STEROID HYDROXYLASES IN THE RAT BRAIN: EVIDENCE OF GENE EXPRESSION AND ENZYME ACTIVITY

by

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Abstract

Aldosterone and corticosterone are the main mineralocorticoid and glucocorticoid products of the rat adrenal cortex. They share a common biosynthetic pathway until the final stage where the substrate deoxycorticosterone (DOC) is converted to either aldosterone or corticosterone by the actions of the enzymes aldosterone synthase (CYP11B2) or 11β-hydroxylase (CYP11B1) respectively. These enzymes are the products of highly homologous genes whose expression, along with that of other components of the corticosteroidogenic pathway, was long thought to be confined to the adrenal cortex. In recent years, however, due to the advent of more sensitive molecular biological techniques, evidence has accumulated to suggest that this is not the case and that certain extra-adrenal tissues may be capable of autonomous aldosterone and corticosterone production.

In chapter 3, RT-PCR was used to detect transcripts from the *CYP11B1* and *CYP11B2* genes in a number of different tissue types. The transcription of genes encoding other components of corticosteroidogenesis, such as the side-chain cleavage enzyme and adrenodoxin, was also examined. Of the tissues examined, only brain tissue was found to contain transcripts from all these genes.

Chapter 4 describes attempts using an SDS-polyacrylamide gel electrophoresis and immunoblotting technique to show that these transcripts are translated within brain tissue to result in the enzymes themselves. This utilised two monoclonal antibodies raised against non-homologous regions of the rat aldosterone synthase and 11β-hydroxylase enzymes. However, no evidence of translation in extra-adrenal tissue was obtained using this technique, despite the identification of a positive band corresponding to 11β-hydroxylase in adrenal tissue fractions.

In chapter 5, the same antibodies were used to detect aldosterone synthase and 11β-hydroxylase within extra-adrenal tissues by immunostaining methods. Using adrenal tissue sections, the two antibodies were found to be highly specific for their respective antigens and apparently free of the cross-reactivity which might be expected between such highly homologous enzymes. Positive staining was also produced in brain tissue, where the enzymes were found to colocalise within the hippocampus and the cerebellum. Although previously published work had established that steroidogenesis occurs in the glial cells of the central nervous system, strong positive staining within the Purkinje cells of the cerebellum in this study presented strong evidence for neuronal expression. Rigorous control experiments confirmed the specificity of these results. Attempts to produce specific staining within heart tissue were unsuccessful.

Primary cultures of rat fetal hippocampal neurons permitted the study of *CYP11B1* and *CYP11B2* expression within a homogenous neuronal cell type. This work is described in chapter 6. *CYP11B1* and *CYP11B2* transcription and translation within these neuronal cells was demonstrated by the techniques used previously. In addition, the substrate DOC was incubated with the cells for 24-hour periods. Upon extraction and partial purification, the aldosterone and corticosterone content of the cell medium was measured by radioimmunoassay and shown to be significantly higher than that of control medium incubated in the absence of DOC.

In summary, this thesis provides compelling evidence of the production of aldosterone and corticosterone within the rat central nervous system. It also includes detailed information concerning the distribution of the aldosterone synthase and 11β-hydroxylase enzymes that produce these steroids. Finally, it demonstrates that

neuronal cells cultured from the fetal rat hippocampus are capable of converting DOC into their respective products.

These findings could have profound implications for the diverse physiological processes that are regulated by corticosterone and aldosterone.

Declaration

I declare that, unless specified otherwise in the text, the work presented in this thesis is my own.

Scott M. MacKenzie

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Dedication

I dedicate this thesis to my parents William and Beryl with thanks for their constant support.

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I am extremely grateful to my supervisors Dr Eleanor Davies and Professor John M.C. Connell for their invaluable help and advice throughout the course of this project. In addition, I thank Dr Catherine J. Clark for sharing her knowledge of immunoblotting and immunostaining techniques, Professor Robert Fraser and Miss Mary Ingram for performing the analysis of cell media described in chapter 6, and Dr Wai Kwong Lee for solving the numerous computing problems I encountered. I am also indebted to Professor Fraser, Dr Davies, Dr Clark and Professor Connell for the suggestions, proofreading and editing which shaped this thesis into its present form.

I thank the University of Glasgow for providing me with funding through its CMPU scholarship and Professor John L. Reid for making available to me the facilities within the Department of Medicine and Therapeutics.

The antibodies used for the immunoblotting and immunostaining procedures were a kind gift from Professor Celso E. Gómez-Sánchez of the Department of Internal Medicine, University of Missouri-Columbia, MO. Mr Niall Whyte of the Western Infirmary's Department of Pathology prepared the tissue sections for subsequent immunostaining. Dr Margaret Lai of the Molecular Medicine Centre at Edinburgh's Western General Hospital provided the primary cultures of fetal rat hippocampal neurons which made the experiments in chapter 6 possible. Professor Jonathan R. Seckl, also of that department, made valuable suggestions regarding experimental design. My thanks to them all.

Publications

MacKenzie SM, Clark CJ, Fraser R, Gómez-Sánchez CE, Connell JMC, Davies E. (2000) Expression of 11β-hydroxylase and aldosterone synthase genes in the rat brain. *Journal of Molecular Endocrinology* **24**, 321-328.

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Abbreviations

3β-HSD	3β-Hydroxysteroid dehydrogenase
5HT	Serotonin (5-hydroxytryptamine)
11β-HSD	11β-Hydroxysteroid dehydrogenase
ABC	Avidin-biotin complex
ACE	Angiotensin-I converting enzyme
ACTH	Adrenocorticotrophic hormone (corticotrophin)
ADX	Adrenalectomised
AHP	Afterhyperpolarisation
AngII	Angiotensin II
AP-1	Activator protein 1
ATF-1	Activating transcription factor 1
AVP	Arginine vasopressin
CamK kinase	Ca^{2+}/calmodulin-dependent protein kinase
CAMP	Cyclic AMP
CBP	CREB binding protein
CNS	Central nervous system
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor I
CRAC	Calcium release-activated calcium channels
CRE	cAMP-response element
CREB	cAMP-responsive element binding protein
CRH	Corticotrophin-releasing hormone
CSA	Catalysed signal amplification
CYP11A1	Gene encoding side-chain cleavage enzyme

CYP11A1	Side-chain cleavage enzyme
CYP11B1	Gene encoding 11β-hydroxylase
CYP11B1	11β-Hydroxylase
CYP11B2	Gene encoding aldosterone synthase
CYP11B2	Aldosterone synthase
DA	Dopamine
DAG	1,2-Diacylglycerol
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DOC	11-Deoxycorticosterone
ECL	Enhanced chemiluminescence
EPSP	Excitatory postsynaptic potential
G protein	Guanine nucleotide-binding regulatory protein
GABA	γ-Aminobutyric acid
GAPDH	Glyceraldehyde phosphate dehydrogenase
GCMS	Gas chromatography mass spectrometry
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal axis
HRE	Hormone response element
icv	Intracerebroventricular
IP3	Inositol 1,4,5-triphosphate
IPSP	Inhibitory postsynaptic potential
LDL	Low density lipoprotein
LTP	Long-term potentiation
LVH	Left ventricular hypertrophy

MR	Mineralocorticoid receptor
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate
P45011β	11β-Hydroxylase
P450aldo	Aldosterone synthase
P450c17	17α-Hydroxylase
P450c21	21-Hydroxylase
P450scc	Side-chain cleavage enzyme
PBS	Phosphate-buffered saline
PKA	cAMP-dependent protein kinase A
POMC	Pro-opiomelanocortin
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF-1	Steroidogenic factor 1
SHRSP	Stroke-prone spontaneously hypertensive rats
StAR	Steroidogenic acute regulatory protein
TBS	Tris-buffered saline
TBST	Tris-buffered saline/Tween 20
VDCC	Voltage-dependent calcium channel
VDKC	Voltage-dependent potassium channel
VDSC	Voltage-dependent sodium channel
VIP	Vasoactive intestinal peptide
VSMC	Vascular smooth muscle cell

Chapter 1

Corticosteroid Production In Adrenal And Extra-Adrenal Tissues: A Review

1.1 Introduction

The steroid hormones are among the most important primary regulators of metabolism. They act principally to switch the expression of genes on or off, thus regulating the synthesis of proteins, although other mechanisms of control have now been discovered. The estrogens, androgens and the corticosteroids were long assumed to be the exclusive products of specific endocrine glands from which they were secreted into the circulation to act at a distance on target organs. Exciting recent evidence now strongly suggests that they may also be produced and act locally in target organs. This raises the possibility that their synthesis may be controlled differently at these sites and that their effects may be qualitatively different. The implications for explaining disease processes and for treating them are clearly immense.

