to liver, kidney and other tissues (Glock & McLean, 1955), it is likely that brain, a tissue that is largely metabolically-dependent on the circulation, will resynthesise rather than store energy-pathway related molecules such as NADP⁺. Thus the large discrepancy between basal and NADP⁺-supplemented 11β-OHSD activity in brain in vitro may reflect rapid depletion of endogenous dinucleotide in the reaction mixture and this might be responsible for previous negative findings (Edwards et al, 1988; Funder et al, 1988). Whether limitation of NADP⁺ concentrations plays any role in vivo in the regulation of 11β-OHSD activity remains to be determined.

Lakshmi et al (1991), also found highest NADP⁺-dependent activity in cerebellum, hippocampus, cortex and pituitary. They showed that these tissues contain 11β-reductase as well as 11β-dehydrogenase activity, although in every brain subregion the ratio is in favour of dehydrogenation. They also show that cortisol is a poor substrate for rat hippocampal 11β-OHSD, providing another explanation for Funder et al (1988) negative results, and suggesting species and tissue differences in 11β-OHSD specificity.

11β-OHSD in all brain areas investigated showed expression of an apparently single mRNA species of identical size to that found in liver. In addition, Western blot analysis revealed a 34 kD immunoreactive species as found in liver but an additional 26 kD species was also detected using one of the antibody (56-125). However, this 26 kD species seems to have no 11β-OHSD activity (Lakshmi et al, 1991).

In situ hybridization localised 11β-OHSD mRNA expression to pyramidal and granule cell neuronal layers of the hippocampus with highest expression in the CA3 field. Whether 11β-OHSD functions to regulate access of corticosterone to hippocampal glucocorticoid and/or MRs, and thereby affects the functions of either or both receptors,
remains to be determined. However, as 11β-OHSD, MR and GR mRNAs are expressed in the majority of hippocampal neurons co-localisation is very probable. Furthermore, utilisation of the quantitative autoradiographic [14C]2-deoxyglucose uptake technique in conscious rats, provided evidence for 11β-OHSD in vivo relevance. Indeed, it has been shown that inhibition of 11β-OHSD by glycyrrhetinic acid in vivo, increases glucose utilisation in hippocampus, particularly in CA3 field, and also in several regions of the hypothalamus including the pre-optic area, the arcuate nucleus and paraventricular nucleus (Seckl et al, 1991). Glucocorticoids affect behaviour and memory via the hippocampal corticosteroid receptors. Impairment of these functions in rat emerge with age or after exposure to chronic stress, due to a decrease in GR concentrations in the hippocampus, which leads to dysfunction of the HPA axis and hypersecretion of glucocorticoids. Sustained exposure to glucocorticoids, in turn, produces a cascade of metabolic effects that can result in neural degeneration and a loss of cognitive function (Sapolsky et al, 1986). A possible role for 11β-OHSD could be to protect hippocampal pyramidal and granules cells from glucocorticoid neurotoxic effects. Also, not all aldosterone binding to hippocampal MR is readily displaced by excess corticosterone and aldosterone may antagonise the MR-mediated actions of corticosterone on hippocampal serotonin turnover (De Kloet et al, 1983, McEwen et al, 1986a). Thus, there may be a subpopulation of hippocampal MR aldosterone-selective that are protected, at least in part, from corticosterone by 11β-OHSD.

In cerebellum and neocortex there were also high levels of 11β-OHSD bioactivity and mRNA expression. These regions have much GR immunoreactivity and mRNA expression (Sousa et al, 1989), but little MR expression (Arriza et al, 1988). In addition, the post-adrenalectomy rise in GR levels in these tissues is down-regulated by very low-dose dexamethasone but not corticosterone (Brinton & McEwen, 1987). An alternative explanation to that evoked by Luttge et al, (1989), is the presence of active 11β-OHSD which metabolises corticosterone but not dexamethasone.

Much pharmacological and ligand-binding data suggest that periventricular areas of the hypothalamus are the location of the central aldosterone-mediated effects on salt and water homeostasis (see introduction). We have found only very modest 11β-OHSD mRNA expression
and bioactivity in whole hypothalamic extracts, in vitro, but in situ hybridisation demonstrated localised higher 11β-OHSD mRNA expression in the most anterior region of the medial preoptic area/OVLT and the periventricular posterior part of the arcuate nucleus. Thus, 11β-OHSD predominates in those hypothalamic areas where MRs are thought to be aldosterone-selective and where central action of aldosterone on salt appetite and blood pressure regulation are thought to take place. At this sites 11β-OHSD could play the same role that it does in kidney, i.e. conferring aldosterone specificity to the MR. Therefore, abnormalities of 11β-OHSD activity might allow excessive binding of corticosterone to normally "aldosterone-selective" hypothalamic MRs leading to neurogenic hypertension. However, Gómez-Sánchez (1991), found that icv infusion of carbonexolone (an 11β-OHSD inhibitor) produces hypertension in rat, which can be blocked by administration of the antimineralocorticoid RU28318. This effect is surprising since chronic icv infusion of corticosterone has no effect and icv infusion of a glucocorticoid agonist (RU 28362) decreases blood pressure. Furthermore, concomitant icv infusion of corticosterone and aldosterone result in corticosterone counteracting the pressor response to aldosterone. Enhanced binding of corticosterone to the MR at this site, due to 11β-OHSD inhibition, would thus be expected to decrease blood pressure. Since it is impossible to know which sites are affected by icv infusion of a particular compound, further study is necessary to interprete this effect of carbenoxolone. In particular, carbenoxolone is not very lipid-soluble and might not access equally all areas of the brain. It would be of interest to see if GE, which is much more lipid-soluble would produce the same effects as carbenoxolone. Finally, carbonexolone is not a specific inhibitor of 11β-OHSD, and for example it may inhibit 5β-reductase (Latif et al, 1990), which would prolonged the half-life of aldosterone and tetrahydroaldosterone, thus increasing mineralocorticoid activity.

Moderately high 11β-OHSD mRNA expression (and bioactivity) was also found in anterior pituitary, suggesting that 11β-OHSD might influence the access of corticosterone to receptors at this site, and providing a possible explanation for observed preferential binding of dexamethasone, compared with corticosterone, to pituitary (De Kloet et al, 1975). Lakshmi et al (1991) found very high 11β-OHSD activity in the pituitary and they postulated that presence of 11β-OHSD might provide a short term
mechanism for preventing prolonged retention of active glucocorticoids in the gland and thus sensitivity to short term fluctuations of circulating steroid may be enhanced. Any role for 11β-OHSD in the regulation of the hypothalamic-pituitary adrenal axis by physiological corticosteroids remains to be determined.

Very recently, Sakai et al. (1992), reported the distribution and localisation of 11β-OHSD-like labelling in rat forebrain by immunocytochemistry. They used two antisera (56-125 & 56-126) directed against different epitopes on 11β-OHSD. Both antisera gave virtually identical staining patterns, although on Western blot 56-125 recognises an additional species of 26 Kd in the brain. Their study largely confirmed our in situ hybridisation data, showing higher labelling in neurons of the hippocampus and cerebral cortex. 11β-OHSD immunoreactivity was also detected in the preoptic area, central nucleus of the amygdala and bed nucleus of the stria terminalis, but there, preferentially in the glial cells.

In summary, high 11β-OHSD bioactivity, mRNA expression and immunoreactivity is found in rat brain. In brain subregions where MR retain aldosterone specifically in vivo, 11β-OHSD role may be similar to that described in kidney. However, presence of 11β-OHSD in tissues that do not contain MR but where GR is very abundant, or in tissues where MR does not display specificity for aldosterone, suggests that 11β-OHSD regulates the access of glucocorticoids to GR at these sites as it does in the skin (Teelucksingh et al, 1990) or in testis (Phillips et al, 1989).

It remains to be seen if 11β-OHSD and MR (and GR) co-localise to the same cells in the brain areas, whether this co-presence confers specificity to aldosterone on these cells and whether the more prevalent glial cell localisation has any significance.
Chapter 4: Tissue-specific regulation of 11β-OHSD
4.1 After corticosteroid manipulation

4.1.1. Introduction

The brain is extremely sensitive to glucocorticoids and variations within the normal physiological range produce detectable changes in the threshold to certain sensations as well as higher functions, such as concentration, memory and intellectual performance. In general, deficiency depresses cerebral activity, causing apathy and lassitude, loss of weight and appetite. Glucocorticoid excess, however, leads to mental overstimulation, resulting in a marked feeling of well-being, hyperactivity, increased appetite, reduced sleep and sometimes tension and irritability. It is therefore unsurprising to find a high incidence of neuropsychiatric disorders both in patients with Cushing's syndrome and Addison's disease (von Zerssen, 1976). Glucocorticoid hypersecretion emerges with age or as a result of chronic stress, and alters the sensitivity of the system of feedback regulation. This negative feedback is mediated by the pituitary, hypothalamus, and a number of suprahypothalamic sites, including the hippocampus. Involvement of the hippocampus is supported by studies showing that lesions in the hippocampus produce glucocorticoid hypersecretion and negative feedback insensitivity (Sapolsky et al, 1986). The inhibitory effects of glucocorticoids at the hippocampus appear to be mediated by the GR. Changes in GR concentrations in hippocampus but not in hypothalamus or pituitary are found as an effect of age (decrease of GR) or after neonatal handling (increase of GR) (Meaney et al, 1988).

In Chapter 3 we reported 11β-OHSD mRNA expression, immunoreactivity and bioactivity in rat brain, with particularly high expression in cerebellum, hippocampus and neocortex, and hypothesised that 11β-OHSD might control the access of corticosterone or cortisol to brain receptors and thereby modulate the effects of glucocorticoids on cerebral function. It is particularly interesting that 11β-OHSD is present in hippocampus where it could perhaps protects sensitive neurons from the deleterious effects of glucocorticoid hypersecretion. To investigate this further, we have examined the effects of acute and chronic corticosteroid manipulation on 11β-OHSD in brain and kidney.
4.1.2. Results: 11β-OHSD is up-regulated by glucocorticoids in brain

Five groups of rats were used for this study (n=3-6 per group). One group was adrenalectomised in order to clear endogenous corticosteroids, a second group was adrenalectomised and replaced with dexamethasone (0.2 mg/kg), by sub-cutaneous injection every day to study the effect of glucocorticoids, a third group was adrenalectomised and replaced with aldosterone (20 µg/kg), by sub-cutaneous injection every day, to study the effect of mineralocorticoids, a fourth group of intact animals was treated with the antiguclocorticoid RU38486 (10 mg/kg) and the control group was sham-operated and received vehicle. Rats were sacrificed 1 or 10 days after treatment (see section 2.5. for more details on the methods).

Twenty-four hours after adrenalectomy there were no significant changes in 11β-OHSD activity in brain subregions (Fig. 4.1.). By contrast, 10 days after adrenalectomy 11β-OHSD activity had fallen in hippocampus (by 14.4%), parietal cortex (by 12.3%) and cerebellum (by 7.2%), compared to sham-operated rats (Fig. 4.2). Dexamethasone replacement led to a reversal of the adrenalectomy-mediated changes in 11β-OHSD bioactivity in all brain regions examined, with rises in hippocampus (by 37.2%), parietal cortex (33.1%) and cerebellum (12.7%), when compared to adrenalectomy alone. In both hippocampus and parietal cortex, but not cerebellum, dexamethasone treatment significantly elevated 11β-OHSD activity above sham-operated control values. Treatment of intact rats with the antiguclocorticoid RU38486 for 10 days led to a marked significant decrease of 11β-OHSD activity in hippocampus (28.2% fall). Aldosterone replacement had no effect on hippocampal enzyme activity in adrenalectomised rats. None of the treatments altered renal 11β-OHSD activity (Fig. 4.1 and 4.2).

To test whether the changes in hippocampal 11β-OHSD activity occurred at the level of transcription, we analysed the effects of 10-day adrenalectomy and dexamethasone replacement by Northern blots (this experiment was done by Susan Low). RNA was extracted from a set of tissue of the 10-day experiment animals (n=5 per group) and hybridised with a [32P]-labelled 11β-OHSD cDNA probe under stringent conditions (last post-
Figure 4.1: Effect of acute (24 h) corticosteroid manipulations on 11β-OHSD bioactivity in rat brain subregions and kidney. Adx=adrenalectomy, sham=sham-operated controls, adx+dex=adrenalectomy and dexamethasone replacement.

Figure 4.2: Effect of chronic (10 days) corticosteroid manipulations on 11β-OHSD bioactivity in rat brain subregions and kidney. Adx=adrenalectomy, sham=sham-operated controls, adx+dex=adrenalectomy and dexamethasone replacement, RU38486 was given to intact rats, adx+aldo=adrenalectomy and aldosterone replacement. *p<0.05 compared with sham-operated control.
Figure 4.3: Effects of sham-operation (Sh) or adrenalectomy (Adx) ± dexamethasone replacement (Dex) for 10 days on 11β-OHSD mRNA expression in rat hippocampus. Autoradiograph of a Northern blot hybridised with $^{32}$P-labeled 11β-OHSD cDNA probe (solid arrow), then rehybridised with a similarly labelled probe to 7S RNA (open arrow) to control for RNA loading (experiment done by Susan Low).

Figure 4.4: Effects of sham-operation (SHAM) or adrenalectomy (ADX) ± dexamethasone replacement (DEX) for 10 days on 11β-OHSD mRNA expression in rat hippocampus. 11β-OHSD mRNA levels are shown as percentage expression in sham-operated controls (control mean normalised to 100%).
hybridisation wash in 0.2 x SSC, 60°C for 30 min.). The same Northern blot was reprobed with a cDNA encoding for 7S (Balmain et al, 1982) using the same conditions of hybridisation, to control for RNA loading. 11β-OHSD mRNA expression fell by 23% after adrenalectomy (compared to sham-operated animals) and rose by 70% (compared to adrenalectomised rats) after replacement with dexamethasone (Figs. 4.3 and 4.4), thus paralleling the changes in bioactivity.

4.1.3. Discussion

Previous studies have suggested that 11β-OHSD activity is under complex hormonal control, with tissue-specific regulation by thyroid hormones, sex steroids and insulin. L-thyroxine injected intraperitoneally in rat in vivo inhibits 11β-OHSD activity in liver but not in kidney; it requires several days before the effect on hepatic 11β-OHSD could be detected (40% decrease after 7 days, 80% decrease after 17 days), and the thyroid hormone is ineffective in vitro (Koerner & Hellman, 1964; Zumoff et al, 1983). In addition, 11β-OHSD activity was found higher in male than in female rat liver and kidney homogenates (Koerner & Hellman, 1964). Gonadectomy operated on rat male decrease 11β-OHSD in liver to female levels and replacement with testosterone in male restore the activity to normal control values and increase 11β-OHSD activity in female to male values. Hypophysectomy has no effect on 11β-OHSD activity in male rat but increase the activity in female to male values, suggesting that the pituitary exert a repressive action on 11β-OHSD activity in female (Lax et al, 1978). Induction of 11β-OHSD activity by estrogen has been shown in baboon placenta; 11β-OHSD activity in baboon placenta at midgestation was increased 3-fold by androstenedione treatment (an estrogen precursor), and fetectomy markedly reduced placental 11β-OHSD activity compared to intact untreated baboons near term. In addition cortisone and especially progesterone showed a dose dependent competitive inhibitory effect on placental 11β-OHSD (Baggia et al, 1990). Induction of 11β-OHSD activity by glucocorticoids has been reported in cultured human skin fibroblasts. In human fibroblasts 11β-reductase activity is higher than 11β-dehydrogenase activity but both activities were induced by treatment of the cells by dexamethasone, an effect blocked by glucocorticoids antagonists. In contrast 8-bromo-cAMP, phorbol esters, or insulin
decrease both 11β-OHSD activities in these cells (Hammami & Siiteri, 1991). Levels of 11β-OHSD activity in rat kidney were found unaltered by dexamethasone treatment or thyroxine administration in vivo or by castration in either sex; in contrast, estrogen administration increases enzyme activity in male rats (Smith & Funder, 1991).

Here we confirm the absence of corticosteroid regulation of renal 11β-OHSD activity but, by contrast, show regulation of 11β-OHSD activity in subregions of the rat brain in vivo by chronic, but not acute manipulations of glucocorticoid levels.

Adrenalectomy significantly attenuated enzyme activity in each brain region studied, an effect presumably due to depletion of glucocorticoids since (i) it was mimicked by treatment of adrenally-intact rats with RU38486 and (ii) it was reversed by dexamethasone, but not by replacement with aldosterone. Indeed, supraphysiological replacement with dexamethasone, a synthetic (non-metabolised) glucocorticoid, potentiated 11β-OHSD activity (compared with control) in hippocampus and parietal cortex, but not cerebellum. These subregional differences in the effects of dexamethasone might be due to tissue-specific regulatory factors. Since acute (24h) adrenal steroid alterations had no effect on 11β-OHSD activity, the chronic effects of glucocorticoids on cerebral 11β-OHSD may be indirectly mediated, i.e. via a second messenger, or perhaps turnover of the protein is slow. Nevertheless, the Northern analysis demonstrates that glucocorticoid effects on hippocampal enzyme activity occur, at least in part, at the level of 11β-OHSD gene transcription. Similar glucocorticoid-mediated up-regulation of 11β-OHSD in cell culture also suggests a direct effect (Hammami & Siiteri, 1991). Furthermore, both human and rat 11β-OHSD gene promoter regions contain sequences resembling glucocorticoid response elements (Tannin et al, 1991; Moisan et al, 1992b, Chapter 5). Interestingly, 4 days of treatment with thyroxine produces no effect on rat liver 11β-OHSD, but decrease in 11β-OHSD activity is detected after 7 days of thyroxine administration.

Since either chronic excess and deficiency of circulating glucocorticoids exert deleterious effects on the brain, including neuronal loss particularly in the hippocampus (Sapolsky et al, 1986; Sloviter et al, 1989), glucocorticoid-mediated regulation of 11β-OHSD activity may function to protect sensitive neurons from long-term elevation or fall of glucocorticoid levels, i.e. glucocorticoid regulation of brain 11β-OHSD may
maintain optimal exposure of neurons to glucocorticoids and preserve essential "tonic" neurochemical functions, whilst not attenuating the effects of the diurnal variation or acute stress.