The corticosteroids are products of the adrenal cortex. The following review considers the evidence that they are synthesised and act locally in other organs, particularly the brain. In order to do this, a brief preliminary survey of their structure, synthesis and control in the adrenal cortex and their action in target organs is presented.

1.2 Corticosteroid Biosynthesis

1.2.1 The corticosteroids

The common and systematic names of various steroids are listed in **table 1.2**. The structures of the more important steroids are shown in **figure 1.2a**. The corticosteroid hormones are separated into mineralocorticoids (aldosterone, DOC) and glucocorticoids (cortisol, corticosterone). The mineralocorticoids cause the distal renal tubules and other ion-transporting epithelia, such as the large bowel and salivary gland, to retain sodium in exchange for the loss of K^+ and H^+. The glucocorticoids assist in the control of carbohydrate metabolism but they also have a significant effect on blood pressure regulation (see **section 1.5.4**). The production of the main mineralocorticoid, aldosterone, is confined to the adrenal zona glomerulosa. The zona fasciculata-reticularis is the source of the main glucocorticoid which in man is cortisol and in the rat is corticosterone.

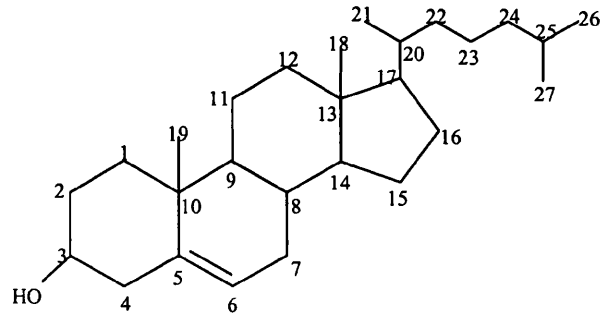
1.2.2 Cholesterol translocation

Before steroidogenesis can proceed, cholesterol must first be delivered to the mitochondrion. Cholesterol may be synthesised from acetate *de novo* within the adrenal cortex, although the majority comes from cholesterol esters stored within intracellular pools. These pools are created by the endocytosis of circulatory lipoproteins, in particular low density lipoprotein (LDL) which, once internalised, release cholesterol esters into the cell. High density lipoprotein (HDL) is a primary source of cholesterol for steroidogenesis in rodents (Simpson *et al.* 1989). It is from such sources that the lipid droplets visible within the cells of the zona fasciculata-reticularis are created, and these must be mobilised for steroidogenesis. This is done

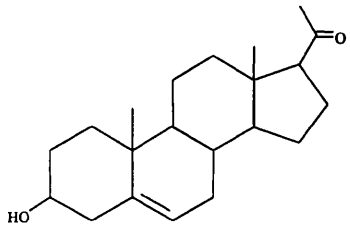
by the enzyme cholesterol ester desmolase, which hydrolyses cholesterol esters in response to hormonal stimulation, mobilising free cholesterol for steroid synthesis.

Common Name	Systematic Name
Cholesterol	Cholest-5-en-3β-ol
Pregnenolone	3β-Hydroxy-pregn-5-en-20-one
17-Hydroxypregnenolone	3β,17α-Dihydroxy-pregn-5-en-20-one
Dehydroepiandrosterone (DHEA)	3β-Hydroxyandrost-5-en-17-one
Progesterone	Pregn-4-ene-3,20-dione
17-Hydroxyprogesterone	17α-Hydroxypregn-4-ene-3,20-dione
11-Deoxycorticosterone (DOC)	21-Hydroxypregn-4-ene-3,20-dione
11-Deoxycortisol (S)	17α,21-Dihydroxypregn-4-ene-3,20-dione
Cortisol (F)	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione
Cortisone (E)	17α,21-Dihydroxypregn-4-ene-3,11,20-trione
Corticosterone (B)	11β,21-Dihydroxypregn-4-ene-3,20-dione
11-Dehydrocorticosterone	21-Hydroxypregn-4-ene-3,11,20-trione
18-Hydroxycorticosterone (18-OHB)	11β,18,21-Trihydroxy-4-ene-3,20-dione
18-Hydroxydeoxycorticosterone (18-OHDOC)	18,21-Dihydroxy-4-ene-3,20-dione
Aldosterone (Aldo)	11β,21-Dihydroxy-3,20-doxopregn-4-en-18-al (as 11→18-hemiacetal)

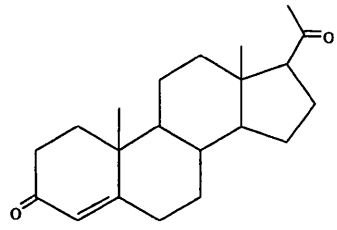
Table 1.2 Common and systematic names of adrenal steroids.



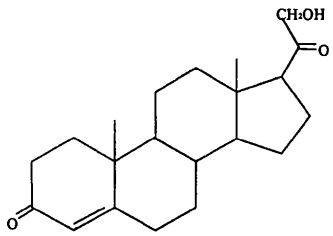
Cholesterol



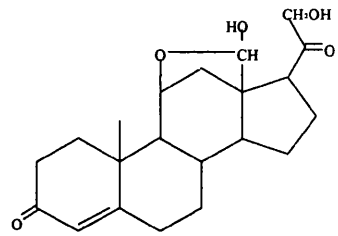
Pregnenolone



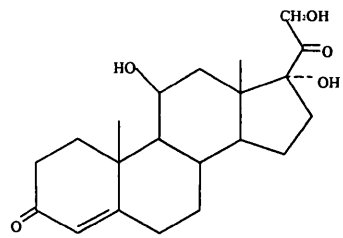
Progesterone



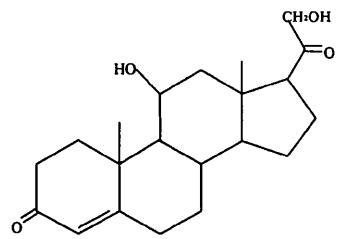
11-Deoxycorticosterone
(DOC)



Aldosterone



Cortisol (F)



Corticosterone (B)

Figure 1.2a Steroid structures and common names.

The rate-limiting step of steroidogenesis is the delivery of mobilised cholesterol to the side-chain cleavage enzyme (see **section 1.2.4**) on the inner mitochondrial membrane. A number of translocation systems have been identified, including sterol carrier protein 2 and the peripheral benzodiazepine receptor (PBR), but the most important involves the steroidogenic acute regulatory protein (StAR).

StAR is essential for efficient adrenal and gonadal steroidogenesis. Congenital lipoid adrenal hyperplasia (lipoid CAH), a condition especially common in Japanese and Korean populations, is caused by mutation of the StAR gene and results in the impairment or lethal absence of steroidogenesis (Miller, 1997). Targeted disruption of the StAR gene in rats results in a similar phenotype. Although the mechanism of StAR-mediated translocation is not fully understood, it appears to act on the outside of the mitochondrion. Despite having a mitochondrial targeting sequence, cleavage of this sequence from the protein does not affect steroidogenesis (Arakane *et al.* 1998). StAR expression can be controlled in order to regulate steroidogenesis (see **section 1.4**).