The absence of glucocorticoid regulation of kidney 11β-OHSD provides further support to the growing body of data suggesting there is a distinct high activity renal 11β-OHSD isoform showing little or no regulation by hormones (Krozowski et al, 1990) or during ontogenesis (Moisan et al, 1992a, section 4.2). Teleologically this would accord with the need for renal 11β-OHSD to protect MR in the distal convoluted tubule and cortical collecting duct from any prevailing level of circulating glucocorticoid, thus ensuring selective aldosterone access even during chronic stress or in states of excess endogenous or exogenous cortisol.

MR in the hippocampus are apparently non-selective and bind corticosterone in vivo. However, these data have been derived from in vivo administration of radio-labelled ligands to animals which have undergone prior adrenalectomy to clear endogenous corticosteroids. Often, but not always, adrenalectomy has been performed several days before administration of steroid and any activity of cerebral 11β-OHSD may have been attenuated. Thus there may be a subpopulation of hippocampal MR that are aldosterone-specific and protected, at least in part, from corticosterone by 11β-OHSD.

4.2 During development

4.2.1. Introduction

Glucocorticoids play an important role during development, affecting the growth and differentiation of a number of tissues and organs, including the central nervous system (for review see De Kloet et al, 1988). High dose glucocorticoid administration during the early postnatal period in rodents leads to permanent inhibition of brain growth, with reduced neurogenesis and glial proliferation, attenuated dendrite formation and behavioural and neuroendocrine impairments (Meyer, 1985). Conversely, adrenalectomy of 11-day-old rats is followed by neuronal and glial proliferation (Yehuda et al, 1989) which is reversed by corticosterone replacement. Hence corticosterone is thought to exert an antiproliferative effect on postnatal neurogenesis and glial mitosis in the normal intact
animal. These effects are most marked during the first two weeks of life and thus close control of circulating glucocorticoid levels during this time appears critical for normal brain development.

Low and constant levels of circulating glucocorticoids were thought to be ensured by the hyporesponsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to stressful stimuli during the first two weeks of life in the rat, and the absence of circadian fluctuations in plasma glucocorticoid concentrations in neonates (Schapiro, 1962). However it has been recently found that various stressors trigger an adult-like pituitary ACTH response in 10-day-old rats, the magnitude of which is affected by corticosterone (Walker et al, 1991). Although the corticosterone response is smaller than that seen in the adult, CBG levels are very low in the neonate and free glucocorticoid levels may be similar. Brain MR which, at least in part, mediate corticosterone feedback on the HPA axis (Levin et al, 1988), are at near adult levels within 3 days of birth, and show the same topography than in the adult, i.e. hippocampus, septum, amygdala and cortical layer II (Rosenfeld et al, 1990; Sarrieau et al, 1988). In contrast, GR levels are low after birth and reach adult levels after 4 weeks of age. Immunocytochemistry of GR protein showed that the staining intensity was high at birth and then fell to minimal levels at day 12 (Rosenfeld et al, 1988 & 1990; Sarrieau et al, 1988). Thus the MR is thought to be the receptor responsible for the physiological effects of corticosterone during brain development, and GR may become important when glucocorticoid levels are elevated, e.g. after stress. Consequently, it remains unclear how the brain is protected from elevated glucocorticoid secretion following stress during this critical period of development.

Similarly to the adult, 11β-OHSD might also regulate glucocorticoid access to corticosteroid receptors during postnatal development. Indeed, the importance of 11β-OHSD has been described in placenta and in foetal lung (see introduction, section 1.3.6.). The ontogeny of 11β-OHSD mRNA expression has been studied previously using the cloned hepatic 11β-OHSD cDNA (Agarwal et al, 1989) in 1,2,3,4,8, and 16-week-old rats in kidney, liver and lung and showed a tissue-specific developmental pattern (Krozowski et al, 1990). In this study, we have examined 11β-OHSD bioactivity and mRNA expression in the brain, in comparison with kidney, during the first two weeks of life to determine whether 11β-OHSD might
affect cerebral glucocorticoid exposure during this crucial period of development.

4.2.2. Results: tissue-specific pattern of 11β-OHSD activity and mRNA during development

Tissues were examined for 11β-OHSD activity (see section 2.4) at various times in neonates and compared to adult values (Fig.4.5). 11β-OHSD activity in the kidney was already high at birth (69±3%) and rose significantly to adult values (80±1%) by postnatal day 5. By contrast, activity in hippocampus and cortex, though moderately high at birth (46±4% and 48±5% respectively), fell significantly to a nadir at postnatal day 10 and then rose gradually to adult values. Cerebellar 11β-OHSD activity was high at birth (61±3%), rose significantly to a peak at postnatal day 10 then fell to adult values by postnatal day 15.

Northern blot analysis (see section 2.3.2.) was used to investigate the postnatal development of 11β-OHSD mRNA expression. Kidney and hippocampal RNA extracts were run on a single gel and are thus directly comparable (Fig.4.6). In addition, the blot was reprobed with human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA (Edwards et al, 1985) using the same conditions of hybridisation, to control for RNA loading. We observed very high levels of 11β-OHSD mRNA expression in hippocampus at every age investigated. However only weak 11β-OHSD mRNA expression was detected in neonatal kidney, compared to contemporaneous hippocampus or adult kidney. Densitometry against GAPDH cDNA expression revealed 11β-OHSD mRNA was 2.5-3 times more abundantly expressed in neonatal hippocampus than in kidney. In adults, by contrast, 11β-OHSD mRNA expression was 1.5-2 times higher in kidney than hippocampus. Adult hippocampus showed a single 11β-OHSD mRNA species which was of similar size to the single species present in neonatal hippocampus. Total RNA from adult kidney was deliberately underloaded to allow differentiation of the multiple 11β-OHSD mRNA species (Fig 4.6). We detected four species confirming previous data (Krozowski et al, 1990) but found that one species gave a much more intense signal than the others. Only two or three species were detected in neonatal kidney.
Figure 4.5. Ontogeny of 11β-OHSD activity in rat brain subregions and kidney. * p< 0.05 compared to adult values (n=4-8 per group)

Figure 4.6. Ontogeny of 11β-OHSD mRNA expression in rat kidney and hippocampus. Autoradiograph of a Northern blot hybridised with 32P-labelled 11β-OHSD cDNA probe. 15 μg of total RNA were loaded per lane, except adult kidney (5 μg). Ad= adult rat.
Figure 4.7: Localisation of 11β-OHSD mRNA expression in neonatal brain. (a) Autoradiograph of a sagital section of 10-day-old rat brain hybridised with $^{35}$S-labelled 11β-OHSD cRNA probe. Note the high expression in dorsal (dh) and ventral hippocampus (vh), cerebellar cortex (cc) and neocortex (nc).

Figure 4.8: Localisation of 11β-OHSD mRNA expression in neonatal kidney. Autoradiograph of longitudinal kidney sections hybridised with $^{32}$P-labelled 11β-OHSD cRNA probe. All sections were hybridised with the same probe and exposed to Kodak XAR film simultaneously for 2 days; expression is thus directly comparable.
In situ hybridisation confirmed high expression of 11β-OHSD mRNA in neuronal layers of the hippocampus, cerebellar cortex and neocortex in the 10-day-old rat (Fig. 4.7). In neonatal kidney, only low expression of 11β-OHSD mRNA could be detected, predominantly in the outer cortex. Renal cortical expression of 11β-OHSD mRNA increased with age, but was much less than adult levels (Fig. 4.8).

4.2.3. Discussion

The presence of 11β-OHSD activity in perinatal brain has been reported in previous work, using homogenates or subcellular fractions of whole brain. 11β-OHSD activity was detected in cerebellum, cortex, brain stem and hypothalamus of fetal baboons, and was particularly high in the cerebellum of the third trimester primate brain, with 50% conversion of cortisol to cortisone (Grosser & Axelrod, 1968). 11β-OHSD activity was also detected in fetal mouse brain (Tye and Burton, 1979) and in fetal sheep tissues 10 days before term where 11β-dehydrogenase was only found in kidney, cerebrum and cerebellum (Smith et al. 1981). In this report we showed a subregionally-specific developmental pattern of 11β-OHSD activity in hippocampus, cortex and cerebellum, associated with high expression of a single species of 11β-OHSD mRNA.

11β-OHSD mRNA expression is high in neonatal hippocampus and cerebellum, but does not vary in parallel with the changes in activity. This might reflect developmental differences in mRNA stability, translation or post-translational modifications; the latter are known to affect the bioactivity of 11β-OHSD (Agarwal et al, 1990). However, the association of very low 11β-OHSD mRNA expression with high bioactivity in the early postnatal kidney indicates that another protein with identical activity may exist, as suggested to occur in parotid, mammary gland and mesenteric arcade (Krozowski et al, 1990).

The biological importance of the various ontogenic patterns of 11β-OHSD activity in the central nervous system remains to be determined. We have describe in section 4.1 how 11β-OHSD activity is affected by glucocorticoid levels in the adult rat brain but not kidney. Since the developmental patterns of 11β-OHSD activity in the neonatal hippocampus and cortex parallel changes in circulating corticosterone levels this may merely
reflect glucocorticoid regulation of enzyme activity in the neonatal period. However, 11β-OHSD activity in neonatal cerebellum shows a distinct pattern, although adult cerebellar 11β-OHSD activity is also affected by glucocorticoid levels. Therefore, other tissue-specific regulatory factors may be of importance.

Corticosteroid receptors display a considerable degree of plasticity during development. MR and GR show a different ontogeny in the developing rat brain and changes in receptor number and capacity are observed as a consequence of neonatal manipulation (De Kloet, 1991). For example, handling of neonatal rats, e.g. daily placing the neonatal animals in a new environment for 15 min., is associated with increased concentrations of GR in the hippocampus and in the amygdala but no changes elsewhere in the brain. This increased GR concentration led to greater hippocampal sensitivity to glucocorticoids and enhanced negative feedback efficacy in the handled rats. This, in turn, retard hippocampal neuron loss and spatial memory deficits associated with age in non-handled rats (Meaney et al, 1988).

The subregional differences in 11β-OHSD activity during development may also relate to a local and time-specific requirement for corticosterone. The enzyme may provide protection of sensitive neurons, glia and/or dendrites from elevated corticosteroid levels, for example during stress. By contrast, reduced 11β-OHSD activity may allow exposure of receptors to physiological corticosteroids ensuring inhibition of cell proliferation at appropriate times. Interestingly, the effects of synthetic (non-metabolised) glucocorticoids and corticosterone differ in their actions on cerebral neurogenesis, perhaps reflecting the activity and importance of the enzyme in vivo. Administration of dexamethasone or prednisone to pregnant rat, mice or monkeys result in a lowered body weight of the pups whereas treatment of rat with low dose of corticosterone cause trophic effects on the cerebellum at least until 12 days of age (Velasquez & Romano, 1987).
Chapter 5: Structure of rat 11β-OHSD gene
5.1 Introduction

The non-uniform tissue distribution of 11β-OHSD as well as its tissue-specific response to corticosteroid manipulation and during development suggest a complex regulation of the expression of 11β-OHSD gene. To understand the molecular mechanisms underlying this regulation, we decided to isolate the gene encoding rat 11β-OHSD.

Another reason for cloning 11β-OHSD gene was the growing body of evidence suggesting the presence of multiple 11β-OHSD isoforms. Immunohistochemical studies using the two antisera raised against the purified 11β-OHSD hepatic enzyme (Lakshmi & Monder, 1988) produced some surprising results. Although MR abound in distal renal tubules, immunoreactive enzyme was found only in proximal tubules (Edwards et al., 1988; Rundle et al., 1989). By contrast, enzyme bioactivity has been demonstrated both proximally and distally (Edwards et al., 1988; Bonvalet et al., 1990; Náray-Fejes-Tóth et al., 1991). Furthermore, Western blots showed different sizes of immunoreactive proteins in different tissues (Monder & Lakshmi, 1990). The first antiserum (56-125) identified a 34 kD protein in liver, an additional species of 40 kD in kidney, 26 kD in brain, and 47 kD in testis. The second antiserum (56-126) identified a 34 kD protein in all tissues, additional 68 kD proteins in liver and kidney (possibly a dimer), and again a 40 kD protein in kidney. Using the purified rat liver 11β-OHSD protein, an 11β-OHSD cDNA has been cloned from a liver cDNA library (Agarwal et al., 1989). This predicts the amino acid sequence of a protein of only 31 kD. This discrepancy can be accounted for by the glycosylation of the protein since when the cDNA was expressed in vitro a 34 kD protein was produced (Agarwal et al., 1990) but it was reduced to 31 kD when glycosylation was prevented by a tunicamycin homologue added to the cells.

Rat liver 11β-OHSD cDNA hybridises to a single major mRNA species of 1.7 kb in most tissues including liver, whereas three additional mRNA species are found in kidney (1.5 kb, 1.6 kb, and 1.9 kb) that are not explained by differences in polyadenylation (Krozowski et al., 1990) and a 3 kb mRNA was found in colon (Whorwood et al., 1991). In situ hybridisation using the liver cDNA localises 11β-OHSD mRNA in kidney to proximal, distal and cortical collecting duct epithelia (Yau et al., 1991a;
Stewart et al., 1991a) consistent with the bioactivity of the enzyme but in contrast with the immunohistochemical distribution.

More recently, cDNA clones have been isolated from a rat kidney library which are not contiguous with the liver cDNA clone in their 5' extremity (Krozowski et al., 1992). One of these has been sequenced and shows an identical open reading frame to the liver cDNA downstream from amino acid Met 27 (Agarwal et al., 1989). This deleted sequence encodes hydrophobic amino acids which constitutes a putative signal peptide for membrane insertion, suggesting that the isoforms may differ in their intra-cellular localisation.

Kinetically, distinct isoforms have also been described, in particular, the apparent Km of 11β-OHSD for corticosterone in intact cortical collecting duct cells is a 100-fold lower than the Km of liver 11β-OHSD (Náray-Fejes-Tóth et al., 1991).

Thus it appears that kidney contains several isoforms of 11β-OHSD which are kinetically and antigenically distinct from the liver form and may be encoded by alternative transcripts from the same gene as the liver form or may be products of an alternative gene. One of our aims was to determine whether the liver and kidney mRNA isoforms could be encoded by a single gene.

5.2 Results

5.2.1. Isolation of rat genomic clones and restriction endonuclease mapping

A Sprague-Dawley rat genomic library was screened by hybridisation to a 32P radiolabelled cDNA probe (see section 2.1.4.). As we were mainly interested in the 5' flanking region of the gene, we used the most 5' EcoRI-EcoRV fragment (460 bp) of the rat cDNA (Argawal et al., 1989) as a probe. The initial screening identified 4 putative positive clones but one of them was lost during the second round of screening. Three rounds of screening were necessary to obtain pure clones. DNA from the three clones were prepared as described in section 2.1.3.3. and were characterised by restriction endonuclease mapping. Double digestions with SacI and NotI, followed by hybridisation of the digests with either T3 or T7
Sacl+ NotI digests

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<th>λD</th>
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<td>5.5</td>
<td>5.5</td>
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<tr>
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* fragment that hybridises to T7 oligonucleotide

# fragment that hybridises to T3 oligonucleotide

for each clone, fragments 5.5 and 0.6 hybridise to cDNA probe

---

**Figure 5.1.** restriction analysis of the three positives λ clones
oligonucleotides or the cDNA, showed that the 3 clones are nearly identical (Fig. 5.1) and that a 15 kb clone (λA) encompasses the other two. λA was thus retained for further analysis. Further digestions with enzymes present in the vector (λ DASHII) polylinker or cutting rarely permitted a detailed restriction map of λA (Table 5.1. and Fig.5.2.) to be drawn.

5.2.2. Sequence analysis

In order to start the sequence analysis of this clone, various subclones in pBluescript vector were constructed (Fig. 5.2.) and oligonucleotides were derived from the cDNA sequence previously published (Argawal et al, 1989).

Sequence of oligonucleotides used in this study were as follows:-
OL-A=5'AGCTCTGTAGGACACACAAAGAAAACCTGCAGCTCTTTC3' (+36_+74)
OL-B= 5'GGAAAGCACAAGCAAGATGAGGTGCCCAAAAGGCTTGCTT3' (+831_+870)
OL-C= 5' GATTGGCTTGGTGGGATCC3' (-88_-68)
OL-D= 5' AACTGCCGTCACACAACTGGACCTGCC3' (+81_+105)
OL-E= 5' CACTTTCTCTCGCAATGACGT3' (+876_+896)
B739= 5' GGAAGAGAAATGGCATATCAT 3' (+924_+944)
942= 5' GCCAGGTCCCTGTTGGACGGCAGTT 3' (+81_+105)
M13 universal primer=5' GTAAAACGACGGCCAGT 3'
M13 reverse primer= 5' AACAGCTATGACCAGT3'

The sequencing strategy is depicted in Fig. 5.3. Most of the reactions were performed with the M13 universal or the reverse primer present at the flanking sites of insertion in the various subclones (Fig.5.2). The other oligonucleotides used are indicated.
**Single digests:**

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N.B. fragments larger than 9 kb are not shown

*Table 5.1: λA restriction endonuclease mapping;*
Figure 5.2: Restriction map of λ A genomic clone and description of the various subclones generated from it.
Total identity between the nucleotide sequence of the first and second exons of our clone and the sequence of the rat liver cDNA showed that this clone represents the rat 11β-OHSD gene. The nucleotide sequence of exons 1 and 2, the first intron and 890 bp of 5’ flanking region is shown in Fig. 5.4 - the nucleotide numbering system employed denotes the predominant transcriptional start site in liver as +1.

There are a number of noteworthy sequence elements within the sequenced region, including a poly(dT) stretch at -416 to -391 and (dA-dC) repeats from +1089 to +1131 in intron B. There is no apparent TATA box but the sequence contains a number of putative transcription factor binding sites; a CCAAT box at -74 (GCCAAT) which could also be described as a palindromic CAAT sequence from -72 to -64 (CAATATTG), 6 CACCC-box elements at -476, -466, -427, -290, -282, and -82, a GC box (SP1 consensus binding site) at -299, sequences corresponding to the consensus half site glucocorticoid responsive element (GRE) TGT^/CCT at +61 and +250, and the pentanucleotide TGACC, in common to estrogen (ERE) and thyroid (TRE) responsive elements, at positions -839, +635, and +1295.

5.2.3. Identification of 11β-OHSD transcription start sites

Primer extension analysis (see section 2.3.4.) was carried out on RNA isolated from liver and kidney to determine the start point(s) of transcription. Oligonucleotides first used for analysis were OL-D, complementary to bases +81 to +105, and OL-A (+36 to +74), both within
Figure 5.4:

Nucleotide sequence of the rat 11β-OHSD 5'-flanking region and the first 2 exons. Vertical arrows represent probable starts of transcription. All numbers are relative to the major transcription start site in liver (+1). Putative transcription factor binding sites are underlined. Putative hormone responsive sites are identified by horizontal arrows.
A) Primer extension analyses:
(The asteriks indicate extended fragments obtained with RNA from kidney only.)