1.2.3 Cytochrome P450 enzymes

Corticosteroid hormones are synthesised from cholesterol by a series of enzyme-catalysed reactions, progressively shortening the side chain and adding substituents in a locus- and stereo-orientation-specific manner. These reactions occur in the mitochondria and the microsomes of the steroidogenic tissues, necessitating translocation between these two compartments (Miller, 1988).

Two types of enzyme are involved: a hydroxysteroid dehydrogenase enzyme, bound to the endoplasmic reticulum, and the mixed function oxidases, or cytochrome P450 enzymes with which this work is principally concerned. They possess haem

prosthetic groups and their apoprotein moieties all have molecular weights of around 50,000. This protein consists of well-defined helical and β-pleated sheet regions arranged in such a way as to leave a cavity through which the substrate may bind to the catalytic site at the centre of the molecule (von Wachenfeldt and Johnson, 1995). The widest sequence diversity is found within the substrate-binding regions of the cytochrome P450s, accounting for the great substrate specificity often demonstrated by these enzymes.

The overall reaction performed by the cytochrome P450s involves the enzyme's insertion of one atom from molecular oxygen into the substrate; the other atom combines with hydrogen to form water:

$$NADPH + H^+ + O_2 + RH \rightarrow R—OH + H_2O + NADP^+$$

The complex pathway by which this is accomplished by the cytochrome is described by Dawson (1988). The reducing power for the reaction is derived from the nicotinamide adenine dinucleotide phosphate (NADPH) + H^+ and is supplied to the cytochrome P450 enzymes through one of two electron transport systems, depending upon whether the cytochrome P450 in question is mitochondrial or microsomal. Mitochondrial systems (e.g. 11β-hydroxylase) require the non-haem iron protein, adrenodoxin, while microsomal systems (e.g. 21-hydroxylase) use NADPH cytochrome P450 reductase to transfer electrons from NADPH to the enzyme (Kominami *et al.* 1984). This is summarised in **figure 1.2b**.

1.2.4 Biosynthetic pathways

The sequence of reactions which converts cholesterol to corticosteroids in the human adrenal cortex is summarised in **figure 1.2c**. The sequence in the rat adrenal cortex, which lacks 17α-hydroxylase, is shown in **figure 1.2d**.

Side-chain cleavage enzyme (P450scc) This enzyme catalyses 20α-hydroxylation, 22-hydroxylation and the cleavage of the cholesterol side-chain between carbons 20 and 22 to yield isocaproic acid and pregnenolone. P450scc is the product of the *CYP11A1* gene. It is a multisubunit protein bound to the inner mitochondrial membrane, possessing a single active site in contact with the membrane. Its low V_{max} leads to an accumulation of reaction intermediates in the mitochondrion (Morisaki *et al.* 1980).

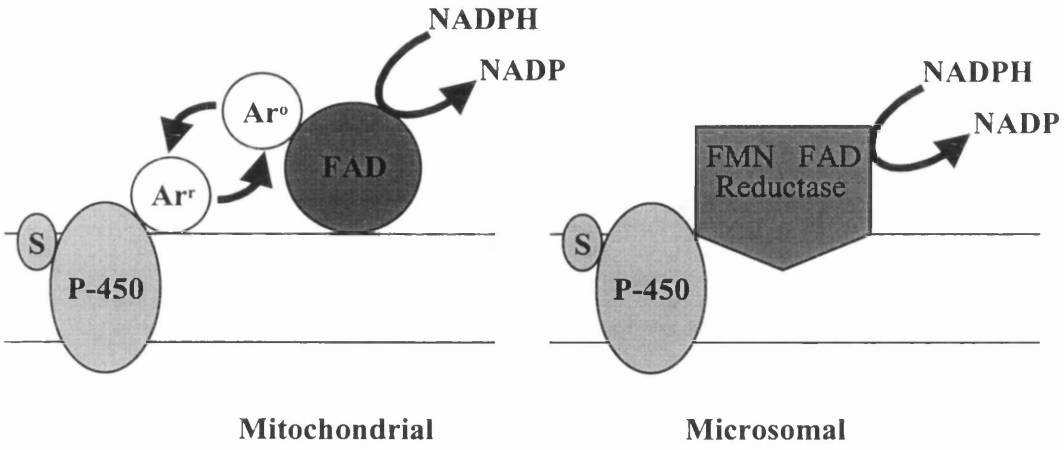


Figure 1.2b Electron transfer systems present in mitochondria and microsomes. In mitochondria, the FAD-containing adrenodoxin reductase binds adrenodoxin (Ar) and transfers to it electrons derived from NADPH. The reduced Ar forms a new complex with cytochrome P450 to which the steroid substrate (S) is bound. After reduction of P450, the oxidised Ar returns to the reductase. In microsomes, the FMN- and FAD-containing NADPH cytochrome P450 reductase tranfers electrons from NADPH to cytochrome P450 (Kominami *et al.* 1984).

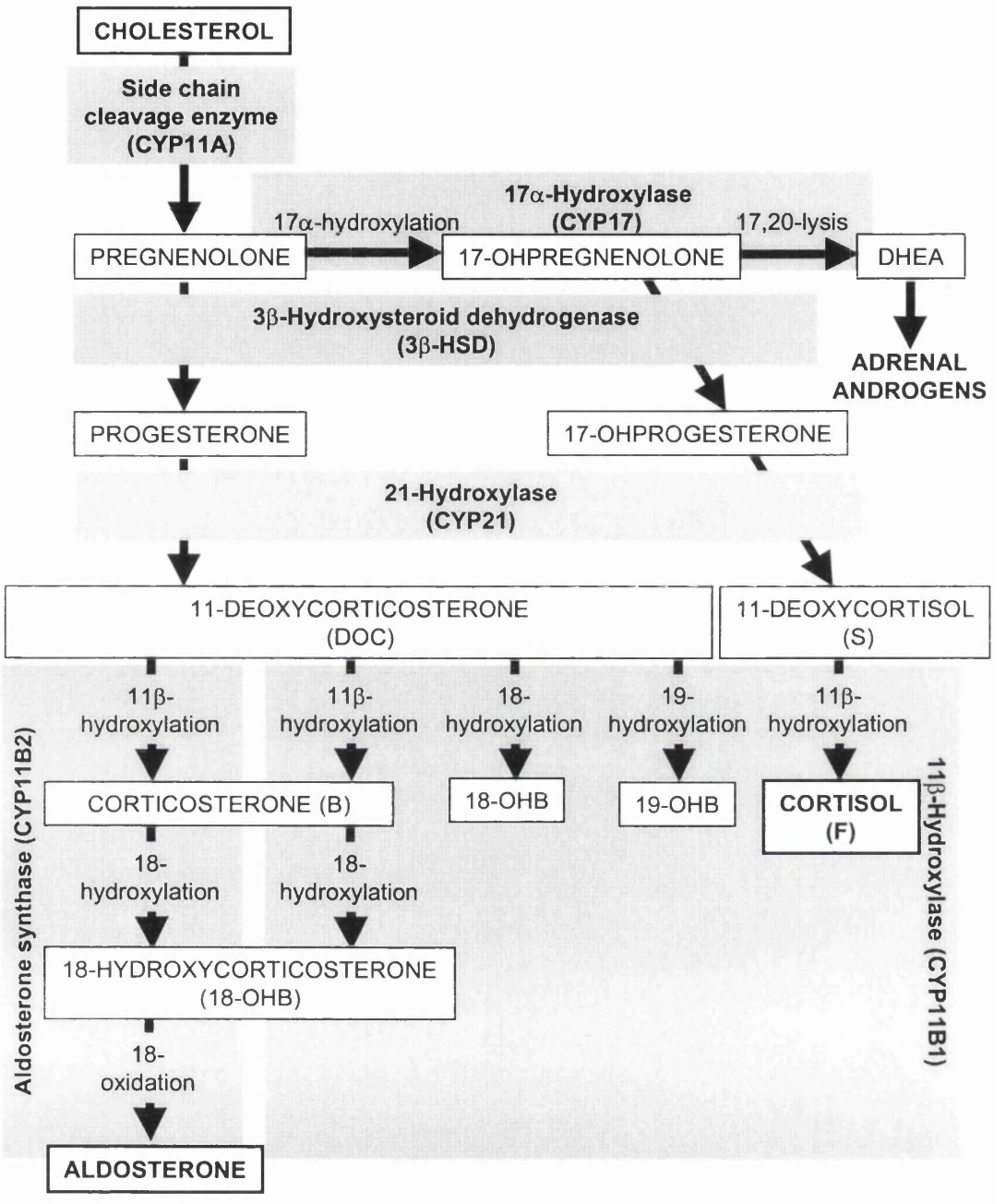


Figure 1.2c Corticosteroidogenic reactions in the human adrenal cortex.

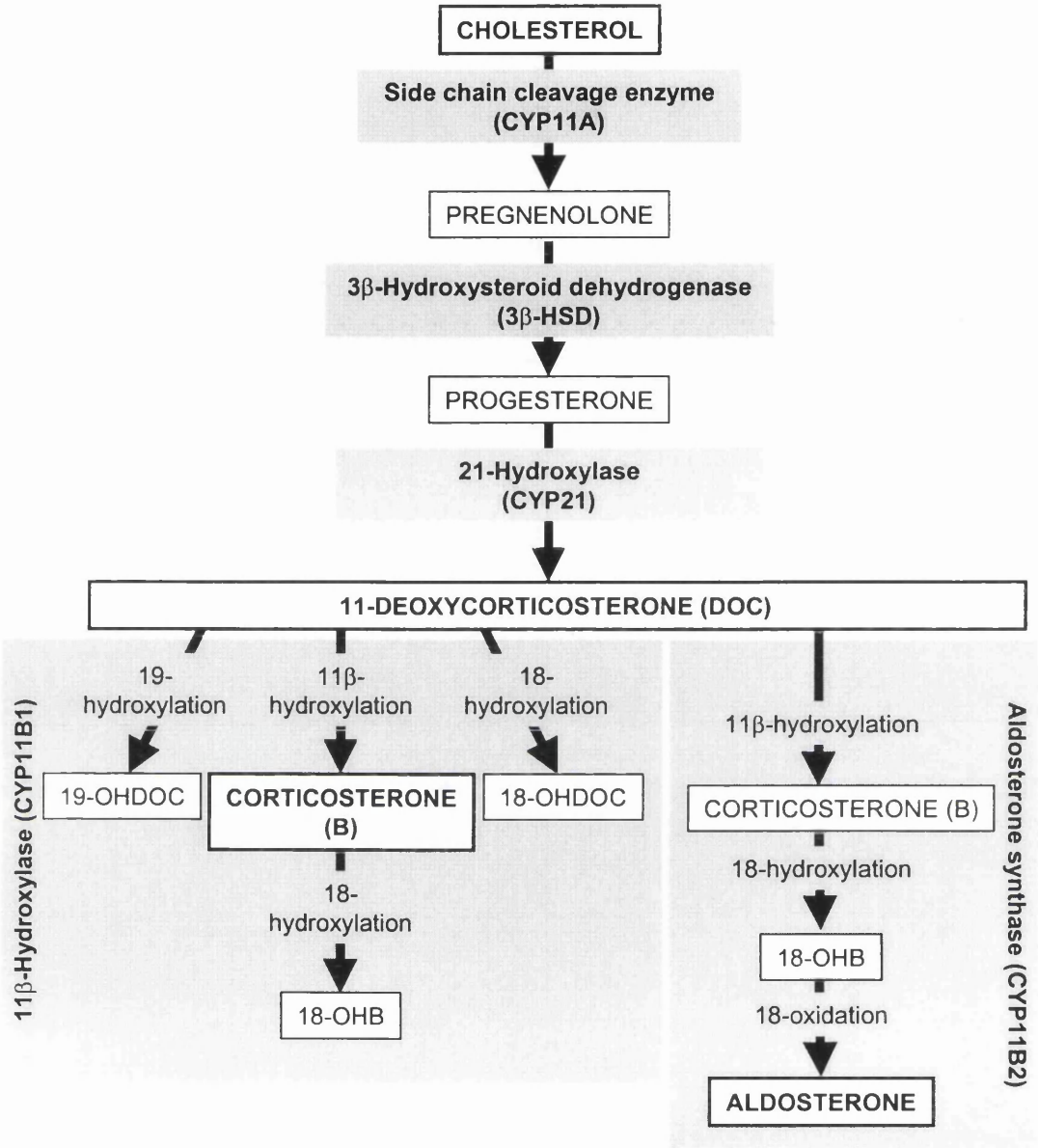


Figure 1.2d Corticosteroidogenic reactions in the rat adrenal cortex.

3β-Hydroxysteroid dehydrogenase/isomerase (3β-HSD) Pregnenolone must pass into the smooth endoplasmic reticulum to be converted to progesterone by 3β-HSD. It is not a cytochrome P450 enzyme and has several isoforms whose expression is tissue-specific (Zhao *et al.* 1991). The enzyme is also able to convert 17α-hydroxypregnenolone to 17α-hydroxyprogesterone. The enzyme does not need adrenodoxin or NADPH cytochrome P450 reductase in order to function although the presence of certain phospholipids is required.

17α-Hydroxylase (P450c17) Pregnenolone and progesterone may both undergo 17α-hydroxylation to form 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively. These reactions are performed by P450c17, a microsomal cytochrome P450 enzyme. 17α-Hydroxypregnenolone can then be converted to 17α-hydroxyprogesterone by 3β -HSD (see above). 17α-Hydroxypregnenolone and 17α-hydroxyprogesterone are precursors for glucocorticoids. Alternatively, they may be converted to adrenal androgens by P450c17's 17,20-lyase activity. The zona glomerulosa contains no 17α-hydroxylase activity.

21-Hydroxylase (P450c21) This microsomal enzyme mediates the conversion of progesterone to 11-deoxycorticosterone (DOC) and 17α-hydroxyprogesterone to 11-deoxycortisol (S). DOC must be transported back to the mitochondrion for the subsequent reactions.

11β-Hydroxylase (P45011β) The actions of P45011β and aldosterone synthase (P450aldo) were originally attributed to a single enzyme encoded by a single gene locus (Miller, 1987). This remains true of the bovine, porcine and frog P45011β (Ogishima *et al.* 1989; Nonaka *et al.* 1991). However, in man, the rat and the mouse, the final stages of glucocorticoid and mineralocorticoid synthesis are performed by

two different enzymes (Okamoto *et al.* 1995). P45011β catalyses the conversion of 11-deoxycortisol to cortisol and 11-deoxycorticosterone to corticosterone. It is a mitochondrial cytochrome P450 found only in the zona fasciculata-reticularis (Curnow *et al.* 1991; Ogishima *et al.* 1992). It is this that limits glucocorticoid production to the inner regions of the adrenal cortex. P45011β can also catalyse the 18- and 19-hydroxylations of DOC, resulting in 18-OHDOC (18-hydroxy-11-deoxycorticosterone) and 19-OHDOC (19-hydroxy-11-deoxycorticosterone), respectively as well as the conversion of corticosterone, to 18-OHB (18-hydroxycorticosterone) (Okamoto and Nonaka, 1992).

Aldosterone synthase (P450aldo) In human subjects and the rat and mouse, DOC produced in the zona glomerulosa undergoes three reactions, all catalysed by P450aldo, to become aldosterone. The reactions occur in the sequence:

1) 11β-hydroxylation to form corticosterone (as with P45011β in the rat zona fasciculata-reticularis).
2) 18-hydroxylation, producing 18-hydroxycorticosterone.
3) 18-oxidation, resulting in aldosterone.