![Diagram of primer extension analyses]

B) RNAse protection analyses:
(The asteriks indicate protected fragments obtained with RNA from kidney only.)

![Diagram of RNAse protection analyses]
exon 1 (Fig. 5.5.). Extension of OL-D and OL-A indicated that major start site in liver, also used in kidney, is 105 bp 5' of the start of translation in liver, in agreement with published findings (Argawal et al) (Fig 5.6.A). An additional larger extension product was seen with kidney RNA. Extension of OL-C (-88 to -68) was used to confirm and to map more accurately the start of this mRNA (Fig. 5.6.B). OL-C was extended to a 196 bp fragment, indicating that this kidney mRNA initiates at -264.

In order to confirm the transcription start points identified by primer extension, we performed RNase protection assays (see section 2.3.5.). Two probes were generated for this purpose complementary to bases -521 to +74 (riboprobe 1 synthesised from subclone pMP5, Fig. 5.5.) and -88 to +310 (riboprobe 2 synthesised from subclone pMP4, Fig. 5.5.). Using riboprobe 1 the predicted 74 bp protected fragment, resulting from hybridisation to mRNA initiated from the major start site at +1, was observed in liver and kidney. In addition a 338 bp protected fragment was detected in kidney confirming that the 5' end of this renal mRNA maps around -264 (Fig.5.7.A). There is no in-phase open reading frame in this sequence suggesting that this transcribed sequence is not translated. Analysis using riboprobe 2, entirely covering exon 1 (Fig. 5.5.), resulted in the predicted 180 bp protected fragment corresponding to exon 1, in both liver and kidney, and an additional 270 bp protected fragment in kidney which probably corresponds to exon 1 plus the 5' 88 bp of the probe (5.7.B).

The cloning of a variant 11β-OHSD cDNA from a rat kidney library was reported while we were analysing our genomic clone (Mercer et al, 1991). This cDNA lacks the 5' region encoding the first 26 aminoacids. Because our sequence analysis revealed that this deleted region was contained in exon 1 (Fig.5.4.), we tested the possibility that this mRNA might be initiated in intron A. For this, we performed RNase protection assays using an antisense probe complementary to bases +758 to +1389 (riboprobe 3 synthesised from subclone pMP7; Fig. 5.5.). We detected the predicted protected fragment (135 bp) corresponding to exon 2 in liver and kidney. An additional 196 bp protected fragment was observed in kidney, suggesting the presence of a transcript containing 64 bp of intron A (Fig. 5.8.A). This additional fragment is clearly detected in kidney but is also apparent in liver, as a minor product, with longer exposure of autoradiographs (data not shown).
Figure 5.6:

(A) Primer extension analysis using OL-D (left) and OL-A (right). L = liver total RNA, K = kidney total RNA, Y = yeast tRNA. 50 μg aliquots were run per lane in parallel with a sequencing reaction. Autoradiograms were exposed for a week at -70°C.
Figure 5.6:
(B) Primer extension analysis using OL-C. K = kidney total RNA, Y = yeast tRNA. 50 μg aliquots were run per lane in parallel with a sequencing reaction. Autoradiograms were exposed for a week at -70°C
Figure 5.7:
(A) RNase protection assay using riboprobe 1 generated from pMP5 (-521 to +74). $K_t=$ whole kidney RNA, $K_m=$kidney medulla RNA; 25 μg aliquots were run per lane and the autoradiogram exposed for 20 h. (B) Similar experiment using riboprobe 2 generated from pMP4 (-88 to +310).
Figure 5.8:

(A) RNase protection assay using riboprobe 3 generated from the AflII-PstI fragment of pMP7 (+758 to +1389). L= liver total RNA, K=kidney total RNA, Y=yeast tRNA; 50 µg per lane run with a sequencing reaction. Autoradiogram was exposed for 60h.
Figure 5.8: 
(B) Primer extension analysis using OL-E; L= liver total RNA, K=kidney total RNA, Y=yeast tRNA; 50 µg/lane. Autoradiogram was exposed for 1 week.
The existence of this mRNA was confirmed by primer extension analysis using OL-E, an oligonucleotide complementary to the beginning of exon 2 (+934 to +954; Fig. 5.5.). OL-E was extended to give 2 major products in kidney, one of 204 bp corresponding to the major start site (+1) and a smaller one of 89 bp, corresponding to initiation at +808, giving rise to an mRNA with a 65 bp 5' extended exon 2 (Fig. 5.8.B).

5.2.4. Northern analysis of 11β-OHSD transcripts

Having identified at least 3 transcription start sites we wanted to determine the corresponding transcripts. To study this we performed Northern blot analysis with oligonucleotides specific to particular transcripts (OL-A, OL-B and OL-C; Fig 5.5.). Hybridisation with a full-length cDNA showed a single major transcript of 1700 nucleotides (nt) in liver (on shorter exposure) and 3 mRNA species of 1500 nt, 1600 nt and 1900 nt in kidney (Fig. 5.9.). OL-A, which is complementary to exon 1 (+36 to +74), hybridised to kidney mRNAs identical in size to the 1900 nt and 1600 nt species seen with full-length cDNA, and with liver RNA gave a virtually identical pattern of hybridisation to that obtained with cDNA. OL-B, corresponding to intron A sequence (+ 831 to +870) hybridised to a single species in kidney, identical in size to the 1500 nt species seen with cDNA; a weak band of the same size is apparent in liver. OL-C, complementary to bases -88 to -68, hybridised to a 1900 nt species in kidney; no signal was detected in liver. In summary, the 1900 nt species corresponds to mRNA initiated at -264 and the 1500 nt species to mRNA initiated at + 808. The 1600 nt species in kidney most probably corresponds to mRNA initiated at +1. Interestingly, the size of the corresponding mRNA in liver is 1700 nt. Several smaller hybridizing species are seen using OL-A probe, especially in liver. They may correspond to related transcripts but have not been further investigated.
Figure 5.9:

Autoradiograms of Northern blots of liver (L) and kidney (K) RNA. The probes used in each hybridization are indicated at the top of the panel. 25 μg of RNA were loaded per lane. After OL-A hybridisation, RNA was stripped by boiling and reprobed with the full-length cDNA. Hybridisation with OL-B and OL-C were performed on RNA run on adjacent lanes of the same gel used for OL-A/cDNA so that all 4 autoradiograms could be aligned. Autoradiograms were exposed 20 h for cDNA, 2 days for OL-A, and 4 days for OL-B and OL-C. The numbers on the right indicate the approximative sizes (nt) of the various species.
5.3 Discussion

We have isolated a rat 11β-OHSD genomic clone and determined the structure of its 5' flanking region.

Using both primer extension and RNase protection analyses we found 3 separate sites of initiation of transcription in kidney, whereas in liver only one site predominates. The major liver transcript corresponds to the previously cloned rat liver and human testis 11β-OHSD cDNAs (Agarwal et al, 1989; Tannin et al, 1991). The open reading frame begins with an ATG codon lying 105 bp downstream of the Cap site. The first 40 amino acid residues predicted by this open reading frame are identical to the actual N-terminal amino acid sequence of the purified hepatic 11β-OHSD (Agarwal et al, 1989; Lakshmi & Monder, 1988). The same promoter (+1) is also used in kidney, although our Northern analyses show that the mRNA species produced is 100 nt shorter (1600 nt instead of 1700 nt) than that initiating from this start site in liver. Tissue-specific differences in polyA tail length may explain this since deadenylation of 11β-OHSD mRNA from liver and kidney converts both the 1700 nt and 1600 nt species to a 1500 nt species (Krozowski et al, 1990). An additional 1700 nt species previously reported in renal papilla (Krozowski et al, 1990) was not detected in our Northern blot experiments, either hybridising with full-length cDNA or oligonucleotides.

The longest renal mRNA species initiates at -264 resulting in a 1900 nt transcript. This extended mRNA and the 1600 nt kidney mRNA initiating at +1 (corresponding to the 1700 nt species in liver) are likely to encode identical proteins since the extra 5' sequence has no potential in-frame ATG initiation codon. This long 5' untranslated sequence may be associated with differences in mRNA stability and translatability; formation of secondary structure (eg stem-loop) within palindromic or complementary stretches of 5' untranslated region RNA prevents the 40S ribosomal subunit from migrating to the AUG codon, however long leader sequences may result in loading of extra 40S ribosomal subunits and thus improvement of the translation (Kozack, 1991). A low translatability of this mRNA is more likely since its 5' untranslated sequence is GC-rich, which would favour a stable potential hairpin, and it contains 7 ATG triplets, all before the 5' end of the +1 mRNA, which could inhibit initiation at the proper downstream starting codon. Only 5-10% of
Eukaryotic mRNAs possess ATG triplets upstream of the true ATG starting codon (Kozack, 1991). The purpose of this long transcript is therefore unclear; Northern analysis show that this mRNA is less abundant than the +1 mRNA in kidney, maybe reflecting a shorter mRNA half-life or a weaker promoter; its expression does not show a developmental pattern that could have explained the presence of this additional promoter (Krozowski et al, 1990, Moisan et al, 1992a). Moreover, a large variety of tissues has been examined for 11β-OHSD mRNA by Northern blot analysis and this 1900 nt has never been found elsewhere than in kidney. Heterogeneity of 5' untranslated region mRNA length has been described in other steroid dehydrogenases, e.g. human estradiol 17β-dehydrogenase but the corresponding mRNA is expressed abundantly in a number of tissues (Luu-The et al, 1989 & 1990).

In addition to the two promoters upstream of exon 1, kidney also exploits a promoter in intron A, with transcription initiating at +808, 65 nucleotides upstream of exon 2. The resulting 1500 nt mRNA transcript is also detected in liver, albeit as a minor product. Interestingly, rat liver contains a 11β-OHSD isoform, accounting for less than 10% of the total enzyme activity, that has the same low apparent Km as the 11β-OHSD activity described in renal cortical collecting duct cells (Monder & Lakshmi, 1989; Náray-Fejes-Tóth, 1991). This shortest mRNA is predicted to encode a protein lacking the first 26 hydrophobic amino acid residues of the full-length "liver-type" enzyme. The lack of the putative membrane signal sequence may, of course, alter the intracellular location of the enzyme and is likely to result in differential post-translational modification. Indeed, when the "liver-type" 11β-OHSD cDNA was expressed into human osteosarcoma cells using recombinant vaccinia virus, it was found that treatment with A1-tunicamycin (an inhibitor of glycosylation) markedly reduced 11β-dehydrogenase activity (by 50%) whereas 11β-reductase was not affected (Agarwal et al, 1990). Since this mRNA species has only been detected in kidney, it is tempting to speculate that it corresponds to the glycosylated form that would mainly function as an 11β-dehydrogenase. This hypothesis would fit with the fact that 11β-OHSD in renal aldosterone target cells requires a strong 11β-dehydrogenase activity that would completely inactivate the circulating glucocorticoids. Whether this isoform is specifically located in the aldosterone target cells
is not yet known, but the tools required to perform the experiment that would answer this question are now available, i.e. in situ hybridisation using the isoform-specific oligonucleotides. A cDNA apparently corresponding to this transcript has been cloned and the 5' sequence of this variant renal cDNA was recently published (Krozowski et al, 1992). Comparison of our intron A sequence and the 5' untranslated end of the cDNA clone (11-HSD1B) revealed differences. It is unclear whether the two transcripts truly differ or if a rearrangement has occurred in 11-HSD1B, but riboprobes spanning +758 to +1389 were protected in our RNase protection assay (Fig. 5.8.A) indicating that the sequence presented here is likely to be correct. In contrast to the rat, only one human kidney 11β-OHSD mRNA species has been detected, corresponding to initiation at a single site identical to the major start site in liver (+1). This discrepancy may be due to true species differences or might reflect changes in pathological human material or the different experimental conditions employed. The amount of RNA used in primer extension experiment was 5-fold greater than that used by Tannin et al (1991) and the autoradiograms shown in this study have been exposed to film for a much longer time (1 or 2 weeks compared to 20 h).

Though the direct relationship between the putative DNA regulatory elements and the 3 transcripts remains to be defined, some idea of the possible regulatory and tissue-specific transcriptional mechanisms can be inferred. Thus, the two first promoters do not contain any TATA-box. Often, TATA-less promoters have sequences flanking the site of initiation (called initiator) that are important for basal transcription. A sequence 2 bp downstream of the -264 start site (GCCATTCTTG) closely resembles the initiator element of the murine terminal deoxynucleotidyl transferase gene (GCCATTCTGG), which itself is related to the adenovirus major late promoter and the immunodeficiency virus-1 promoter (Smale et al, 1989). Recently, a protein (called TFII-I) has been characterised that recognises this type of initiator and promotes basal transcription of the corresponding mRNA (Roy et al, 1991). It would be of interest to test if TFII-I binds to this initiator-like sequence by band-shift assay and induces transcription from the -264 promoter in an in vitro transcription system.
The sequence surrounding the +1 promoter (TGATGTC*ACAATTCAGA) is identical to that found in the homologous human 11β-OHSD gene (Tannin et al., 1991) and may also bind a promoter or tissue-specific protein complex. The palindromic CCAAT element is in a favorable position to regulate the liver-type promoter (+1), but not the other promoters. The predominant induction of transcripts arising from the CCAAT-box associated promoter (+1) in liver may be due to the very high liver content of CCAAT-binding factors (McKnight et al., 1989), which have been implicated in the regulation of genes encoding enzymes involved in energy metabolism (a hepatic function also ascribed to glucocorticoids). The GC box and the CACCC elements are well located to regulate the longest transcript, as well as the +1-derived transcript. The CACCC-box elements, especially those arranged in tandem, are very similar to the ones found in the β-globin promoter (Dierks et al., 1983). The most upstream CACCC-box tandem is in an antisense orientation, as found in the tryptophan oxygenase promoter where these sequences bind a transcription factor involved cooperatively in steroid hormone (glucocorticoid) induction (Schule et al., 1988). There are 2 putative GREs within the transcribed region which might regulate any of the 3 transcripts. Although glucocorticoid regulation of 11β-OHSD in liver and kidney has not been shown, we have demonstrated glucocorticoid induction of 11β-OHSD activity and mRNA expression in hippocampus (Chapter 4), confirming biochemical findings in cultured fibroblasts (Hammami & Siiteri, 1991); the major 11β-OHSD mRNA species in hippocampus is similar in size to the liver transcript. In between the CACCC boxes lies a run of 25 thymines followed by 13 guanines; a polydT sequence has the potential to form a tighter DNA helix (10 bp per turn instead of 10.6 in B DNA) and has been reported to influence nucleosome positioning (Peck & Wang, 1981; Prunell, 1982), in a way suggesting that sequences flanking this polydT stretch are potential binding sites for regulatory proteins. A polydT sequence of the same length is found in the 5′ flanking region of the angiotensinogen gene (Brasier et al., 1989). Finally, 3 ERE/TRE half-sites are present, and sex steroids and perhaps thyroid hormones are known to affect 11β-OHSD activity (Koerner & Hellman, 1964; Lax et al., 1978; Zumoff et al., 1983; Baggia et al., 1990; Pepe et al., 1988; Smith & Funder, 1991)

The +808 promoter has a run of 5 AT bp (TTTTA) 31 nucleotides 5′ of the Cap site, and an AT-rich region (TTATGAATAA) 62 bp upstream of the Cap
site; either might function as a TATA sequence. An element resembling a CCAAT box (GGCCAAA) is present 87 bp upstream of this third start site. Functional analysis in an in vitro system will clarify the importance of these elements.

In addition, Krozowski et al (1992) reported that the sequence analysis of the 3' end of renal 11β-OHSD clones showed the presence of three alternate sites of poly(A) addition. No correlation was apparent between the site of poly(A) addition and one or the other type of 11β-OHSD cDNA. The insertion of an AU-rich sequence into the 3' non-coding region of genes has been shown to destabilise transcripts (Shaw & Kamen, 1986). These observations suggest that 11β-OHSD mRNA can be regulated at the level of mRNA turnover by the selection of different 3' untranslated sequences.

In summary, 3 promoters permit independent transcriptional regulation of each 11β-OHSD mRNA and 3 alternative polyadenylation sites can further influence the turn-over of these mRNA, producing greater flexibility of control. Thus, tissue-specific and ontogenetic regulation of 11β-OHSD expression and activity may reflect the different array of regulatory factors present. At least two proteins are produced from this gene, with differences which probably influence their intracellular location. A cytosolic or membrane-bound location might, in turn, result in differential post-translational modification, such as glycosylation, that modulates the function of the enzyme by selectively stabilising the 11β-dehydrogenase activity. The role of the most 5' promoter is unexplained and might simply represent an ancestral promoter. Whether additional genes also encode further isoforms of this crucial enzyme remain to be determined.

* These three promoters still remain putative; and further studies are required to demonstrate their function.
Chapter 6: General discussion and perspectives
Our perception of the physiological role of 11β-OHSD has rapidly evolved in the last past four years, thanks to studies carried out all around the world and employing a variety of approaches and techniques including clinical science, enzymology, protein chemistry, immunology, physiology in animals or in cell systems and molecular biology.

It is now thought that this versatile enzyme plays a central role in influencing the expression of corticosteroid-dependent processes in all tissues in which it is found. 11β-OHSD mediates the amount of intracellular glucocorticoid accessible to the receptor from the variable supply from the circulation. In aldosterone-selective cells, 11β-OHSD is very active and unidirectional in order to metabolise completely the glucocorticoid that is in competition with aldosterone. In cells containing only GR or both corticosteroid receptors, the purpose of 11β-OHSD would rather be to adjust the glucocorticoid concentrations to physiological limits by partially inactivating or activating circulating glucocorticoids. An example of this situation is the putative role of 11β-OHSD in brain as described in Chapters 3 & 4.

The importance of 11β-OHSD in the brain has yet to be determined but the work presented in this thesis along with studies carried out elsewhere (Lakshmi et al, 1991; Seckl et al, 1991; Sakai et al, 1992) provide good evidence for a biological role of 11β-OHSD in this tissue. In Chapter 3, I reported our work showing 11β-OHSD in vitro activity and mRNA expression in the central nervous system, particularly in hippocampus, cortex, cerebellum, pituitary and in some nuclei of the hypothalamus. This distribution was subsequently confirmed by an immunocytochemical study using two different antisera raised against the hepatic 11β-OHSD (Sakai et al, 1992). Thus, the presence of the enzyme in the central nervous system is now clearly established and preliminary studies like those described in Chapter 4 aim to define the role(s) of 11β-OHSD in the brain. We have found that 11β-OHSD is induced by glucocorticoids in brain, particularly in hippocampus. Furthermore, 11β-OHSD is highly expressed in hippocampus, cortex and cerebellum at the early stages of development when maturation of the brain occurs. The differential pattern of expression during development suggest that the enzyme has locally defined functions that vary from region to region, but the working hypothesis is that 11β-OHSD provides a tissue-specific mechanism to adjust the concentrations of glucocorticoids to the level required by a given
tissue at a given stage of development or in response to changes in the environment that affect glucocorticoid levels. In addition to this work, a possible function for 11β-OHSD in brain in vivo has also been suggested by the following studies: i) using \([^{14}\text{C}]\) 2-deoxyglucose uptake it has been found that inhibition of 11β-OHSD in conscious rats leads to increased metabolic activity in regions of the brain exhibiting high enzyme activity and mRNA expression (Seckl et al, 1991); ii) using an in vivo autoradiographic technique we found that inhibition of cerebral 11β-OHSD by intracerebroventricular administration of glycyrrhetinic acid led to increased corticosterone uptake in the hippocampus, suggesting that 11β-OHSD regulates the access of corticosterone to, at least, part of the hippocampal MR (Seckl et al, unpublished data); iii) intracerebroventricular infusion of carbonexolone (an 11β-OHSD blocker) increased blood pressure in rat (Gómez-Sánchez, 1991).

It would be of particular interest to extend these studies in hippocampus and in the brain areas thought to be involved in salt appetite and blood pressure. One way to determine the functional importance of 11β-OHSD is to examine the effect(s) of local inhibition of the enzyme on a well-characterised feature or function of the organ under test. For example, a study looking at the effects of inhibition of 11β-OHSD in hippocampus on the survival of the hippocampal neurons in chronically stressed animals would help to determine whether 11β-OHSD protects the hippocampus from the neurotoxic effects of glucocorticoid hypersecretion. Another example is studying the effect of 11β-OHSD inhibition in hippocampus on learning and spatial memory as can be assessed using a Morris swim maze or a multiple radial-arm maze. A correlation between 11β-OHSD activity (and mRNA expression) and the protective effect of postnatal handling on glucocorticoid hypersecretion would be very informative. Aldosterone specific actions in the brain could be tested by looking at the binding of glucocorticoid or mineralocorticoid agonist and antagonist compounds in the presence or absence of 11β-OHSD inhibition in the organs known to be aldosterone-specific.

However much remains to be learnt about the 11β-OHSD enzyme itself. We now know that the various 11β-OHSD mRNA species detected in kidney are generated from a single gene by differential promoter usage (Chapter 5), but their physiological significance, subregional distribution
and regulation remain to be determined. For example, since glycosylation stabilises the 11\(\beta\)-dehydrogenase but has no effect on the 11\(\beta\)-reductase component of the enzyme, it would be informative to express the truncated 11\(\beta\)-OHSD protein in vitro using the corresponding cDNA and to determine what activity it produces knowing that in the same conditions the full-length 11\(\beta\)-OHSD cDNA produces both 11\(\beta\)-reductase and 11\(\beta\)-dehydrogenase activities. Also, now that we have derived oligonucleotides probes specific to a given 11\(\beta\)-OHSD mRNA species, their respective tissue distribution and putative co-localisation with MR or GR can be examined. It would be interesting to know whether a specific isoform locates only in mineralocorticoid target cells. Transfection into cells is another way to examine whether 11\(\beta\)-OHSD functions in a paracrine or autocrine manner. Cells would be cotransfected with i) a vector expressing 11\(\beta\)-OHSD, ii) a vector expressing MR, iii) a vector containing a reporter gene sensitive to ligand-bound MR. Arriza et al (1987) transfected the two latter vectors into CV1 cells and showed that the reporter gene is equally sensitive to aldosterone and corticosterone bound to the MR. It would thus be interesting to see if high levels of 11\(\beta\)-OHSD activity confer aldosterone specificity to MR in these cells in the presence of competitive concentrations of glucocorticoids.

Determination of the tissue-specific and hormonal regulation of the cloned 11\(\beta\)-OHSD gene would obviously be of great importance. The techniques that can now be used include i) gel retardation assays using nuclear extract of various cell types and oligonucleotides derived from the sequenced promoter region. This would indicate the DNA sequences that interact with proteins and maybe determine areas interacting with tissue-specific factors. ii) promoter assays whereby potential regulatory regions are inserted upstream of a reporter gene, such as luciferase, whose activity is easily assayable. Then these regulatory regions would be further analysed by deletion or mutation. Ultimately, the promoter region could also be studied in transgenic animals where the transfected gene would have the advantage to be packaged in chromatin therefore eliminating artefact due to a vector system as well as revealing if tissue-specific factors are involved.

Given the discrepancies between enzyme activity and mRNA levels in parotid and mammary gland (Krozowski et al, 1990) and in kidney during the course of development (Moisan et al, 1992a), it is likely that
there is another gene for 11β-OHSD. This possibility is currently being investigated in this laboratory and the first step of the study is the purification of a NAD-dependent human placental 11β-OHSD enzyme. It is hoped that this enzyme will be different from the previously purified rat hepatic 11β-OHSD and that the corresponding cDNA and gene will be isolated thereafter.

The availability of the 11β-OHSD human gene and cDNA sequence (cloned using the rat hepatic cDNA) will help us to determine the genetic defect involved in the "apparent mineralocorticoid excess" patients, whether it relates to this or another gene, and whether it is likely to result in deficiency of one particular isoform.
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ADDENDUM


Abbreviations

ACE: angiotensin-converting enzyme
ACTH: adrenocorticotropic hormone or corticotropin
ADX: adrenalectomy
AME: apparent mineralocorticoid excess
ANF: atrial natriuretic factor
AR: androgen receptor
AV3V: anteroventral third ventricle
AVP: arginine vasopressin
bp: base pair
BSA: bovine serum albumin
CA1-3: field 1-3 of Ammon's horn
cAMP: cyclic adenosine monophosphate
CAT: chloramphenicol acetyl transferase
CBG: corticosteroid-binding globulin
cDNA: complementary deoxyribonucleic acid
cpm: counts per minute
CRH-41: corticotropin-releasing factor
cRNA: complementary ribonucleic acid
CV1: monkey kidney cell line
DEP: diethyl pyrocarbonate
DEX: dexamethasone
DNA: deoxyribonucleic acid
DNase: deoxyribonuclease
DTT: dithiothreitol
EDTA: ethylene diamine tetraacetic acid
ER: estrogen receptor
ERE: estrogen receptor element
ERR1: estrogen related receptor
GAPDH: glyceraldehyde phosphate dehydrogenase
GI: glycyrrhizinic acid
GE: glycyrrhetinic acid
GR: glucocorticoid receptor
GRE: glucocorticoid receptor element
HPA: hypothalamo-pituitary-adrenal
HPLC: high-pressure liquid chromatography
Hsp90: heat-shock protein 90 kD
kb: kilobase
Kd: dissociation constant
kD: kilodalton
Ki: inhibition constant
Km: Michaelis constant
LDL: low density lipoprotein
MMTV: mouse mammary tumour virus
mol wt: molecular weight
MR: mineralocorticoid receptor
mRNA: messenger RNA
NAD: nicotinamide adenine dinucleotide
NADH: nicotinamide adenine dinucleotide, reduced form
NADP: nicotinamide adenine dinucleotide phosphate
NADPH: nicotinamide adenine dinucleotide phosphate, reduced form
nt: nucleotide
O.D.: optical density
OHSD: hydroxysteroid dehydrogenase
OVLT: organ vasculosum of the lamina terminalis
p: probability
PEG: polyethylene glycol
PepCK: phosphoglucomutase
Pipes: piperazine-N,N'-bis[2-ethanesulfonic acid]
POMC: pro-opiomelanocortin
PR: progesterone receptor
PVN: paraventricular nucleus
RAR: retinoic acid receptor
RNA: ribonucleic acid
RNase: ribonuclease
RNasin: ribonuclease inhibitor
rpm: rotation per minute
SDS: sodium dodecyl sulfate
SEM: standard error of the mean
SRE: steroid response element
SSC: saline-sodium citrate
SV40: simian virus 40
TBE: Tris, borate, EDTA
TE: Tris, EDTA
TEMED: N'-tetramethylethylenediamine
TETH: tetrahydrocortisone
THF: tetrahydrocortisol
TLC: thin layer chromatography
TR: thyroid receptor
TRE: thyroid response element
tRNA: transfer RNA
UV: ultra-violet
VDR: vitamin D₃ receptor
APPENDIX 2
Publications arising from the thesis work

Articles


Abstracts


ABSTRACT. In peripheral aldosterone target sites (e.g., kidney), 11β-hydroxysteroid dehydrogenase (11β-OHSD) metabolizes corticosterone to inactive 11-dehydrocorticosterone and thus protects mineralocorticoid receptors from exposure to corticosterone in vivo. We have investigated whether 11β-OHSD could account for the site-specific differences in corticosteroid receptor sensitivity to corticosterone in rat brain. Enzyme activity, estimated as the percentage conversion of [3H]corticosterone to [3H]11-dehydrocorticosterone in the presence of NADPH (200 μM), was: hippocampus, 55.8 ± 2.7%; cortex, 52 ± 3.1%; pituitary; 40 ± 2%; hypothalamus, 26.1 ± 1.2%; brain stem, 21.4 ± 1.7%; and spinal cord, 12.3 ± 1.8%. Northern blots, using [32P]dCTP-labeled probes from an 11β-OHSD cDNA clone derived from rat liver, showed expression of a single mRNA species in all brain areas, of identical size to 11β-OHSD mRNA in liver and kidney. Highest expression was found in hippocampus and cortex. In situ hybridization, using [32P]UTP-labeled cRNA probes, localized high mRNA expression to cerebral cortex (particularly parietal cortex, layer IV), hippocampus (highest in CA3), hypothalamic medial preoptic area and arcuate nuclei and anterior pituitary. In conclusion, there is localized 11β-OHSD mRNA expression and enzyme bioactivity in rat brain. The distribution of 11β-OHSD corresponds to areas of reduced glucocorticoid or mineralocorticoid receptor affinity for corticosterone. Therefore, 11β-OHSD may regulate the access of corticosterone to cerebral mineralocorticoid and/or glucocorticoid receptors and thus modulate corticosteroid effects on brain function. (Endocrinology 127: 1450–1455, 1990)
in vivo. In kidney, 11\(\beta\)-hydroxysteroid dehydrogenase (11\(\beta\)-OHSD), an NADP\(^+\)-dependent microsomal enzyme, metabolizes corticosterone to inactive 11-dehydrocorticosterone (which cannot bind to mineralocorticoid receptors) (19) and thus protects the nonselective mineralocorticoid receptor from exposure to corticosterone in vivo (14–21). In the congenital absence of 11\(\beta\)-OHSD, cortisol is the predominant glucocorticoid in man; metabolized by 11\(\beta\)-OHSD to inactive cortisone) has potent renal mineralocorticoid effects, leading to hypertension and hyperkalemia (22). Similarly, inhibition of 11\(\beta\)-OHSD by dexamethasone or its active metabolite glycyrrhetinic acid or the derivative carbonoxolone) allows physiological glucocorticoids to exhibit mineralocorticoid activity (23). In addition to kidney and salivary gland, high levels of 11\(\beta\)-OHSD activity have been found in liver, lung and testis (24, 25), but heart has little or no 11\(\beta\)-OHSD activity and cardiac mineralocorticoid receptors bind aldosterone and corticosterone with similar affinity (20, 21).

As hippocampal mineralocorticoid receptors bind corticosterone and aldosterone with equal affinity in vivo, it has been suggested that similar specificity-conferring mechanisms are absent in brain and 11\(\beta\)-OHSD bioactivity was not found in whole hippocampal extracts in vitro (20, 21). However, early studies in a variety of species demonstrated 11\(\beta\)-OHSD bioactivity in whole brain extracts and this activity was dependent on addition of exogenous NADP\(^+\) to the reaction (26, 27). The functional studies outlined above suggest that localized mineralocorticoid specificity-conferring mechanisms are operative within the CNS.

To examine whether 11\(\beta\)-OHSD is also associated with glucocorticoid receptors we recently investigated rat cerebellum (a tissue with high glucocorticoid receptor levels and mRNA expression, but very little aldosterone binding), or mineralocorticoid receptor mRNA expression (4, 7, 15, 18), and found 11\(\beta\)-OHSD mRNA expression and NADP\(^+\)-dependent bioactivity in cerebellar cortex (28) (Moisan, M.-P., J. R. Seckl, L. P. Brett, C. Mondor, A. K. Agarwal, P. C. White, and C. R. W. Edwards, submitted for publication). Clearly 11\(\beta\)-OHSD might be responsible for the localized differences in corticosterone binding to mineralocorticoid and/or glucocorticoid receptors in other regions of the brain. We have now investigated the distribution of 11\(\beta\)-OHSD bioactivity and mRNA expression rat brain.

Materials and Methods

11\(\beta\)-OHSD enzyme bioassay

Rats (Wistar, male, 200–250 g, n = 5) were decapitated and the brains removed and dissected on ice. Tissues were homogenized in Krebs-Ringer bicarbonate buffer (118 mM NaCl, 3.8 mM KCl, 1.19 mM KH\(_2\)PO\(_4\), 2.54 mM CaCl\(_2\), 2H\(_2\)O, 1.19 mM MgSO\(_4\), 7H\(_2\)O, 25 mM NaHCO\(_3\) and 0.2% glucose) using a Dounce tissue grinder. The total protein content was estimated colorimetrically (Bio-Rad protein assay kit), using an aliquot of the homogenate. 0.5 mg of total protein was incubated with NADP\(^+\) (200 μM) and 12 nM (final concentration) 1,2,6,7\(^{3}H\)-corticosterone (specific activity: 84 Ci/mmol, Amersham International) in Krebs-Ringer buffer (0.2% BSA) for 1 h at 37 C. After incubation, steroids were extracted with ethyl acetate and separated by thin-layer chromatography. The percentage conversion of [\(^{3}H\)]corticosterone to [\(^{3}H\)]11-dehydrocorticosterone was calculated from the radioactivity of each fraction (20).

RNA preparation

Male rats were decapitated and the brain was rapidly removed and dissected on ice. Tissue was immediately frozen in liquid nitrogen. Control tissues included liver (a rich source of 11\(\beta\)-OHSD and the tissue of origin of the cDNA probe used), heart (an organ with negligible 11\(\beta\)-OHSD activity and nonselective mineralocorticoid receptors in vivo), kidney, testis and lung (the latter tissues are areas where in vitro and in vivo roles for 11\(\beta\)-OHSD have been established or proposed). Total RNA was extracted with guanidium thiocyanate as described (29). Briefly, 0.1–0.2 g of tissue was homogenized in 1–2 ml of 4 M guanidium thiocyanate, 0.25 M sodium citrate, 5.5% sarcosyl and 0.1 M β-mercaptoethanol. DNA was precipitated by addition of sodium acetate (0.2 M; pH 4) and proteins were removed by phenol/chloroform extraction. The aqueous phase containing RNA was precipitated twice with isopropanol and resuspended in diethylpyrocarbonate-treated H\(_2\)O. RNA concentration and purity was assayed spectrophotometrically and aliquots stored at −70 C prior to use. Equivalence of loading onto gels was confirmed visually by ethidium bromide staining under ultraviolet illumination.

Northern blot and cDNA/mRNA hybridization

An aliquot of 20 μg of total RNA was fractionated on 2% agarose-2.2 M formaldehyde gels and blotted onto nitrocellulose (Hybond C extra, Amersham International, UK) by capillary transfer at 4 C overnight. The nitrocellulose membrane was prehybridized in 50% formamide, 5 × SSPE, 5 × Denhardt’s solution, 200 μg/ml denatured hering testis DNA, 0.1% SDS, and 1 mM EDTA. Hybridization was performed in identical buffer containing radioactive labeled 11\(\beta\)-OHSD cDNA probe. The 1.2-kb 11\(\beta\)-OHSD probe, subcloned in a pBluescript KS vector (30) was labeled with \(^{32}P\)dCTP (3000 Ci/mmol, Amersham International, UK) by oligonucleotide random priming (BCL, UK) to a specific activity of 1–2 × 10\(^{8}\) cpm/μg DNA and diluted in hybridization buffer (3–6 × 10\(^{8}\) cpm/ml). Hybridization was performed at 42 C overnight and the membrane washed to a final stringency of 0.2 × SSC; 0.1% SDS at 60° C and exposed to Kodak XAR film.

In situ hybridization

Adult male Wistar rats (180–220 g) were decapitated and the brain rapidly removed on ice. The forebrain and hindbrain was separated by a coronal cut of the brain stem and immediately frozen on Dry Ice. Tissue was stored at −85 C prior to sectioning.
in a cryostat at -20 to -22 C. Coronal sections (10 µm) were mounted onto gelatin and poly-L-lysine-coated slides, and stored at -85 C. Tissue sections were fixed in 4% paraformaldehyde/0.1 M phosphate buffer and washed in 2 X SSC made up in diethylpyrocarbonate-treated sterile water. Prehybridization buffer containing 50% formamide, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.5 mg/ml denatured salmon sperm DNA, 0.05 mg/ml total yeast RNA, and 0.05 mg/ml yeast tRNA were pipetted onto the sections and the slides were incubated at 50 C for 2 h in sealed boxes.

T3 polymerase (GIBCO BRL, UK) was used to transcribe 1.2-kb 32P-labeled antisense cRNA probe from SacI-linearized pBluescript vectors containing the 11β-OHSD cDNA insert (30). The proportions of labeled and cold UTP were adjusted to yield probes of specific activity 10 x 10^6 Ci/mmole. The cRNA probe was denatured and added to prehybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.1 mg/ml denatured salmon sperm DNA, 0.05 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 0.1 g/ml Dextran sulfate, and 10 mM DTT) to a final concentration of 0.05 pmol/ml and applied to the slides. The slides were incubated overnight at 50 C in sealed boxes. Following hybridization the slides were rinsed twice in 2 X SSC for 30 min at room temperature prior to RNase A digestion (30 µg/ml for 30 min at 37 C). The slides were washed in reducing salt concentrations to a maximum stringency of 0.1 X SSC at 60 C for 60 min. After dehydration in increasing concentrations of ethanol (maximum 90%) in 0.3 M sodium acetate, sections were air dried and exposed to autoradiographic film (Hyperfilm β-max, Amersham International, UK). Similar sections were dipped in photographic emulsion (Ilford, UK) and stored at 4 C for 20-30 days before being developed and counterstained with haematoxylin and eosin.