It is probable that these reactions occur without intermediate release from the binding site. Structurally, aldosterone synthase is extremely similar to 11β-hydroxylase (see **section 1.3**) and its expression is restricted to the zona glomerulosa (Yabu *et al.* 1991; Vinson *et al.* 1995).

The next section will concentrate on the genes which encode aldosterone synthase and 11β-hydroxylase.

1.3 The *CYP11B* Genes

1.3.1 The human *CYP11B* genes

The human genome contains the genes *CYP11B1* and *CYP11B2* which encode the enzymes 11β-hydroxylase and aldosterone synthase, respectively (Chua *et al.* 1987; Mornet *et al.* 1989; Zhang and Miller, 1996). They are located in tandem on chromosome 8, approximately 40kb apart, with each gene spanning approximately 7kb of genomic DNA. Their exon nucleotide sequences are 95% identical, with homology falling to 90% in the introns. The resulting enzymes are composed of 503 amino acids which share 93% identity (Kawamoto *et al.* 1992).

1.3.2 The rat *CYP11B* genes

The rat possesses four *CYP11B* genes, all on chromosome 7 (Nomura *et al.* 1993; Mukai *et al.* 1993). With the exception of *CYP11B4*, where exon 3 and part of exon 4 have been deleted, all the genes comprise nine exons and eight introns (see **figure 1.3a**). *CYP11B4* is thought to be a pseudogene.

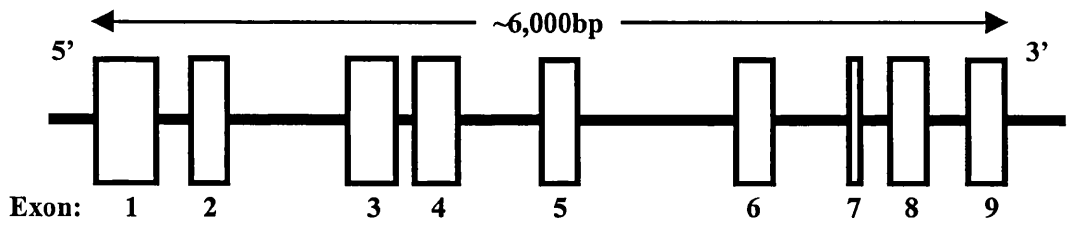


Figure 1.3a Exon-intron arrangements of the rat *CYP11B1* and *CYP11B2* genes (not to scale)(Mukai *et al.* 1993).

CYP11B3 is transcribed in the zona fasciculata-reticularis of the rat adrenal cortex for only a short period after birth. It can be detected in 18-day-old rats but not

in adult or 2-day-old rats (Mellon *et al.* 1995). During this period, levels of *CYP11B3* transcripts in the adrenal gland are higher than those of either *CYP11B1* or *CYP11B2*. *CYP11B3* transcription is regulated by adrenocorticotrophic hormone (ACTH) (see **section 1.4.1**) during this time. Mellon *et al.* (1995) demonstrated that the enzyme resulting from *CYP11B3*, P450c11B3, possesses 18- and 11-hydroxylase activities. *CYP11B3* transcript levels are known to be significantly higher in the 21-day-old spontaneously hypertensive rat (SHR) than in WKY animals of the same age (Malee and Wu, 1999).

CYP11B1 and *-B2* code for 11β-hydroxylase and aldosterone synthase (see **appendix 1**), although the rat forms of these enzymes are smaller than those in man, each containing 499 amino acids. Whereas *CYP11B1*, *-B3* and *-B4* all share at least 95% sequence identity, *CYP11B2* has only 90% identity to the others throughout its coding region (Mukai *et al.* 1993). However, extensive regions of difference occur only in exons 3, 4 and 5. At exon 5, *CYP11B1* has only 65% nucleotide homology to *CYP11B2*, resulting in just 52% identity at the amino acid level. This presumably accounts for the differences in the resulting enzymes' catalytic functions.

Studies of chimeric forms of the rat *CYP11B1* and *CYP11B2* genes have shown that exon 5 is essential for aldosterone synthase activity. If the hybrid form of these genes contains the first five exons of *CYP11B1* and the last four of *CYP11B2*, the expressed protein has 11β-hydroxylase, but not 18-hydroxylase or 18-oxidase, activity (Zhou *et al.* 1994). Similar studies of the human forms of the genes showed that exons 5 and 6 of *CYP11B2* are essential for 18-hydroxylase and 18-oxidase activities. The alteration of just two residues within these exons, from the *CYP11B1* to the *CYP11B2* forms, is sufficient to restore the 18-hydroxylase and 18-oxidase activities (Curnow *et al.* 1997).

1.3.3 The *CYP11B* gene promoters and transcription factors

The promoter or 5'-untranslated regions (5'-UTRs) of the rat *CYP11B1* and *CYP11B2* genes share the least homology of all, which is unsurprising given the great differences in the transcriptional regulation of the two genes. For the first 500 bases of the 5'-flanking regions, the *CYP11B1* and *CYP11B2* sequences are 50% homologous.

The promoter regions of all the *CYP11B* genes investigated so far share six *cis*-elements which appear to be well conserved between species. These regions, first identified in the bovine *CYP11B* gene, were originally named Ad1 to Ad6 (Morohashi *et al.* 1993). See **figure 1.3b**.

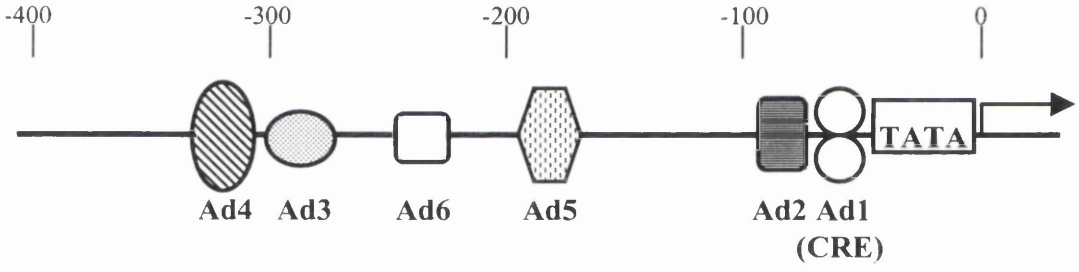


Figure 1.3b Schematic diagram of the bovine *CYP11B* promoter (Morohashi *et al.* 1993).

Despite the marked differences in the regulation of the bovine gene by comparison with the *CYP11B1* and *CYP11B2* genes of various other mammals, conserved sequences can be aligned with relative ease. Only Ad6 appears to have been poorly conserved, while the position of Ad5 has shifted upstream by about 100bp in the human, mouse and rat genes (Nomura *et al.* 1993). See **figure 1.3c**.