Controls, carried out to test the specificity of the hybridization method and the 11β-OHSD probe included pretreatment of sections with RNase A (100 µg/ml for 1 h at 37 C) prior to hybridization with antisense probe and hybridization using labeled-cRNA sense probe (not complementary to 11β-OHSD mRNA, transcribed by T7 polymerase (Promega, UK) and applied at 10 x 10^6 cpm/ml).

Statistics

Data were assessed by ANOVA followed by Scheffe’s test. Significance was set at P < 0.05. Values are expressed as means ± SEM.

Results

In homogenates, 11β-OHSD bioactivity expressed as the percentage conversion of [3H]corticosterone to [3H] 11-dehydrocorticosterone over 60 min in the presence of NADP+ (200 µM) was: hippocampus, 55.8 ± 2.7%; parietal cortex, 52 ± 3.1%; hypothalamus, 28.1 ± 1.2%; brain stem, 21.4 ± 1.7%; spinal cord, 12.3 ± 1.8% and pituitary, 40 ± 1% (variance, P < 0.001; Fig. 1). Hippocampus and cortex showed significantly more activity than all other regions (P < 0.05), but somewhat lower activity than that we have previously described in cerebellum (64.3 ± 2.4%) (28) Moisan, M.-P., J. R. Seckl, L. P. Brett, C. Monder, A. K. Agarwal, P. C. White, and C. R. W. Edwards, submitted for publication. Kidney showed 73 ± 3% conversion and heart 17 ± 2% under identical assay conditions, peripheral tissues of high and low 11β-OHSD bioactivity respectively (20, 21, 28).

Northern blots (20 µg total RNA) hybridized with 32P-labeled 11β-OHSD cDNA probes under stringent conditions showed 11β-OHSD mRNA expression in all regions...
of the brain, although at levels lower than liver and kidney (Fig 2). No expression was found in heart. Testis and lung showed lower expression than brain, although the lung RNA sample showed some degradation and thus the level of 11β-OHSD mRNA in this tissue may have been underestimated. Within the brain, cortex and hippocampus showed high 11β-OHSD mRNA expression, at levels similar to cerebellum (a tissue we have previously shown to have high 11β-OHSD mRNA expression) (28) with lower expression in brain stem, spinal cord, hypothalamus, and pituitary. In situ hybridization using 35S-labeled cRNA antisense probes (Table 1) confirmed localized cerebral 11β-OHSD mRNA expression, with the highest levels in parietal cortex (layer IV) neurons.

Table 1.

<table>
<thead>
<tr>
<th>Region</th>
<th>11β-OHSD expression</th>
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<tr>
<td>Hypothalamus</td>
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</tr>
<tr>
<td>Anterior medial preoptic area</td>
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<tr>
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<td>±</td>
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<tr>
<td>Supraoptic nucleus</td>
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<td>Supraoptic nucleus</td>
<td>+</td>
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<tr>
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<td>±</td>
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<tr>
<td>Medial habenula</td>
<td>++</td>
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<tr>
<td>Lateral habenula</td>
<td>±</td>
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<tr>
<td>Amygdala</td>
<td>+</td>
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<td>Olfactory nucleus</td>
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<tr>
<td>Dentate gyrus (granule)</td>
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<tr>
<td>CA1 (pyramidal)</td>
<td>++</td>
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<tr>
<td>CA2 (pyramidal)</td>
<td>++</td>
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<tr>
<td>CA3 (pyramidal)</td>
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<tr>
<td>Molecular layer</td>
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<tr>
<td>Fimbria</td>
<td>+</td>
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<tr>
<td>Cortex</td>
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<td>Frontal (layer IV)</td>
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<td>Frontal (other layers)</td>
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<tr>
<td>Cingulate</td>
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<td>Piriform</td>
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<tr>
<td>Parietal (layers II and III)</td>
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<td>Parietal (layer IV)</td>
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<td>Parietal (layer VI)</td>
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<td>White matter</td>
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<td>Meninges</td>
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<td>Choroid plexus</td>
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<td>Pituitary (anterior)</td>
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<td>Pituitary (neurointermediate lobe)</td>
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Distribution of 11β-OHSD mRNA expression by in situ hybridization histochemistry in selected forebrain regions and pituitary. Key: −, absent; ±, very weak (just above background); +, weak; ++, moderate; ++++, strong. (++) denotes higher expression in some areas of the structure indicated.

(Fig. 3). Neurons in the other layers of parietal cortex also expressed 11β-OHSD mRNA and modest expression was found in the caudal part of the frontal cortex (layer IV only) cingulate and piriform cortex. Other cortical neurons did not express 11β-OHSD mRNA. Most, if not all, hippocampal neurons showed high expression of 11β-OHSD mRNA (Fig. 3), with the highest levels in CA3 pyramidal cells. Hypothalamus generally showed low 11β-OHSD mRNA expression but localized expression was found in the anterior part of the medial preoptic area (including the organ vasculosum of the lamina terminalis; OVLT) and the posterior part of the arcuate nucleus around the posterior margin of the third ventricle. There was high mRNA expression in anterior lobe of pituitary, but the neurointermediate lobe was negative. Vasculature in the meninges and choroid plexus had high 11β-OHSD mRNA expression paralleling recent reports of high enzyme bioactivity in mesenteric blood vessels (31).

There was no hybridization of ‘sense’ cRNA probes under the stringent conditions (0.0165 M Na+ at 60 C) employed. RNase A pretreatment markedly attenuated the hybridization signal (data not shown).

Discussion

We found 11β-OHSD bioactivity and mRNA expression in rat brain. In forebrain, as in cerebellum (28), 11β-OHSD activity is NADP+-dependent in vitro. In unsupplemented hippocampus or whole forebrain enzyme activity is low (20, 26, 27) but is markedly increased, to levels similar to those found in kidney, when the cosubstrate NADP+ is added to the incubation medium. This suggests that depletion of cosubstrate in vitro might be
In neocortex there were also high levels of 11β-OHSD activity and mRNA expression, predominantly localized to parietal cortex, particularly layer IV. This region processes sensory inputs, and has much glucocorticoid receptor immunoreactivity and mRNA expression (3, 4), but little mineralocorticoid receptor binding (7, 18). Glucocorticoids can modulate sensory processing (35). In addition, the postadrenalectomy rise in cortical glucocorticoid receptor levels is not down-regulated by corticosterone, whereas very low dose dexamethasone treatment leads to down-regulation (7). As dexamethasone is not a substrate for 11β-OHSD these data may suggest that 11β-OHSD could regulate the access of corticosterone to cortical glucocorticoid receptors in vivo. However, in cortex and hippocampus binding of ligand to mineralocorticoid receptors may regulate both mineralocorticoid receptor and glucocorticoid receptor levels, at least in mice (18), and clearly the in vivo interactions between ligand and the two receptor types are complex.

Much pharmacological and ligand-binding data suggest that periventricular areas of the hypothalamus are important for regulating the location of the central aldosterone-mediated effects on salt and water homeostasis. Thus, circumventricular organs and anterior hypothalamus show preferential uptake and metabolism of aldosterone, rather than corticosterone, in vivo (7, 9). Lesions of the anteroventral third ventricle, AV3V (which includes the medial preoptic area and OVLT to the circumventricular posterior part of the arcuate nucleus) but in contrast hippocampectomy (which removes limbic system mineralocorticoid receptors) does not alter the actions of adrenal steroids on salt appetite (36). We have found only very modest 11β-OHSD mRNA expression and bioactivity in whole hypothalamic extracts, in vitro, but in situ hybridization demonstrated localized high 11β-OHSD mRNA expression in the most anterior region of the medial preoptic area/OVLT and the periventricular posterior part of the arcuate nucleus. Thus, 11β-OHSD predominates in those hypothalamic areas where mineralocorticoid receptors are thought to be aldosterone-selective and to mediate the central actions of aldosterone on salt-water homeostasis and neuroendocrine and hemodynamic control. Clearly, abnormalities of 11β-OHSD activity might allow excessive binding of corticosterone to normally ‘aldosterone-selective’ hypothalamic mineralocorticoid receptors leading to neurogenic hypertension. Certainly lesions of AV3V attenuate or prevent the development of blood pressure increases in mineralocorticoid-salt excess and other models of hypertension in rats (36, 38, 39).

Moderately high 11β-OHSD mRNA expression (and bioactivity) was also found in anterior pituitary, suggesting that 11β-OHSD might influence the access of corticosterone to receptors at this site, and providing a possible explanation for observed preferential binding of
dexamethasone, compared with corticosterone to pituitary (40). However, any role of 11β-OHSD in the regulation of the hypothalamic-pituitary adrenal axis by physiological corticosteroids remains to be determined.

In summary, we provide evidence for regional 11β-OHSD mRNA expression and bioactivity in rat brain, in vitro. Cerebral 11β-OHSD activity may explain the site-specific differences in binding and actions of physiological corticosteroids at both mineralocorticoid and glucocorticoid receptors, in vivo.

Acknowledgments
We thank Dr. Carl Monder, The Population Council, New York, for generously providing the 11β-OHSD cDNA clone used in these studies.

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Beta-Hydroxysteroid Dehydrogenase Messenger Ribonucleic Acid Expression, Bioactivity and Immunoreactivity in Rat Cerebellum

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Key words: 11β-hydroxysteroid dehydrogenase, cerebellum, steroid receptors, corticosterone, in situ hybridization.

Abstract

β-Hydroxysteroid dehydrogenase (11β-OHSD) metabolizes corticosterone to inactive 11-dehydrocorticosterone and thus protects specific mineralocorticoid receptors from exposure to corticosterone in the kidney in vivo. Clearly, 11β-OHSD might also regulate corticosterone access to glucocorticoid receptors. We have investigated cerebellum, a tissue with high glucocorticoid receptor but very low mineralocorticoid receptor levels and have shown marked 11β-OHSD bioactivity with similar co-substrate affinities and inhibition kinetics to the renal enzyme. 11β-OHSD messenger ribonucleic acid was expressed in cerebellum as localized in Purkinje and granule cells. This distribution was confirmed immunohistochemically. Thus, we provide evidence that 11β-OHSD in cerebellum and suggest that it may regulate the access of corticosterone to glucocorticoid receptors in addition to mineralocorticoid receptors.

There are two types of corticosteroid receptors that have been classically termed type I or mineralocorticoid receptors (MR), those that bind aldosterone with high affinity and type II or glucocorticoid receptors (GR), those that bind corticosterone with much lower affinity. Both types are expressed in many tissues, but particularly high levels of GR are in brain, thymus, leucocytes, liver, kidney, and hypothalamus. In brain, GR are widely distributed in all tissues, but particularly high levels of GR are in Purkinje and granule cells. MR and its mRNA show a more tissue-specific distribution but are found not only in classical aldosterone target tissues such as kidney, parotid and colon but also in heart and brain. MR mRNA is detectable in several brain regions, with the highest levels of expression in the hippocampus and brainstem.

In v. vivo, the physiological glucocorticoids cortisol (man, guinea pig and mouse) and corticosterone (rat) bind with the same affinity as 11-dehydrocorticosterone to purified (3, 4) or recombinant expressed MR (5). However, in v. vivo, although physiological glucocorticoids circulate at 10- to 100-fold molar excess to aldosterone, they do not bind to MR in target tissue such as kidney or parotid, nor do they metabolize little mineralocorticoid activity. In heart and hippocampus, MR bind corticosterone with high affinity, in v. vivo. Indeed, MR may even antagonize the actions of corticosterone at non-selective sites (6). In addition to corticosterone-prefering binding sites in the limbic system, in v. vivo autoradiographic techniques have also defined aldosterone-preferring binding sites in periventricular areas of the brain (7). Despite the different in v. vivo specificities of the MR in kidney and hippocampus, the receptors are structurally identical and are translated from identical mRNA transcripts (2). Until recently the mechanisms conferring tissue-specific selectivity to MR in v. vivo were unclear.

11β-Hydroxysteroid dehydrogenase (11β-OHSD) is an NADP+-dependent microsomal enzyme which catalyses the metabolism of cortisol in man (or corticosterone in the rat) to inactive cortisone (11-dehydrocorticosterone) (8). Enzyme activity is found in liver, lung, kidney, testis, colon and salivary gland (9, 10). We have recently demonstrated the crucial role of 11β-OHSD in determining MR specificity in v. vivo (11). Thus, in aldosterone-selective tissues (kidney, parotid and colon), 11β-OHSD rapidly metabolizes cortisol to cortisone and protects MR from exposure to glucocorticoid. We have shown that the apparent mineralocorticoid excess in congenital 11β-OHSD deficiency is due to the mineralocorticoid activity of circulating glucocorticoids (12). Furthermore, inhibition of 11β-OHSD in normal subjects with liquorice, (or its major active component β-glycyrrhetinic acid or the hemsuccinate derivative curbenoxolone) reveals potent renal and colonic mineralocorticoid actions for cortisol (13). In the rat kidney, in v. vivo autoradiography has demonstrated that 11β
OHSD inhibition leads to a pattern of [3H]corticosterone binding indistinguishable from [3H]aldosterone binding (11). Thus, 11β-OHSD appears to protect the non-specific renal MR from exposure to glucocorticoids in vivo.

Recent data have shown that 11β-OHSD bioactivity is absent in hippocampus, which has high levels of both MR and GR (11, 14), apparently confirming some earlier studies using whole brain homogenates (15). However, experiments performed more than 20 years ago demonstrated NADP*-dependent 11β-OHSD bioactivity in rodent brain (16, 17). Subregions of brain, other than hippocampus, have not been studied. In addition to the role of 11β-OHSD in regulating cortisol-MR interactions we have recently found evidence that this enzyme may act to modulate the access of cortisol to GR in human skin (18) and clearly any 11β-OHSD activity in the nervous system may relate to either or both receptors. We have now investigated whether 11β-OHSD bioactivity and or mRNA expression are found in rat cerebellum, a region which contains high levels of GR and MR mRNA but very little MR and its mRNA (2, 19, 20). An abstract of aspects of this work has been presented to the Physiological Society.

Results

In vitro bioactivity

Cerebellar homogenates showed modest 11β-OHSD activity in the absence of NADP*, converting 18.3 ± 1% of [3H]cortico- 

sterone to [3H]1-dehydrocorticosterone in 60 min. This was significantly more than heart (negative control tissue with non- 

selective MR) which showed 0.8 ± 0.5% conversion (indistinguishable from the assay blank values). Kidney showed 63.4 ± 3.7% 

conversion under these conditions. As brain contains very little 

NADP* (21), the co-substrate for 11β-OHSD, the incubations 

were repeated in the presence of excess (200 µM) NADP*. This 

led to a modest increase in activity in kidney (by 15.6 ± 3%) and 

heart (by 14.2 ± 3%) but a marked increase in 11β-OHSD activity 

cerebellum (by 46 ± 2.9%) (all P < 0.001; ANOVA followed by 

unpaired student's t-test) (Fig. 1). Addition of β-glycyrrhetinic acid to the reaction significantly reduced the 11β-OHSD activity 
cerebellar homogenates to 36 ± 2.5% (10⁻⁸ M), 10.8 ± 0.6% 

(10⁻⁷ M) and 2.2 ± 0.6% (10⁻⁶ M) (Fig. 2). The kinetics of 

inhibition are similar to those of kidney homogenates (22).

Fig. 1. 11β-OHSD in vitro activity in homogenates of rat cerebellum compared to kidney and heart (n=5). 0.5 mg of total protein was 

incubated with 12 nM [3H]corticosterone for 60 min. The co-substrate 

NADP* was added at a final concentration of 200 µM in the second 

experiment.

Messenger RNA expression and localization

The expression of 11β-OHSD mRNA was determined in 20 total RNA extracted from rat liver, testis and cerebellum and fractionated on denaturing agarose gels. 11β-OHSD mRNA expression was detected by Northern blot hybridization (Fig. 4). Liver is the original tissue from which the cDNA has been isolated and cloned (23) and testis is an area where the importance of the enzyme has been recently described (24). A single band 

hybridization was detected in liver, testis and cerebellum. The 

intensity of the signal was stronger in cerebellum than in testis 

confirming a high level of 11β-OHSD mRNA expression in 

cerebellum, although lower than liver.

In situ hybridization with a 1.2 kb [35S]UTP-labelled cRNA 

antisense probe transcribed in vitro from the cDNA confirmed 

the presence of 11β-OHSD mRNA in cerebellum. 11β-OHSD 

mRNA was localized to the cerebellar cortex with highest 

expression in the Purkinje and granule cell layers (Fig. 4). Hybridization 

was markedly attenuated (> 50-fold) with RNase A pretreatment (data not shown). No specific hybridization with 'sense' cRNA probe was seen under the stringent conditions employed (Fig. 4). Microscopy of emulsion-coated 

sections, counterstained with haematoxylin and eosin, revealed 

moderate silver grain intensity over granule cell bodies, with 

higher expression in Purkinje cells (Fig. 5). Very low (but absent 

background) expression was found in most other neurons but 

not over white matter. There was no difference in the distribution 

or relative regional intensity of hybridization using 1.2 or 0.6 

probes (data not shown).

Immunocytochemical studies with two separate 11β-OHSD 

antisera (1:2-300 dilution) revealed positive staining of Purkinje 

and granule cell cytoplasm in the cerebellar cortex (Fig. 4). 

Further moderately positive immunostaining was located in large 

neurons in the basal cerebellar nuclei. Other cells, including those in the molecular layer and the underlying brainstem nuclei, were 

largely negative. Diffuse moderately positive staining was found 

in most axons and dendrites, but myelin was negative. Similar 

results were found with both fixatives and with both antisera. 

Preabsorption of the antisera with concentrated antigen 

(sonicated rat liver microsomes) prevented immunostaining.
11β-hydroxysteroid dehydrogenase in rat cerebellum

Fig. 3. 11β-OHSD mRNA expression in liver (L), testis (T) and cerebellum (C). Each lane contained 20 μg total RNA.

Fig. 4. Distribution of 11β-OHSD mRNA in rat cerebellum by in situ hybridization, using a [35S]-labelled cRNA antisense probe (A). The most intense expression is seen in the Purkinje cell layer (P), with moderate expression in the granule cell layer (G), but expression in the molecular layer (M) and white matter (W) is low. BS—underlying brainstem. A control section hybridized with sense cRNA probes is shown to demonstrate non-specific binding (B).

Purkinje and granule cells (and renal tubules) but only partially attenuated staining in molecular layer dendrites (Fig. 6). Therefore, the immunostaining in dendrites and axons was considered to be non-specific, presumably analogous to that previously described in kidney using one of these antisera (25). Furthermore, 11-OHSD activity in soluble (non-microsomal) fractions has not been reported; the enzyme would therefore not be expected to be located in neuronal processes. Preimmune rabbit serum showed no staining (Fig. 6). Kidney immunostaining corresponded to our previous findings (11), with positive staining of tubular cells and negative staining of glomeruli.

Discussion

We have demonstrated 11β-OHSD mRNA expression and in vitro bioactivity in rat cerebellum. Enzyme mRNA expression and immunoreactivity are localized in the cerebellar cortex.

In the absence of exogenous NADP+ cerebellar homogenates showed modest enzyme activity (18.3 ± 1% conversion) compared with 63.4 ± 3.7% conversion of corticosterone to 11-dehydrocorticosterone in kidney, a tissue where the biological importance of 11β-OHSD in vivo has been established (11). However, addition of excess NADP+, the enzyme co-substrate, increased cerebellar
11β-OHSD activity to 64.3 ± 2.9%. NADP+ led to only a small increase in renal enzyme activity and conversion remained low in the negative control heart tissue. Levels of adenine dinucleotides are low in brain (21), but neuronal concentrations and any variations of levels in brain subregions are unknown. It is likely that brain, a tissue that is largely metabolically-dependent on the circulation, will resynthesize rather than store energy-pathway-related molecules such as NADP+. Thus, the large discrepancy between basal and NADP+–supplemented 11β-OHSD activity in cerebellum in vitro may reflect rapid dephosphorylation of endogenous dinucleotide in the reaction mixture. Whether limitation of NADP+ concentrations plays any role in vivo in the regulation of 11β-OHSD activity remains to be determined. Nevertheless, the maximum cerebellar 11β-OHSD activity is similar to that found in kidney, suggesting that the enzyme may have important physiological effects in cerebellum.

The biological activity of 11β-OHSD in cerebellum appears to be mediated by a similar enzyme to that found in kidney and liver. Thus, not only do these activities have the same co-substrate requirement, but also both renal and cerebellar enzymes show similar inhibition kinetics with β-glycyrrhetinic acid (22). Furthermore, mRNA for the enzyme is expressed in cerebellum with a transcript size identical to that found in liver and kidney. However, enzyme identity between tissues must be confirmed at the post-translational level. The level of 11β-OHSD mRNA expression in cerebellum is at least as high as in testes, a tissue with well documented enzyme activity (9, 24). Cerebellar mRNA expression is less than that found in liver, the major site of corticosteroid degradative metabolism.

Immunohistochemically, 11β-OHSD was localized to cerebellar cortex, with the highest staining found in granule and Purkinje cells. There was also non-specific immunostaining in most, if not all, axons and dendrites (presumably analogous to the non-specific binding reported using 11β-OHSD antiserum in kidney (25)). In situ hybridization showed a distribution of 11β-OHSD mRNA similar to that of enzyme immunoreactivity. This is not surprising for a microsomal enzyme, which would not be expected to be exported from the cell of biosynthesis. The specificity of hybridization was clearly confirmed by the absence of signal when sense control probes were employed.

The cerebellar cortex contains very high levels of GR and 11β-OHSD mRNA. In contrast, MR mRNA expression is very low, which, when compared quantitatively (by RNase protection assay) with kidney, hippocampus, cortex, diencephalon, midbrain and pons (2), confirming results from ligand binding studies (19). It is unclear whether 11β-OHSD in cerebellum relates to MR or GR. Cerebellum, unlike most brain subregions including hippocampus and cerebral cortex, does not show down-regulation of GR by corticosterone–MR binding, suggesting that in cerebellum MR and GR are not significantly colocalized or co-regulated (7, 15). Taken together with the high levels of in vitro enzyme activity and the very low concentration of MR these data suggest that the major importance of cerebellar 11β-OHSD may be in regulating access of glucocorticoids to GR. Further studies to determine the role of 11β-OHSD in regulating the actions of corticosteroids in cerebellum are in progress.

Materials and Methods

11β-OHSD enzyme assay

Adult male Wistar rats (180 to 220 g) were decapitated and the brain rapidly removed and dissected on ice. The cerebellum was separated from the brainstem by severing the cerebellar peduncles; kidney and heart were used as positive and negative controls, respectively (11, 14). Tissue was homogenized in 5 ml of Krebs-Ringer bicarbonate buffer (118 mM NaCl, 3.8 mM KCl, 1.19 mM KH2PO4, 2.54 mM CaCl2, 2H2O, 1.1 mM MgSO4, 7H2O, 25 mM NaHCO3 and 0.2% glucose) using a dounce tissue grinder. The total protein content of the homogenate was estimated colorimetrically (Bio-Rad protein assay kit).

Total protein (0.5 mg) was then incubated with a final concentration of 12 nM 1,2,6,7,11H]corticosterone (specific activity 84 Ci/mmol; Amersham International, UK) in Krebs-Ringer buffer (+0.2% bovine serum albumin) for 60 min at 37 °C. In some experiments, the enzyme co-substrate NADP+ was added before incubation, at a final concentration of 200 μM. After incubation, steroids were extracted with ethyl acetate and then separated by thin-layer chromatography. 11β-OHSD activity was determined as the percentage conversion of [11H]corticosterone to [11H]11-dehydrocorticosterone (11).

Enzyme inhibition studies

The 11β-OHSD inhibitor β-glycyrrhetinic acid was diluted in ethanol to a concentration of 10−5 M and subsequently diluted in water. β-Glycyrrhetinic acid was added to cerebellar homogenates at 10−4 M, 10−5 M, 10−6 M final concentration and 11β-OHSD activity assayed as above.
11β-hydroxysteroid dehydrogenase in rat cerebellum

DNA preparation from cerebellum

Male Wistar rats (180 to 220 g) were decapitated, the cerebellum removed as above and immediately frozen in liquid nitrogen. Total RNA was extracted with guanidium thiocyanate, as described (26). Briefly, 0.5 g of cerebellum was homogenized in 2 ml of 4 M guanidinium thiocyanate, 0.025 M sodium citrate, 0.5% sarcosyl and 0.1 M β-mercaptoethanol. DNA was precipitated by addition of sodium acetate (2 M; pH 4) and proteins were removed by phenol/chloroform extraction. The aqueous phase containing the RNA was precipitated twice with isopropanol and resuspended in diethylpyrocarbodine-treated H2O.

Northern blot and cDNA/mRNA hybridization

Twenty μg total RNA was fractionated on a 1% agarose-2.2 M formaldehyde gel and blotted onto to nitrocellulose (Hybond C extra; Amersham International) by capillary transfer at 4 °C overnight. The nitrocellulose membrane was prehybridized in 50% formamide, 5× SSPE, 5× Denhardt's solution, 200 μg/ml denatured herring testis DNA, 0.1% SDS and 1 mM EDTA. Hybridization was performed in identical buffer containing radiolabelled 11β-OHSD cDNA probe. The 1.25 x 106 cpm/ml transcribed by T7 polymerase from the cDNA. Secondly, fixed sections were pretreated with RNase A (100 μg/ml for 1 h at 37 °C) prior to hybridization with antisense probe. Control experiments were carried out to test the specificity of both the hybridization method and the 11β-OHSD cDNA probe. First, cerebellar sections were treated as before but hybridized with a sense probe (not complementary to 11β-OHSD mRNA, 10 x 106 cpm/ml) transcribed by T7 polymerase from the cDNA. Secondly, fixed sections were pretreated with RNase A (100 μg/ml for 1 h at 37 °C) prior to hybridization with antisense probe. Finally, to ensure there was good tissue penetration of the 1.2 kb cDNA probe, we performed similar in situ hybridization studies with a 597 bp (115)UTP-labelled sense cRNA, using Sty 1-linearized 11β-OHSD plasmid.

Immunohistochemistry

Male rats were deeply anaesthetized with pentobarbitone and perfused through the ascending aorta with 250 ml ice-cold phosphate-buffered saline followed by 500 ml ice-cold 0.1 M paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or Bouin's solution (24). Tissue was removed and postfixed overnight. Sections (4 μm) were cut and immunostained as previously described (11) using two separate polyclonal rabbit antisera (both at 1:2,300 dilution) raised against purified rat liver 11β-OHSD (8). Detection was by the horseradish peroxidase-labelled streptavidin-biotin method (reagents from DAKO Ltd, UK). Control sections were immunostained with preimmune rabbit serum. Kidney sections were also immunostained to give positive (tubules) and negative (glomeruli) tissue controls. Specificity of immunostaining was controlled by preabsorption of the
858 11β-hydroxysteroid dehydrogenase in rat cerebellum
antiserum (1:400 dilution) with sonicated purified rat liver microsomes
(which contain concentrated 11β-OHSD bioactivity) for 24 h at 4 °C.

Acknowledgements
This research was supported by grants to J. R. S. from the Wellcome
Trust Royal Society of Edinburgh and the Scottish Hospitals' Endowment
Research Trust.

Accepted 2 July 1990

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11β-Hydroxysteroid dehydrogenase mRNA expression in rat kidney

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11β-Hydroxysteroid dehydrogenase (11β-OHSD) protects non-
mineralocorticoid receptors from exposure to aldosterone. Although 11β-OHSD bioactivity and aldosterone binding sites are found in distal tubular cells, mineralocorticoid receptor and 11β-OHSD immunoreactivities are not colocalized. However, there are several kidney isoforms of 11β-OHSD, not all of which may be immunoreactive, whereas only a single mRNA has been described. Using in situ hybridization we found 11β-OHSD mRNA is highly expressed in all renal tubular epithelia in the rat. It is therefore likely that 11β-OHSD is localized with mineralocorticoid receptors in distal tubular cells.

Recombinant or recombinant expressed mineralocorticoid receptors are nonspecific and bind the physiological glucocorticoids corticosterone (rat) or cortisol (humans) and mineralocorticoid aldosterone with equal affinity in vitro (2, 7). However, although corticosterone circulates at femtomolar levels, no biochemical evidence has been obtained for mineralocorticoid receptors in vivo. The apparent paradox lies in the activity of 11β-hydroxysteroid dehydrogenase (11β-OHSD) which catalyzes the conversion of corticosterone to 11-dehydro-
corticosterone, or cortisol to cortisone (the 11-dehydro-
corticosteroids do not bind to mineralocorticoid receptors). In mineralocorticoid-selective target tissues such as kidney, 11β-
OHSD acts to protect nonspecific mineralocorticoid receptors from exposure to corticosterone (or cortisol) in vivo (4, 6). Patients with congenital 11β-OHSD deficiency exhibit a syndrome of apparent mineralocorticoid resistance in which cortisol acts as a mineralocorticoid causing hypercortisolism and hypokalemia (12). Similarly, inhibition of 11β-OHSD with licorice lead glucocorticoids to develop renal mineralocorticoid activity (13). In the rat, renal binding of [3H]corticosterone in vivo (normally minimal) becomes indistinguishable from [3H]aldosterone binding when 11β-OHSD is inhibited (4).

Establishing the tissue localization of 11β-OHSD in kidney has been problematic. Immunohistochemical studies using nonspecific polyclonal antisera raised against purified rat liver 11β-OHSD suggest that the enzyme and mineralocorticoid receptors are not colocalized, with mineralocorticoid receptor immunoreactivity and aldosterone binding sites in distal tubular and cortical collecting duct cells but not in medullary interstitial cells (4, 11). Therefore a hypothesis of paracrine protection of renal mineralocorticoid receptors by 11β-OHSD was proposed (4). However, corticosteroids diffuse readily through tissues, and a paracrine mechanism is unlikely to completely prevent access of glucocorticoids to mineralocorticoid target cells.

After the recent isolation of an cDNA encoding rat liver 11β-OHSD (1), expression of 11β-OHSD mRNA was demonstrated in a variety of rat tissues including the kidney (1, 8). Interestingly, 11β-OHSD mRNA and mineralocorticoid receptor mRNA were demonstrated in most, if not all, pyramidal neurons in the CA3 subregion of the hippocampus, suggesting probable colocalization, at least in brain (8). We have now examined the distribution of 11β-OHSD mRNA in the kidney using in situ hybridization histochemistry.
METHODS

Male rats (250–350 g) were anesthetized with pentobarbital sodium and perfused intracardially with 250 ml sterile ice-cold physiological saline followed by 500 ml ice-cold 4% paraformaldehyde-0.1 M phosphate buffer (pH 7.4) containing 0.02% diethylpyrocarbonate. The kidneys were removed and were postfixed in paraformaldehyde solution for 2–4 h and then in 15% sucrose/0.1 M phosphate-buffered saline at 4°C overnight. Tissue was frozen, and cryostat sections (10 μm) were cut, mounted onto gelatin and poly-L-lysine-coated slides, and stored at −85°C. Slides were washed twice in 2× saline sodium citrate (SSC) containing 0.02% diethylpyrocarbonate, treated with proteinase K (1 μg/ml) for 30 min at 37°C, and washed in 2× SSC before hybridization. In parallel studies rats were decapitated, and the kidneys were immediately frozen on dry ice and stored at −85°C. Fresh frozen sections were postfixed in 4% paraformaldehyde-0.1 M phosphate buffer containing 0.02% diethylpyrocarbonate and washed twice in 2× SSC containing 0.02% diethylpyrocarbonate before hybridization.

For isotopic probes, T3 polymerase (GIBCO) was used to transcribe 598 bp 35S-uridine triphosphate (UTP)-labeled antisense cRNA probes from Sty I linearized pBluescript vector containing the 1,265 bp 11β-OHSD cDNA (1). RNA probes (sp act 10 × 10⁴ Ci/ml, final concentration 0.05 pmol/ml) were denatured, added to hybridization buffer (50% formamide, 0.6 M NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.5, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, 0.05 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 0.1 g/ml dextran sulfate, and 10 mM dithiothreitol (DTT)) and applied to the slides as described (8). Slides were incubated overnight at 50°C, rinsed twice in 2× SSC, treated with RNase A (30 μg/ml, 30 min at 37°C), and washed to a maximum stringency of 0.1× SSC at 60°C for 60 min. After dehydration in increasing concentrations of ethanol, slides were exposed to autoradiographic film (Hyperfilm Bmax , Amersham International). For microscopic localization, slides were dipped in photographic emulsion (Ilford), exposed in a light-tight box for 3 wk before being developed (D19; Ilford) and counterstained with hematoxylin and eosin. Controls included pretreatment with RNase A (100 μg/ml for 1 h at 37°C) and hybridization with noncomplementary “sense” probes.

To confirm the microscopic distribution of 11β-OHSD mRNA expression biotinylated 11β-OHSD cRNA probes were used, as these gave better cellular resolution, although a lower signal, than isotopic probes. Best results were obtained with transcripts of 118 bp Pst I-linearized 11β-OHSD cDNA and prefixed material. Biotin-11-UTP (1 mM) was added to a standard in vitro transcription reaction (Promega). Sections were hybridized and washed as with isotopic probes and were then rinsed in phosphate-buffered saline (PBS). Biotin was detected by exposure to avidin-biotin peroxidase complex (ABC, Vector Elite kit; Vector Laboratories, Peterborough, UK) for 30 min. Sensitivity was increased by subsequent incubation with biotinylated antidig (1:250; Vector Lab-
11β-OHSD mRNA LOCALIZATION IN KIDNEY

Fig. 2. A: photomicrograph of rat kidney sections hybridized with unlabeled 11β-OHSD antisense cRNA probe, visualized by horseradish peroxidase, and counterstained with methyl green. Signal is seen in cytoplasm of renal tubular epithelial cells of proximal (P) and distal (D) convoluted tubules. Intersitial and glomerular (G) cells are negative. Original magnification × 180. B: 11β-OHSD antisense cRNA probe, visualized by photographic emulsion counterstained with hematoxylin and eosin. 11β-OHSD mRNA expression was seen in all renal tubular epithelial cells of cortex and medulla, including those of proximal (P) and distal (D) convoluted tubules.

Comparison between the immunohistochemical localization of 11β-OHSD and our in situ hybridization findings. First, the 11β-OHSD mRNA expressed in distal tubular cells may not be translated. However, major nontranslated splicing variants are unlikely, as the only single mRNA band has been found in Northern analysis of RNA from rat kidney and other tissues (1, 8). Alternatively, 11β-OHSD might be synthesized in distal tubules but subsequently degraded or rapidly transported to other sites. However, 11β-OHSD activity is concentrated in the microsomal fraction, and the enzyme is thus unlikely to be exported. Furthermore, high 11β-OHSD bioactivity with similar enzymatic characteristics to recombinant expressed 11β-OHSD has been found in distal tubular preparations (3, 4, 10).

Second, the existing 11β-OHSD antisera may not recognize the enzyme form(s) found in distal tubular epithelia. 11β-OHSD immunoreactivity is present as 34,000, 40,000, and 68,000 species in kidney (9). Further isoforms are found in other tissues, including a 26,000 species in brain and a 47,000 band in testis (9). These data suggest that the enzyme undergoes tissue-specific posttranslational processing; sequence analysis of cloned rat 11β-OHSD cDNA shows potential N-glycosylation sites (1). The current antisera do not bind to the active site of 11β-OHSD (9), and other biologically active but nonimmunoreactive isoforms of the gene product may occur. Thus the antisera may only recognize 11β-OHSD forms present in proximal, but not distal, tubule cells.

Finally, the 11β-OHSD activity in distal tubular cells may represent a separate gene product (5). Genomic blot hybridization has suggested the presence of more than one related gene in both rat and human genomes (1). However, the presence of a single mRNA band in Northern blots (1, 8) and high levels of 11β-OHSD mRNA in both distal and proximal tubule cells in situ under the stringent conditions of this study suggest that additional gene products need not be invoked to explain 11β-OHSD activity in aldosterone-target cells of the tubular epithelium.

Corticosteroid 11β-dehydrogenases may play a variety of roles in the kidney. It is likely that the enzyme in distal tubular epithelia protects colocalized mineralocorticoid receptors from exposure to circulating glucocorticoid. In contrast, the proximal tubular form might regulate access of corticosterone to glucocorticoid receptors, analogous perhaps to 11β-OHSD activity recently described in human skin (14). Alternatively, one renal 11β-OHSD form might be relatively inactive under basal conditions but could be potentiated by elevated (stress) levels of glucocorticoid, as occurs for hippocampal 11β-OHSD activity (unpublished observations).