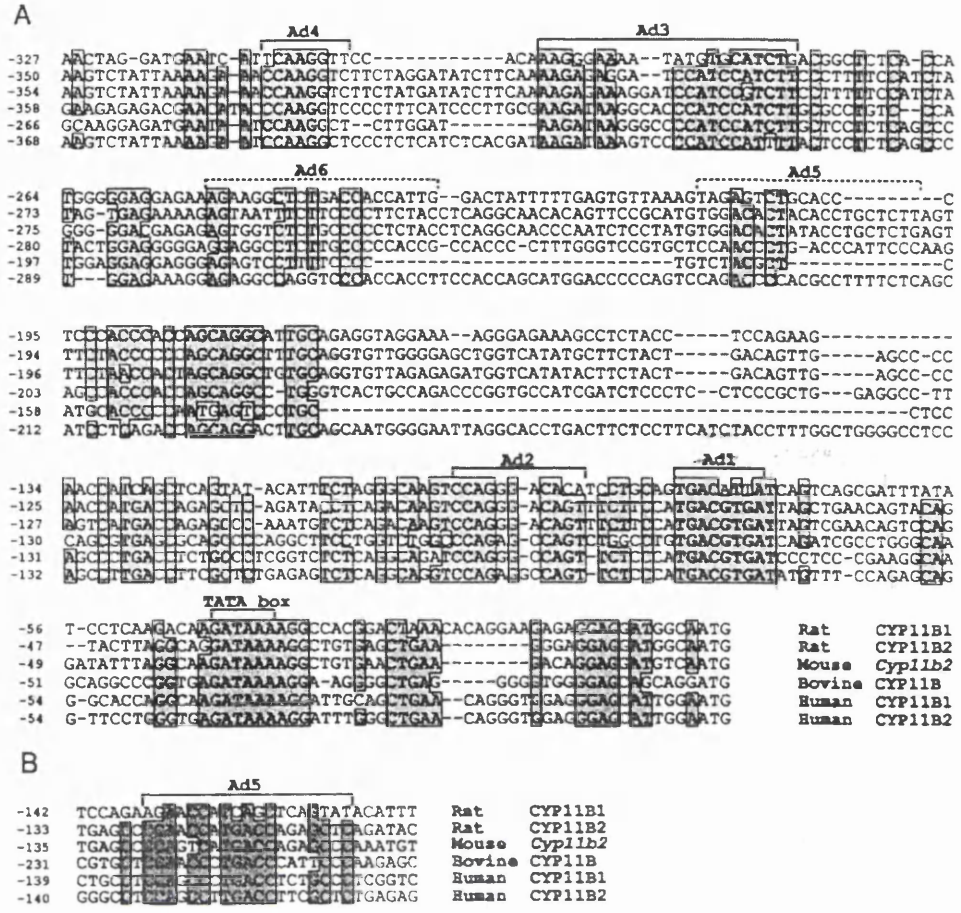


Figure 1.3c Comparison of the 5' upstream regions of the *CYP11B* genes. Nucleotides are numbered relative to the first translated codon and conserved bases are shaded. Panel A shows the best alignment for Ad1–4. Panel B shows the best alignment for Ad5 (Nomura *et al.* 1993).

Some of the factors that bind these and other promoter sites are as follows:

The cAMP-responsive element-binding protein (CREB) Ad1 is now known to be a cAMP-responsive element (CRE), which has the consensus sequence TGACGTCA. CREs are to be found in many other genes whose transcription is also dependent on cyclic-AMP levels. The cAMP cascade activation is summarised below, and in **figure 1.3d**.

1) An extracellular hormone binds to a transmembrane receptor, which is coupled to a guanine nucleotide-binding regulatory protein (G protein). G proteins exist in two forms: the stimulatory form (G_s) and the inhibitory form (G_i). Here, hormone binding to the G_s protein causes it to bind GTP instead of GDP. This change stimulates adenylate cyclase activity, increasing intracellular cAMP production and thus activating cAMP-dependent protein kinase (PKA).

2) The PKA catalytic subunit enters the nucleus and phosphorylates transcription factors of the CREB/ATF1 (CRE-binding protein/Activating transcription factor-1) family.

3) Phosphorylation permits CREB also to bind the CREB binding protein (CBP) (Groussin and Bertherat, 1998). CBP may be a component of certain RNA polymerase II holoenzyme complexes so, when phosphorylated, CREB's binding to the CRE DNA site, causes the transcriptional machinery to assemble at the start site (Montminy, 1997).

CREB phosphorylation can also be regulated by CaM kinases via the Ca^{2+} pathway (see **section 1.4.2**).

Steroidogenic factor 1 (SF-1) The Ad4 element confers tissue-specific cAMP responsiveness upon the bovine *CYP11B* promoter in the presence of the Ad1(CRE) region. A nuclear hormone receptor subsequently purified from steroidogenic tissue, the Ad4 binding protein (Ad4BP), has since adopted the additional name steroidogenic factor 1 (SF-1). It binds the Ad4 and, less strongly, Ad5 sequences in a number of steroidogenic promoters from a variety of species, including rat, human and mouse, recognising the sequences CCAAGGTC and AGGTCA (Morohashi *et al.* 1992). SF-1 has been regarded as an orphan nuclear receptor as its ligand was

unknown, although 25-, 26- and 27-hydroxycholesterols may fulfil this role (Lala *et al.* 1997; Bertherat, 1998).

It is clear that SF-1 is an important transcription factor, regulating the expression of genes for cytochrome P450s and StAR (Sandhoff *et al.* 1998). Little is known about its regulation.

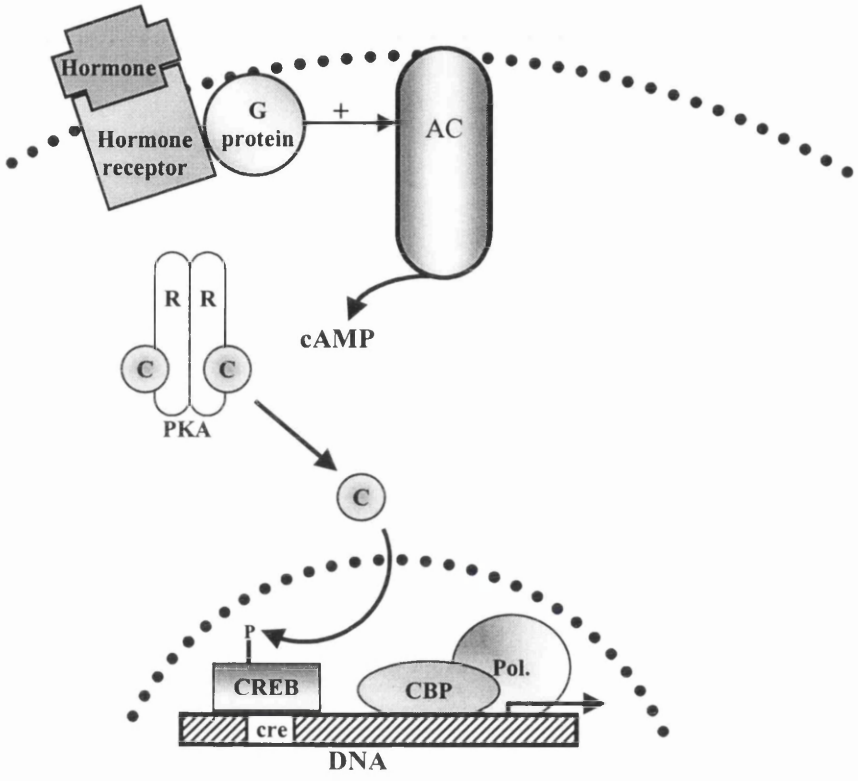


Figure 1.3d The cAMP signalling pathway (Groussin and Bertherat, 1998).

Chicken ovalbumin upstream promoter-transcription factor-I (COUP-TF) COUP-TF is an orphan receptor belonging to the steroid receptor superfamily. It was observed to be overexpressed in a nonfunctioning adrenocortical tumour, a DOC-producing adenoma and a tumour from a patient with Cushing's syndrome but its levels were low in aldosterone-producing and corticosterone-producing adenomas (Shibata *et al.* 1998). COUP-TF may therefore be of importance in the regulation of

steroidogenesis. The same study showed that *CYP17* mRNA was low in those tumours that overexpressed COUP-TF, and vice versa. The *CYP17* promoter has a COUP-TF binding site which overlaps an SF-1 site. Thus, the two transcription factors may compete for the same site, one acting as a repressor, the other as a promoter of gene transcription. The Ad5 region of the human *CYP11B2* promoter has recently been shown to contain sites for SF-1, COUP-TF and one other unidentified protein (Clyne *et al.* 1999).

Activator protein-1 (AP-1) The Jun family of proteins (Jun, JunB, JunD) and Fos family of proteins (c-Fos, FosB, Fra-1, Fra-2) can homo- or heterodimerise in a variety of combinations to form the transcription factor called AP-1 which is associated with the regulation of *CYP11B1* expression and appears to be responsible for 11β-hydroxylase's zonal distribution within the adrenal cortex (Mukai *et al.* 1995). The Jun/Fos composition of AP-1 may be important for the activation of *CYP11B1* transcription in response to ACTH (see **section 1.4.1**) (Mukai *et al.* 1998). An SF-1 site is immediately adjacent to this AP-1 element but its presence is not required for *CYP11B1* transcription. In the rat *CYP11B2* promoter, the SF-1 site is conserved but the AP-1 site is not. The mechanism by which AP-1 achieves tissue-specific expression of *CYP11B1* and the role of SF-1 at this site are still unclear.

Clearly, the factors which control gene expression must interact with these or as yet undiscovered sites in the promoter. While the control of extra-adrenal corticosteroidogenesis is not understood, control in the adrenal cortex has received intense scrutiny. This is the subject of the next section.

1.4 Regulation of corticosteroid secretion

The different physiological effects of the mineralocorticoids and the glucocorticoids requires that their secretion from the adrenal cortex is regulated separately. Detailed evidence from studies of the rat adrenal (Oertle and Müller, 1993) confirms that this is the case. Intra-adrenal storage of the corticosteroids is minimal. Control therefore operates at the level of corticosteroid synthesis and stimulation of zonal growth. The mechanisms include second messenger-activated protein activation which result in immediate – i.e. acute – stimulation of synthesis and secretion together with induction of the synthesis of new proteins such as growth factors. These latter proteins contribute to adrenocortical growth due to both hypertrophic and hyperplastic changes, a chronic response which increases overall adrenocortical capacity. A number of factors are concerned with this control. Since several recent reviews are available, these factors are briefly summarised with particular attention to their modes of interaction with the genome.

1.4.1 Adrenocorticotrophic hormone (ACTH)

Glucocorticoid secretion by the zona fasciculata-reticularis is regulated almost exclusively by the hypothalamic-pituitary-adrenal axis. See **figure 1.4a**.

Hypophysectomy reduces glucocorticoid levels to 5 percent of basal values within two hours. This fall can be prevented by the administration of ACTH (also known as corticotrophin), a peptide hormone secreted by the anterior pituitary (Vinson *et al.* 1992). ACTH's precursor molecule, pro-opiomelanocortin (POMC), is synthesized within the corticotroph cells of the anterior pituitary and gives rise to a number of biologically active peptides, including ACTH, melanocyte-stimulating hormone and β-endorphin. ACTH is a single peptide chain of 39 residues, released

from POMC by enzymatic cleavage. ACTH's activity resides within the highly-conserved first 24 amino acids (Vinson *et al.* 1992).

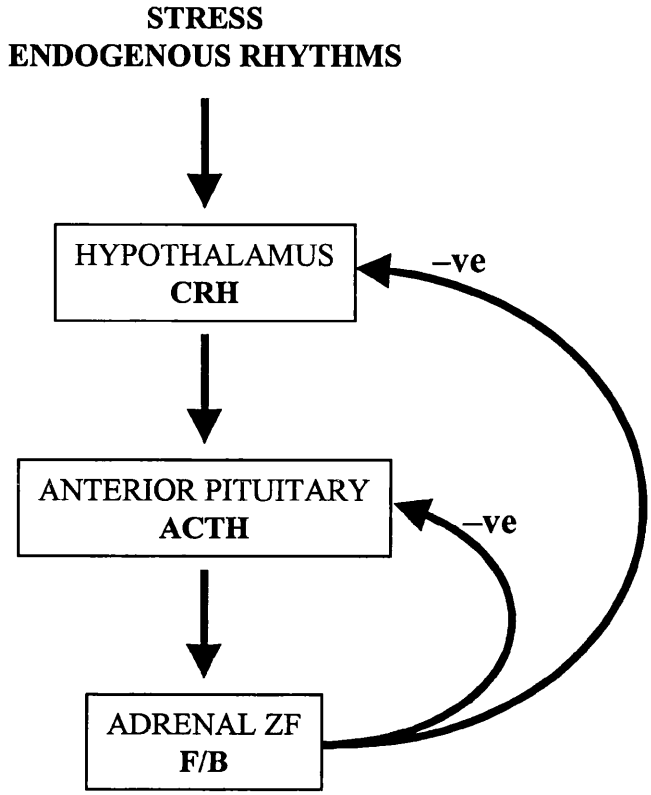


Figure 1.4a The hypothalamic-pituitary-adrenal (HPA) axis.

The secretion of ACTH from the anterior pituitary is pulsatile and obeys a circadian rhythm. In the rat, which is nocturnal, ACTH levels peak in the late afternoon. In human subjects, it peaks between 6–9a.m., with the lowest point being in the evening. ACTH secretion is also subject to stimulation by a variety of 'stresses' such as physical and emotional stress, hypoglycaemia, hypotension, electric shock and surgical procedures which induce hypothalamic neurons to secrete corticotrophin releasing hormone (CRH) from the hypothalamus (see **figure 1.4a**). Arginine vasopressin (AVP) and certain neurotransmitters, such as epinephrine, also

induce ACTH release. The increased corticosteroid production induced by ACTH acts on the hypothalamus and anterior pituitary to inhibit further CRH and ACTH secretion, respectively.

ACTH action on the zona fasciculata-reticularis ACTH's primary action is to increase glucocorticoid secretion from the zona fasciculata-reticularis. It upregulates the expression of adrenodoxin, adrenodoxin reductase, P450scc, P450c21, P450c17 and P45011β, although the extent to which expression is increased is highly variable (e.g. a 14-fold increase in P450c17 mRNA but a less than threefold increase in P450scc mRNA) (Waterman and Simpson, 1985; Raikhinstein and Hanukoglu, 1994; Engeland *et al.* 1997).

Each zona fasciculata cell possesses approximately 7200 high-affinity receptors, as well as some 630,000 low-affinity ACTH-binding sites (Gallo-Payet and Escher, 1985), but only a small receptor occupancy is required to obtain the maximal steroidogenic response. ACTH's most important second messenger, cAMP, exerts its effects via protein kinase A (PKA) phosphorylation of CREB (see **section 1.3.3** and **figure 1.3d**). It also phosphorylates cholesterol desmolase, increasing the levels of free cholesterol within the cell (Boyd *et al.* 1983). Human StAR protein possesses a PKA consensus phosphorylation site which, when phosphorylated, increases its host cell's steroidogenic activity (Arakane *et al.* 1997). cAMP also increases transcription of the StAR gene (Kiriakidou *et al.* 1996; Ariyoshi *et al.* 1998). This StAR response occurs in both the zona glomerulosa and zona fasciculata. The increase is apparently in two phases: an early stimulatory phase, presumably induced by modifications such as phosphorylation, and a more chronic effect which probably relies on induction of new StAR synthesis (LeHoux *et al.* 1998).

Evidence suggests that the ACTH-mediated stimulation of *CYP11B1* expression also involves AP-1 factors. An interesting mechanism has been suggested whereby ACTH stimulates changes in the Jun/Fos composition of AP-1 by inducing the expression of certain members of the Jun and Fos families (see **section 1.3.3**). Although these changes in composition do not affect AP-1's DNA-binding affinity, they may alter its transcriptional activation capabilities (Mukai *et al.* 1998).

The intracellular Ca^{2+} concentration is also important to ACTH-stimulated glucocorticoid production. If free calcium within the cell is chelated or the uptake of calcium into the cells is prevented, then ACTH stimulation of glucocorticoid production is impaired. An influx of extracellular Ca^{2+} is required by ACTH to activate adenylate cyclase (Davies *et al.* 1985).