Thus, we have demonstrated 11β-OHSD mRNA expression in both proximal and distal renal tubular epithelia. Cellular colocalization of 11β-OHSD and mineralocorticoid receptors in the kidney is very likely.

We thank Dr. C. Monder for the 11β-OHSD cDNA clone. This work was supported by a Royal Society of Edinburgh-Wellcome Trust Senior Clinical Research Fellowship to J. Seckl, Stichting Dr. C. L. van Steeden-Fonds (to A. D. van Haast), and grants from the Scottish Hospital Endowment Research Trust and the Sir Stanley and Lady Davidson Medical Research Fund to J. Seckl.

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Received 22 August 1990; accepted in final form 25 January 1991.

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Ontogeny of 11β-Hydroxysteroid Dehydrogenase in Rat Brain and Kidney*

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ABSTRACT. Close regulation of circulating corticosteroid levels during the early postnatal period is crucial for normal development and maturation of the central nervous system. In the first weeks of life cerebral glucocorticoid receptor concentrations are low and the hypothalamic-pituitary-adrenal axis is relatively unresponsive to stress, which might, in part, protect the developing brain from elevated corticosteroid levels. However, central mineralocorticoid receptors are at near adult levels and free glucocorticoid concentrations may approximate adult values as corticosteroid binding globulin is absent. Thus other mechanisms controlling cerebral exposure to corticosteroids may be of importance. 11β-Hydroxysteroid dehydrogenase (11β-OHSD) determines the access of corticosterone to peripheral mineralocorticoid and glucocorticoid receptors in adults in vivo by metabolizing corticosterone to inactive 11-dehydrocorticosterone. The enzyme has recently been demonstrated in brain subregions and may modulate local corticosteroid-receptor interactions. We therefore examined 11β-OHSD bioactivity and messenger RNA (mRNA) expression in the brain, compared with kidney, during the neonatal period. 11β-OHSD bioactivity (expressed as the percentage conversion of corticosterone to 11-dehydrocorticosterone) was moderately high in hippocampus and parietal cortex at birth (46 ± 4% and 48 ± 5%, respectively), fell significantly to a nadir (32 ± 1% and 30 ± 1%, respectively) at postnatal day 10 and then gradually rose to adult values (52 ± 3% and 58 ± 3%). By contrast, 11β-OHSD activity in cerebellum was high at birth (60 ± 3%), fell significantly to a peak at postnatal day 10 (74 ± 3%), and then fell to adult values by postnatal day 15 (64 ± 3%). Renal 11β-OHSD activity was moderately high (69 ± 3%) at birth and reached adult values (80 ± 2%) by postnatal day 5. Northern blots showed high and similar expression of a single species of 11β-OHSD mRNA from birth to adulthood in the hippocampus. Only low expression of 11β-OHSD (two or three separate species) was found in the kidney during the first 2 weeks of life, whereas, in adults high expression of 11β-OHSD mRNA was detected in kidney (four species). Using in situ hybridization high 11β-OHSD mRNA expression was localized to the neuronal layers of the postnatal hippocampus, neocortex, and cerebellum, and low but detectable expression was found in the neonatal renal cortex. Thus, 11β-OHSD is highly expressed in rat brain subregions in the early postnatal period with specific developmental patterns of activity. The discrepancy between 11β-OHSD mRNA expression and bioactivity in the developing kidney is unexplained and may reflect the presence of more than one gene product with similar bioactivity. The enzyme may play an important role in the developing brain by protecting tissues from (or exposing them to) elevated corticosterone levels. (Endocrinology 130: 400-404, 1992)

GLUCOCORTICOIDS play an important role during development, affecting the growth and differentiation of a number of tissues and organs, including the central nervous system (for review see 1). High dose glucocorticoid administration during the early postnatal period in rodents leads to permanent inhibition of brain growth, with reduced neurogenesis and glial proliferation, attenuated dendrite formation and behavioral and neuroendocrine impairments (2). Conversely, adrenalectomy of 11-day-old rats is followed by neuronal and glial proliferation (3) which is reversed by corticosterone replacement. Hence corticosterone is thought to exert an antiproliferative effect on postnatal neurogenesis and glial mitosis in the normal intact animal. These effects are most marked during the first 2 weeks of life and thus close control of circulating glucocorticoid levels during this time appears critical for normal brain development.

Low and constant levels of circulating glucocorticoids were thought to be ensured by the hyporesponsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to stressful stimuli during the first 2 weeks of life and the absence of circadian fluctuations in plasma glucocorticoid concentrations in neonates (4). However, it has been recently found that various stressors trigger an adult-like pituitary ACTH response in 10-day-old rats, the magnitude of which is affected by corticosterone (5). Although the corticosterone response is smaller than that
11β-OHSD ONTOGENY IN BRAIN AND KIDNEY

Northern blot analysis

Kidneys and brains from Wistar rats of various ages were removed, subregion dissected, and immediately frozen on dry ice. Tissues were pooled where necessary to obtain 50-100 mg. Total RNA was extracted by the guanidinium thiocyanate method, as described (13). Approximately 15 µg total RNA from each sample (5 µg adult kidney) were fractionated on 1% agarose-0.7 m formaldehyde gels and blotted onto nitrocellulose (Hybond C extra, Amersham International) by capillary transfer overnight. Hybridization was performed at 42°C overnight in 50% formamide with a random primed 32P-labeled 11β-OHSD complementary DNA (cDNA) probe consisting of the excised p11DH insert (14) and the membrane washed to a final stringency of 0.2 x SSC, 0.1% sodium dodecyl sulfate at 60°C and exposed to Kodak XAR film for 2 days. Filters were rehybridized with human GAPDH cDNA under the same conditions (15). GAPDH mRNA expression represents a sequence unrelated to 11β-OHSD, whose expression is ubiquitous and which does not vary much during development in brain and kidney as judged by our data.

In situ hybridization

Kidneys and brains from Wistar rats of various ages were removed and immediately frozen on dry ice. Sections (10 µm) of kidney were cut longitudinally and brain sagittally and were applied to gelatin and poly-L-lysine-coated slides. Sections were hybridized, as described previously (10), using either 32P or 35S-labeled antisense cRNA probes synthesized from Styl-linearized pl1DH vector (SA, ~106 Ci/mmol). Slides were washed to a maximum stringency of 0.1× SSC at 60°C for an h. Control slides were incubated with radiolabeled sense RNA probes under identical conditions to test the specificity of hybridization.

Statistics

Data were compared by analysis of variance followed by Student’s unpaired t test. Significance was set at P < 0.05. Values are expressed as mean ± SEM.

Results

Tissues were examined for 11β-OHSD activity at various times and compared to adult values (Fig. 1). 11β-OHSD activity in the kidney was already high at birth (69 ± 3%) and rose significantly to adult values (80 ± 1%) by postnatal day 5. By contrast, activity in hippocampus and cortex, though moderately high at birth (46 ± 4% and 48 ± 5%, respectively), fell significantly to a nadir at postnatal day 10, and then rose gradually to adult values. Cerebellar 11β-OHSD activity was high at birth (61 ± 3%), rose significantly to a peak at postnatal day 10 then fell to adult values by postnatal day 15.

Northern blot analysis was used to investigate the postnatal development of 11β-OHSD mRNA expression. Kidney and hippocampal RNA extracts were run on a single gel and are thus directly comparable (Fig. 2). We observed very high levels of 11β-OHSD mRNA expres-

Materials and Methods

11β-OHSD enzyme assay

Wistar rats of various ages (1, 5, 10, 15-day-old, and adult) were decapitated, their brains and kidneys removed, and dissected on ice. Tissues were assayed as described previously (10). In brief, 0.5 mg/ml tissue protein from each tissue homogenate was incubated with 200 µM NADP+ and 12 nM 1,2,6,7,113-OHSD corticosterone (SA, 84Ci/mmol, Amersham International, Aylesbury, UK) in Krebs-Ringer buffer (+0.2% BSA) for 1 h at 37°C. After incubation, steroids were extracted with ethyl acetate and separated by TLC. The percentage conversion of 3H-corticosterone to 3H-11-dehydrocorticosterone was calculated from the radioactivity of each fraction.
OHSD mRNA in neuronal layers of the hippocampus, cerebellar cortex and neocortex in the 10-day-old rat (Fig. 3). In neonatal kidney, only low expression of 11β-OHSD mRNA could be detected, predominantly in the outer cortex. Renal cortical expression of 11β-OHSD mRNA increased with age, but was much less than adult levels (Fig. 4). There was no hybridization of sense RNA probes (data not shown).

Discussion

There is increasing evidence that 11β-OHSD plays an important role in the central nervous system. High levels of enzyme bioactivity, mRNA expression, and immunoreactivity have been detected in the central nervous system, particularly in hippocampus, cortex, and cerebellum (10, 11, 16), and 11β-OHSD immunoreactivity colocalizes with MR-like immunoreactivity in hippocampus, cortex, hypothalamic paraventricular nucleus, and amygdala (17). A possible function for 11β-OHSD in...
11β-OHSD activity in neonatal period. However, 11β-OHSD activity in neonatal cerebellum shows a distinct pattern, although adult cerebellar 11β-OHSD activity is also affected by glucocorticoid levels (25). Therefore other tissue-specific regulatory factors may be of importance. The subregional differences in 11β-OHSD activity during development may also relate to a local and time-specific requirement for corticosterone. The enzyme may provide protection of sensitive neurons, glia, and/or dendrites from elevated corticosteroid levels, for example during stress. By contrast, reduced 11β-OHSD activity may allow exposure of receptors to physiological corticosteroids ensuring inhibition of cell proliferation at appropriate times. Interestingly, the effects of synthetic (nonmetabolized) glucocorticoids and corticosterone differ in their actions on cerebral neurogenesis, perhaps reflecting the activity and importance of the enzyme in vivo (26).

In summary, we have found subregionally specific patterns of development of 11β-OHSD activity in the neonatal brain with high 11β-OHSD mRNA expression during this period. 11β-OHSD may determine glucocorticoid effects in the maturing postnatal brain. The kidney showed high enzyme activity around birth but low mRNA expression, suggesting 11β-OHSD in neonatal kidney may be structurally different from brain.

Acknowledgment

We thank Dr. C. Monder for providing the 11β-OHSD cDNA clone.

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Endocrinology 127:1450-1455
Commentary

Multiple isoforms of the cortisol–cortisone shuttle

B. R. Walker and M.-P. Moisan

Human plasma contains similar free concentrations of the active steroid cortisol and its inactive metabolite cortisone. Interconversion of cortisol and cortisone (or corticosterone and 11-dehydrocorticosterone in rats) has been recognized since the 1950s. Until recently it was thought that separate NADP/NADPH-dependent enzymes were responsible: 11β-dehydrogenase to convert cortisol to cortisone and 11β-reductase for the reverse reaction (Abramowitz, Branchaud & Murphy, 1982; Lakshmi & Monder, 1985). It is now known that both activities can be expressed from the same cDNA clone (Agarwal, Monder, Eckstein & White, 1989; Agarwal, Tusie-Luna, Monder & White, 1990), suggesting that a single 11β-hydroxysteroid dehydrogenase protein (11β-OHSD) catalyses both reactions. Recently, interest in the enzyme was stimulated by observations in patients with congenital 11β-dehydrogenase deficiency (the apparent absence of mineralocorticoid excess) (Ulick, Levine, Gunczler et al. 1979; Stewart, Corrie, Shackleton & Edwards, 1988) and in volunteers given the 11β-dehydrogenase inhibitors liquorice (Stewart, Valentino, Wallace et al. 1987; Monder, Stewart, Lakshmi et al. 1989) or carbenoxolone (Stewart, Wallace, Atherden et al. 1990). In these situations cortisol acted as a mineralocorticoid. It was hypothesized that 11β-dehydrogenase in the kidney is necessary to protect intrinsically non-specific mineralocorticoid receptors in distal renal tubules from exposure to cortisol, and thereby allow specific access for aldosterone. This was confirmed when administration of 11β-dehydrogenase inhibitors to rats resulted in binding of [3H]corticosterone by renal mineralocorticoid receptors (Edwards, Stewart, Burt et al. 1988; Funder, Pearce, Smith & Smith, 1988).

Having established the physiological role of cortisol–cortisone conversion in the kidney, questions were raised about its importance elsewhere. Tissues with mineralocorticoid receptors exhibiting aldosterone specificity (e.g. salivary glands) have high 11β-dehydrogenase activity (Edwards et al. 1988). However, 11β-dehydrogenase is also active in homogenates of a wide variety of tissues (see Walker & Edwards, 1991) where mineralocorticoid receptors are not abundant (e.g. liver), or where mineralocorticoid receptors are present but do not display specificity for aldosterone (e.g. hippocampus: Moisan, Seckl & Edwards, 1990; Lakshmi, Sakai, McEwen & Monder, 1991). In these sites the enzyme may fulfill a different function, perhaps regulating access of cortisol to glucocorticoid receptors. This hypothesis is supported by the correlation between enzyme activity and glucocorticoid receptor-mediated responses in human skin (Teelucksingh, Mackie, Burt et al. 1990), rat testis (Phillips, Lakshmi & Monder, 1989), colon (Fuller & Verity, 1990) and mammary gland (Quirk, Slattery & Funder, 1990). Recently evidence has emerged which suggests that 11β-OHSD in glucocorticoid target tissues may be a different species from that in classical mineralocorticoid targets.

Carl Monder's group in New York used 11β-OHSD purified from rat liver microsomes to raise two rabbit antisera (Monder & Lakshmi, 1990). Subsequent studies of immunohistochemical distribution of the enzyme produced some surprising results. Although mineralocorticoid receptors abound in distal renal tubules, immunoreactive enzyme was found only in proximal tubules (Edwards et al. 1988; Rundle, Funder, Lakshmi & Monder, 1989). By contrast, enzyme bioactivity has been demonstrated both proximally and distally (Edwards et al. 1988; Bonvalet, Doignon, Blot-Chabaud et al. 1990; Naray-Fejes-Toth, Watlington & Fejes-Toth, 1991). Furthermore, Western blots showed different sizes of immunoreactive proteins in different tissues (Monder & Lakshmi, 1990). The first antiserum (56–125) identified a 34 kDa protein in liver, and additional species of 40 kDa in kidney, 26 kDa in brain and 47 kDa in testis. The second antiserum (56–126) identified a 34 kDa protein in all tissues, additional 68 kDa proteins in liver and kidney (possibly a dimer) and again a 40 kDa protein in kidney. Using the purified rat liver 11β-OHSD protein, an 11β-OHSD cDNA has been cloned from a liver cDNA library.
(Agarwal et al. 1989). This predicts the amino acid sequence of a protein of only 31 kDa. This discrepancy can be accounted for by glycosylation of the protein since when the cDNA was expressed in vitro a 34 kDa protein was produced (Agarwal et al. 1990) which was reduced to 31 kDa by deglycosylation with tunicamycin. Interestingly, tunicamycin also altered the relative activities of 11β-dehydrogenase and 11β-reductase (Agarwal et al. 1990).

Kinetic data also suggest the presence of multiple isoforms. This is reflected in experiments examining the cofactor preference of 11β-dehydrogenase for NADP or NAD and by the differences between tissues in the equilibrium between cortisol and cortisone. The reduction of tetrazolium to formazan blue in tissue slices depends on NADPH or NADH generated by 11β-dehydrogenase. Blue staining only occurred in distal tubules supplied with NAD rather than NADP (Mercer & Kroczowski, 1991). Similarly, we found that 11β-dehydrogenase in kidney homogenates utilized NAD and NADP with equal facility, in contrast with most tissues where NADP is preferred. 11β-Reductase activity is relatively labile in vitro (Agarwal et al. 1990). It is therefore hard to establish whether different kinetics in vitro reflect a different cortisol–cortisone balance in vivo. However, in vivo experiments show that human kidney has predominant 11β-dehydrogenase activity (Whitworth, Stewart, Burt et al. 1989) while liver has predominant 11β-reductase, since almost all oral cortisone is converted on first pass to cortisol (Stewart et al. 1990) and hepatic vein cortisone concentrations are much lower than renal vein (authors’ unpublished data). Thus the presence of different isoforms may result in different local concentrations of active and inactive steroid.

The liver cDNA clone has been used as a probe to study 11β-OHSD expression in various rat tissues. On Northern blots it apparently cross-hybridized to a single species of mRNA (approx. 1.7 kb) in most sites except kidney, where at least three other species were detected (1.5 kb, 1.6 kb and 1.9 kb) (Krozowski, Stuchberry, White et al. 1990), and colon where a mRNA of 3 kb was found (Whorwood, Stewart, Franklyn & Sheppard, 1991). More recently cDNA clones have been isolated from a rat library which are not contiguous with the liver cDNA clone in their 5′ extremity (Mercer, Obeyesekere, Smith et al. 1991). One of these has been sequenced and shows an identical open reading frame to the liver cDNA downstream from amino acid Met 27. Our own data derived from cloning of the 11β-OHSD rat gene shows that these two isoforms (with and without amino acids 1–27) are generated by differential promoter usage. The amino acid sequence upstream from Met 27 is very hydrophobic and contains a putative signal peptide for membrane insertion, suggesting that the isoforms may differ in their intracellular localization. In-situ hybridization using the liver probe has shown renal 11β-OHSD mRNA in both proximal and distal tubules (Yau, Van Haarst, Moisan et al. 1991; Stewart, Whorwood, Barber et al. 1991), consistent with the bioactivity of the enzyme but in contrast to the immunohistochemical distribution. It remains to be seen whether renal isoforms will show differential distribution, with a specific isoform located in mineralocorticoid target cells of the distal tubule. Recently, a human cDNA clone derived from a testis library has been isolated, and the human 11β-OHSD gene on chromosome 1 has been characterized (Tannin, Agarwal, Monder et al. 1991). Surprisingly, only one mRNA species was detected in human kidney using this cDNA as a probe. However, the similar structure of the human and rat genes suggests that similar isoforms can be transcribed in both species.

In summary, there is evidence for multiple isoforms of 11β-OHSD with differences in antigenic epitopes, protein size, cofactor binding, substrate/product equilibrium and tissue distribution. At least some of these isoforms are generated from a single gene. We suggest that this complex group of enzymes might dictate the concentration of active steroid in each target site and thereby offer tissue-specific modulation of corticosteroid receptor activation. Furthermore, while 11β-dehydrogenase protects some receptors by inactivation of cortisol to cortisone (e.g. mineralocorticoid receptors in kidney and glucocorticoid receptors in skin), in other sites 11β-reductase may amplify the exposure of relatively low affinity glucocorticoid receptors by activation of cortisone to cortisol. The isoforms responsible may be subject to differential regulation. Future studies will be directed at characterizing more isoforms, particularly those represented by multiple mRNA species in kidney. Also further studies of the gene with functional analysis of the promoter regions may reveal an explanation for tissue-specific changes in enzyme activity during development (Moisan, Edwards & Seckl, 1992) and after glucocorticoid exposure (Moisan, Campbell, Noble et al. 1991).

What implications does the existence of multiple 11β-OHSD isoforms have for clinical research? The analogy between 11β-OHSD dictating tissue sensitivity to cortisol and 5α-reductase dictating sensitivity to testosterone has been drawn before in a Commentary in this Journal (Fraser, 1990). Recently, a second gene for 5α-reductase has been cloned (Andersson, Berman, Jenkind & Russe1, 1991). It codes for a second enzyme which differs from the first in its tissue distribution, kinetics and sensitivity to finasteride inhibition. Furthermore, it is a defect in the second gene which accounts for male pseudohermaphroditism. It seems likely that there is another gene for 11β-OHSD given the discrepancies between enzyme bioactivity and mRNA
levels in rat parotid and mammary gland (Krozowski et al. 1990) and in kidney during the course of development (Moisan et al. 1992). Congenital deficiency of 11β-dehydrogenase (Ulick et al. 1979; Stewart et al. 1988) is independent of 11β-reductase deficiency (Taylor, Bartlett, Dawson & Enoch, 1984; Phillipou & Higgins, 1985) and appears only to affect mineralocorticoid target sites. The availability of the known human gene sequence will help us to establish what genetic defect affects these patients, whether it relates to this or another gene and whether it is likely to result in deficiency of only one isoform. We have recently found evidence of 11β-dehydrogenase deficiency in patients with essential hypertension, but have not shown that this relates to abnormal renal mineralocorticoid receptor activation (Walker, Shackleton & Edwards, 1991a). It is possible that the defect affects an extrarenal isofrom and is linked with hypertension through another mechanism, perhaps involving deficient 11β-dehydrogenase in vascular smooth muscle (Walker, Yau, Brett et al. 1991b). Finally, there is evidence that the metabolic effects of liquorice and carbenoxolone are different, since carbenoxolone apparently inhibits both 11β-dehydrogenase and 11β-reductase and does not produce a fall in plasma cortisone levels or a kaliuresis (Stewart et al. 1990). Thus 11β-OHSD inhibitors may display some isofrom specificity. Perhaps we can exploit this specificity to clarify the physiological roles of multiple tissue-specific shuttle enzymes which are central to the balance of active and inactive corticosteroids.

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Differential promoter usage by the rat 11β-hydroxysteroid dehydrogenase gene.

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Abstract
11β-hydroxysteroid dehydrogenase (11β-OHSD) catalyzes the conversion of physiological glucocorticoids to inactive products, thus protecting non-selective renal mineralocorticoid receptors (MR) from circulating glucocorticoids (ensuring aldosterone-selectivity in vivo) and modulating glucocorticoid access to MR and glucocorticoid receptors (GR) in other tissues. Detection of multiple mRNA and immunoreactive 11β-OHSD species in kidney, but not liver, extracts suggests the presence of tissue-specific isoforms. To determine whether differential promoter usage might explain the mRNA heterogeneity we cloned and sequenced rat 11β-OHSD genomic DNA. Total identity was found between the nucleotide sequence of exons 1 and 2 and the previously published rat liver cDNA. Using both primer extension and RNase protection analyses we found the predominant transcription start site in liver (+1) is 105 bp 5' of the start of translation. In kidney 2 additional Cap sites were detected: (i) 264 bp 5' of exon 1; there is no in-phase open reading frame suggesting the additional 5' sequence is not translated. (ii) 65 bp upstream of exon 2, within intron A; the predicted truncated protein lacks the first 26 hydrophobic residues. Oligonucleotide probes specific to transcripts arising from each promoter confirmed all 3 are employed in kidney, whereas a single predominant species was found in liver, thus demonstrating tissue-specific differential promoter usage of the 11β-OHSD gene.

Introduction
11β-OHSD catalyzes the reversible conversion of physiological glucocorticoids (corticosterone in rats, cortisol in humans) to inactive products (11-dehydrocorticosterone and cortisone respectively). In the kidney 11β-OHSD confers in vivo aldosterone-selectivity on MR which, when purified or expressed in vitro, bind aldosterone, cortisol and corticosterone with equal affinity (1, 2). 11β-OHSD activity is also found in a wide variety of tissues (for review see 3) where MR are not abundant (eg liver) or where MR are present but bind glucocorticoids (eg hippocampus). At these sites the enzyme may play a different role, modulating glucocorticoid access to MR and/or GR.

Liver and kidney 11β-OHSD differ as to number of mRNA and immunoreactive species and kinetic constants (4-7). It appears that kidney contains several isoforms of 11β-OHSD which are distinct from the liver form. In this study our aim was to determine whether the liver and kidney mRNA isoforms could be encoded by a single gene.

Results:
Cloning and sequencing of 11β-OHSD genomic DNA.

A rat genomic library was screened by hybridization to an EcoRI-EcoRV fragment of rat 11β-OHSD cDNA (6). Three positive clones were identified; a 15 kb clone encompassing the other 2 was sequenced. Identity between the nucleotide sequence of the first and second exons of this clone and rat liver cDNA showed that our clone represented the rat 11β-OHSD gene. The nucleotide sequence of exons 1 and 2, the first intron and 890 bp of 5' flanking region is illustrated in Fig 1. Noteworthy sequence elements include a poly(T) stretch at -416 to -391 and (dA-dC) repeats from +1089 to +1131 in intron B. There is no apparent TATA box but the sequence contains a number of putative transcription factor binding sites; a CCAAT box at -74, 6 CACCC-box elements at -476, -466, -427, -290, -282, and -82, a GC box (SP1 consensus binding site) at -299, sequences corresponding to the consensus half site glucocorticoid responsive element (GRE) TGT(T/C)CT at +61 and +250, and the pentanucleotide TGACC, in common to estrogen (ERE) and thyroid (TRE) responsive elements, at positions -839, +635, and +1295.

Identification of 11β-OHSD transcription start sites.

Primer extension analysis was carried out on liver and kidney RNA to determine the start point(s) of transcription. Oligonucleotides initially used were OL-D (complementary to bases +81 to +105) and OL-A (+36 to +74), both within exon 1 (Fig 2). Extension of OL-D and OL-A indicated that the major start site in liver, also used in
kidney, is 105 bp 5' of the start of translation in liver, in agreement with previous findings (6) (Fig 3A). An additional larger extension product was seen with kidney RNA. Extension of OL-C (-88 to -68) (Fig 3B) indicated this kidney mRNA initiates at -264. RNase protection assays confirmed these start sites. Using riboprobe 1 (-521 to +74) the predicted 74 bp protected fragment resulting from hybridization to mRNA initiating from the major start site (+1) was observed in liver and kidney. An additional longer protected fragment was detected in kidney confirming the -264 start site (Fig 3C). Analysis with riboprobe 2 (-88 to +310), entirely covering exon 1 (Fig 2), confirmed the +1 start site in both tissues and a 5'-extended mRNA only in kidney (not shown).

The cloning of a variant 11B-OHSD cDNA from a rat kidney library was reported while we were analyzing our genomic clone (8). This cDNA lacks the 5' region encoding the first 26 amino acids. Since our sequence analysis revealed this deleted region represented exon 1 we tested the possibility that the corresponding mRNA might initiate in intron A. RNase protection using riboprobe 3 (+758 to +1389; Fig 2) demonstrated a protected fragment (135 bp) corresponding to exon 2 in liver and kidney. An additional 196 bp protected fragment was observed in kidney suggesting a transcript containing 64 bp of intron A (Fig. 4A). This mRNA was confirmed by primer extension using OL-E (+934 to +954) complementary to exon 2 (Fig 2). OL-E extended to give 2 major products in kidney, (i) 204 bp corresponding to the +1 start site, and (ii) 89 bp corresponding to initiation at +808, yielding an mRNA with a 65 bp 5' extended exon 2 (Fig. 4B).

Northern analysis of 11B-OHSD transcripts.

Northern analysis demonstrated the presence of transcripts corresponding to the 3 transcription start sites. Hybridization with a full-length cDNA showed a single major 1700 nt transcript in liver and 3 mRNA species of 1500 nt, 1600 nt and 1900 nt in kidney (Fig. 5). OL-A (complementary to exon 1, +36 to +74; Fig 2) hybridized to kidney mRNAs identical in size to the 1900 nt and 1600 nt species seen with full-length cDNA, and with liver RNA gave an identical pattern of hybridization to cDNA. OL-B (intron A sequence, +831 to +870) hybridized to a single species in kidney identical in size to the 1500 nt species; a weak band of this size is apparent in liver. OL-C (-88 to -68) hybridized to a 1900 nt species in kidney; no signal was detected in liver. In summary, the 1900 nt species corresponds to mRNA initiated at -264 and the 1500 nt species to mRNA initiated at +808. The 1600 nt species in kidney most probably corresponds to mRNA initiated at +1; the corresponding liver species is 1700 nt.

Discussion

We have isolated a rat 11B-OHSD genomic clone and determined the structure of its 5' flanking region. This gene generates at least 3 distinct mRNA species by tissue-specific differential utilization of promoters. One of these transcripts is predicted to encode a truncated 11B-OHSD protein. We found 3 separate sites of initiation of transcription in kidney, whereas in liver only one site predominates. The major liver transcript corresponds to the previously cloned rat liver and human testis 11B-OHSD cDNAs (6, 9). The Cap site sequence (TGATGTC*AACAATTGAGA) is identical to that found in human liver from the homologous human 11B-OHSD gene (9). The same promoter (+1) is also used in kidney, although the mRNA species produced is 100 nt shorter than that from liver. Tissue-specific differences in polyA tail length may explain this difference (7). We could not detect the 1700 nt species previously reported in renal papilla (7).

The renal mRNA species initiating at -264 (1900 nt) and +1 (1600 nt) are likely to encode identical proteins since the extra 5' sequence has no in-frame ATG. Heterogeneity of 5' untranslated region mRNA length has been described in other steroid dehydrogenases and may be associated with differences in mRNA stability and translatability (10,11). Kidney also exploits a promoter in intron A, initiating 65 nt upstream of exon 2, which has two possible TATA sequences 31 (TTTATA) and 62 (TTATGAAATGAA) nt 5' of the Cap site and a element resembling a CCAAT box (GCCAAA) 87 bp upstream. This mRNA is predicted to encode a protein lacking the first 26 hydrophobic amino acids of 'liver-type' 11B-OHSD which may alter the enzyme's intracellular localization or post-translational processing, particularly glycosylation, which potently influences 11B-OHSD kinetics and reaction direction (12). A cDNA apparently corresponding to this transcript has been recently cloned (8) and, after submission of our manuscript, its 5' sequence was published (13). Our intron A sequence and the 5' untranslated end of the cDNA clone 11-
HSD1B (13) differ. Since riboprobes spanning +758 to +1389 were protected from RNase (Fig 4A) the sequence presented here is likely to be correct.

Though the relationship between the putative DNA regulatory elements and the 3 transcripts remains to be defined, some idea of possible regulatory and tissue-specific transcriptional mechanisms can be inferred. The CCAAT element is favorably positioned to regulate the +1 promoter, whose predominant use in liver may reflect the high hepatic content of CCAAT-binding factors (14) implicated in the regulation of genes related to energy metabolism (a function of glucocorticoids). The GC box and the CACCC elements are well located to regulate both 1900 and 1600 nt transcripts. CACCC sequences apparently bind a transcription factor involved cooperatively with GR (15); there are 2 putative GREs within the transcribed region and glucocorticoids induce 11B-OHSD activity and mRNA expression in some tissues (16, 17, Moisân et al, unpublished data). Finally, 3 promoters permit independent transcriptional regulation of each, producing greater flexibility of control. Thus, tissue-specific and ontogenetic regulation of 11B-OHSD expression and activity (18) may reflect the different array of transcription factors present. Whether additional genes also encode isoforms of this crucial enzyme remain to be determined.

Materials and methods
Isolation of genomic clones and sequence analysis

Approximately 10^6 plaques of a Sprague Dawley rat genomic library, cloned into λ DASH™II (Stratagene #945501), were screened by hybridization with a 5′ EcoRI-EcoRV fragment of pCI1DH-1 (6) labeled with 32P-dCTP by random priming according to the manufacturer's protocol (Boehringer Mannheim, UK). DNA from positive clones was analyzed by restriction endonucleases; suitable fragments were subcloned into pBluescript SK (Stratagene) and DNA sequences determined by the dideoxy nucleotide chain termination method using a Sequenase (version 1) kit (United States Biochemical).

Sequences of oligonucleotides used in this study were as follows:

OL-A=5'AGCTCTGTAGGACACACAAAAGAAA ACCTGACAGCTCTTC 3'

OL-B=5'GGAAGCAGAAGGAATGTGAC TCCAAAGCTTTGCTCT3'
OL-C=5' GATGATCTTGGGTGGGATCC
OL-D=5' AACTGCGGTCACAGGACCTGGGC 3'
OL-E=5'CACCTTTCCTCCCTGGGACAT3'

Primer extension and RNAse protection analyses

Oligonucleotides were labeled with 32P-γ-[ATP] and T4 polynucleotide kinase to 1-2×10^9 cpm/μg and 10^6 cpm hybridized to RNA samples (50 μg) at 60°C for 1 h in 100 mM KCl, 10 mM MgCl2, 25 mM Tris-HCl, pH 8.5. Primer extension reactions were performed in 30 mM KCl, 8 mM MgCl2, 50 mM Tris-HCl, 500 μM each dNTP, 50 μg/ml actinomycin D, 20 units of RNase A (Promega) and 50 units of AMV reverse transcriptase at 42°C for 30 min. After phenol-chloroform extraction and ethanol precipitation, the reaction product was analyzed on 6% polyacrylamide gels in comparison with a sequencing reaction.

For RNAse protection assays, 32P[U]TP-labeled antisense RNA probes were synthesized using T7 or T3 RNA polymerase. Templates were degraded using DNase I. 5×10^5 cpm of RNA probe was hybridized to RNA samples (25-50 μg) overnight at 46°C in 80% formamide, 0.4 M NaCl, 40 mM Pipes pH 6.7 and 1 mM EDTA. Non-hybridized RNA was degraded with RNases A (40 μg/ml) and T1 (2 μg/ml) for 1 h at 30°C. Following proteinase K treatment and phenol-chloroform extraction, the samples were ethanol precipitated and analyzed by 6% PAGE, as above.

Northern analysis

Total RNA was fractionated on 1.2% agarose-formaldehyde gels and transferred to Hybond N+ (Amersham International). Oligonucleotides (20 ng) were labeled with [32P]-γ-dCTP and terminal deoxynucleotidyl transferase to 2-5×10^9 cpm/μg and added to hybridization buffer (1xSSPE, 2x Denhardt's, 1% nonfat dry milk, 10% dextran sulfate, 2% SDS, 200 μg/ml herring testis DNA, 200 μg/ml polyadenyl acid). Hybridization was performed at 60°C for oligonucleotides OL-A and OL-B, 50°C for OL-C, and final washes in 0.2xSSC, 0.5% SDS at 50°C for 15 min.
Full-length 11β-OHSD cDNA (1265 bp) was labeled to 1x10^9 cpm/µg with [32P]-α-dCTP and hybridized at 50°C in 50% formamide, 5x Denhardt's solution, 5xSSC, 0.5% SDS; final washes were in 0.1xSSC, 0.5% SDS at 60°C, as described (19).

References
FIGURES

Figure 1:
Nucleotide sequence of the rat 11β-OHSD 5'-flanking region and the first 2 exons. Vertical arrows represent starts of transcription. All numbers relate to the major transcription start site in liver (+1). Putative transcription factor binding sites are underlined and putative hormone responsive elements half sites are identified by horizontal arrows.

Figure 2:
Schematic representation of sequenced region and of probes used in the study. Exons are represented by heavy bars. Vertical arrows represent restriction sites and horizontal arrows indicate direction of oligonucleotides used in primer extension and Northern analyses. Riboprobes used in RNase protection assays are shown.

Figure 3:
(A) Primer extension analysis using OL-D (left) and OL-A (right). L= liver total RNA, K= kidney total RNA, Y= yeast tRNA. 50 µg aliquots were run per lane in parallel with a sequencing reaction. (B) similar experiment using OL-C. Autoradiograms were exposed for a week at -70°C. (C) RNase protection assay using riboprobe 1 generated from the 5' most SacI fragment (-521 to +74). Kt= whole kidney RNA, Km= kidney medulla RNA; 25 µg aliquots were run per lane and the autoradiogram exposed for 20 h.

Figure 4:
(A) RNase protection assay using riboprobe 3 generated from the AflIII-PstI fragment (+758 to +1389). L= liver total RNA, K= kidney total RNA, Y= yeast tRNA; 50 µg per lane run with a sequencing reaction. Autoradiogram was exposed for 60h.(B) Primer extension analysis using OL-E; 50 µg/lane. Autoradiogram was exposed for 1 week.

Figure 5:
Autoradiograms of Northern blots of liver (L) and kidney (K) RNA. The probes used in each hybridization are indicated at the top of the panel. 25 µg of RNA were loaded per lane. After OL-A hybridization, RNA was stripped by boiling and reprobed with the full-length cDNA. Hybridization with OL-B and OL-C were performed on RNA run on adjacent lanes of the same gel used for OL-A/cDNA so that all 4 autoradiograms could be aligned. Autoradiograms were exposed 20 h for cDNA, 2 days for OL-A, and 4 days for OL-B and OL-C. The numbers on the right indicate the approximative sizes (nt) of the various species.

Acknowledgements
We thank Drs Carl Monder and Perrin White for generously providing the full-length rat 11β-OHSD cDNA clone, Dr Karen Chapman for helpful criticism of the manuscript and Drs John Mullins, Robert Hill, Anthony Brooks and Ian Jackson for much useful advice and discussion. This work was supported by grants ( to JRS & CRWE) from the Wellcome Trust/Royal Society of Edinburgh, the Scottish Hospital Endowments Research Trust and the Sir Stanley and Lady Davidson Research Fund.