ACTH action on the zona glomerulosa It is surprising that zona glomerulosa cells possess far more ACTH-binding sites than zona fasciculata cells (65,000 high-affinity and approximately 1,000,000 low-affinity sites per glomerulosa cell) (Gallo-Payet and Escher, 1985). Initially, some of the confusion over ACTH's actions in the outer region of the cortex was due to an inability to purify zona glomerulosa cells. Because of this, the rise in aldosterone observed upon acute ACTH infusion was attributed to contaminating zona fasciculata cells. However, in human subjects an acute increase in aldosterone secretion can be achieved by infusion of ACTH, with a maximum response achieved after 15 minutes, as is the case with angiotensin II and potassium (see below), although aldosterone output falls below that of controls within 24 hours (Quinn and Williams, 1988). This aldosterone escape response is observed in many species. In the sheep, cells of the zona glomerulosa adopt an intermediate glomerulosa/fasciculata phenotype after five days of ACTH administration. In the rat, *CYP11B2* transcripts rise and then fall over a 24 hour

period of ACTH infusion (Holland and Carr, 1993). It is therefore apparent that *CYP11B2* transcription is not regulated by cAMP-dependent mechanisms alone.

Vascular effects of ACTH ACTH causes a marked increase in blood flow through the adrenal gland, which has implications for the release of corticosteroids into the bloodstream. If this flow increase is prevented, the rise in corticosteroid secretion is significantly reduced. The response is observed even when steroidogenesis is inhibited, so it is unlikely that corticosteroids are responsible. Instead, it appears probable that it is caused by serotonin and histamine from mast cells within the adrenal capsule (Hinson *et al.* 1989; Vinson *et al.* 1992).

Chronic effects of ACTH The adrenal gland's weight and blood content increase as a result of chronic ACTH administration. These effects can be seen within 1–2 days. The response of individual steroidogenic genes to prolonged ACTH treatment is variable. Adrenodoxin, adrenodoxin reductase, P450scc, P450aldo and P450c17 gene transcripts fall back to their basal levels after 48 hours (Holland and Carr, 1993; Raikhinstein and Hanukoglu, 1994). Transcripts for 11β-hydroxylase, however, have been shown to remain elevated for at least four days, and may even be increasing throughout this period. This would be consistent with its unique regulatory mechanism utilising AP-1 (see **section 1.3.3**). *In situ* hybridisation work has shown that the inner fasciculata cells, which normally do not express *CYP11B1*, are recruited for 11β-hydroxylase expression after long periods of stress or ACTH activation (Engeland *et al.* 1997). There is evidence to suggest that the fasciculata may also expand outward under such conditions, altering cells from a glomerulosa to a fasciculata phenotype, with all the morphological and biosynthetic alterations this implies (Vazir *et al.* 1981).

1.4.2 Angiotensin II (AngII)

The most important stimulus to aldosterone secretion is an alteration in electrolyte and fluid levels. Restriction of dietary sodium causes hypertrophy of the zona glomerulosa, increasing the adrenal cortex's capacity for aldosterone secretion, while glucocorticoid levels remain unaffected. Reduced plasma sodium does not work directly on the cells of the zona glomerulosa. Instead, a number of factors are involved in modulating aldosterone secretion, including the renin-angiotensin system which is illustrated in **figure 1.4b**. Ang II is the major active product of this system but the heptapeptide, Ang III, and the hexapeptide, Ang IV, have also received some attention (Semple and Morton, 1976; Peach *et al.* 1976).

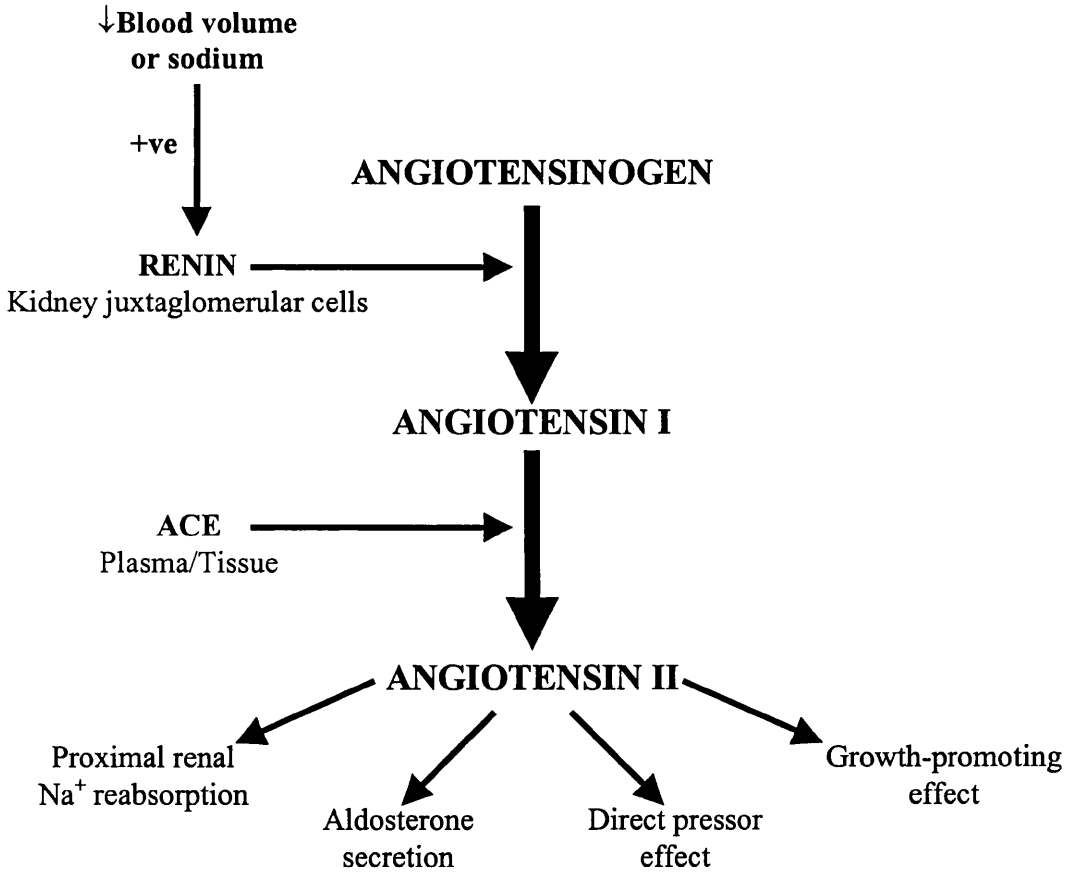


Figure 1.4b The renin-angiotensin system (RAS).

Angiotensin II action on adrenal cortex AngII receptors are found in two forms on the surface of adrenocortical cells: a high-affinity receptor, AT1, and a less numerous, lower affinity receptor, AT2, which is believed to be of less physiological importance (Matsusaka and Ichikawa, 1997). Two forms of AT1, AT1A and AT1B, are present in the rat. AT1 is found in especially high levels on renin-producing cells, including juxtaglomerular cells, and may play a role in a negative feedback mechanism. AT1 receptors are found in the inner adrenocortical regions, but they are most numerous in the zona glomerulosa where they mediate the AngII regulation of aldosterone secretion. Sodium depletion causes an increase in the concentration and affinity of AngII receptors, increasing the sensitivity of the adrenal cortex to AngII stimulation, and AngII has been reported to upregulate its own receptors. Chronic ACTH administration and dietary sodium loading have the opposite effect (Vinson *et al.* 1992).

AngII stimulates the transcription of *CYP11B2* (Imai *et al.* 1992). It exerts its intracellular effects via membrane receptors. These are linked to a G protein system that generates the second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) which activate protein kinases and release Ca^{2+} from intracellular stores, respectively. See **figure 1.4c**.

Ca^{2+} is released into the cytosol from the extracellular medium or from intracellular stores. Adrenal glomerulosa cells possess T-type and L-type voltage-operated Ca^{2+} channels at their surface which are activated by raised extracellular potassium levels (see **section 1.4.3**). Ca^{2+} entering into the cell via L-type channels passes freely into the cytosol, raising the cytosolic calcium ion concentration ($[Ca^{2+}]_{ic}$). However, Ca^{2+} arriving through the T-type channels may be delivered

directly to the mitochondrial matrix via a 'pipeline' (possibly part of the endoplasmic reticulum) without any apparent rise in $[Ca^{2+}]_c$ (Rossier, 1997).

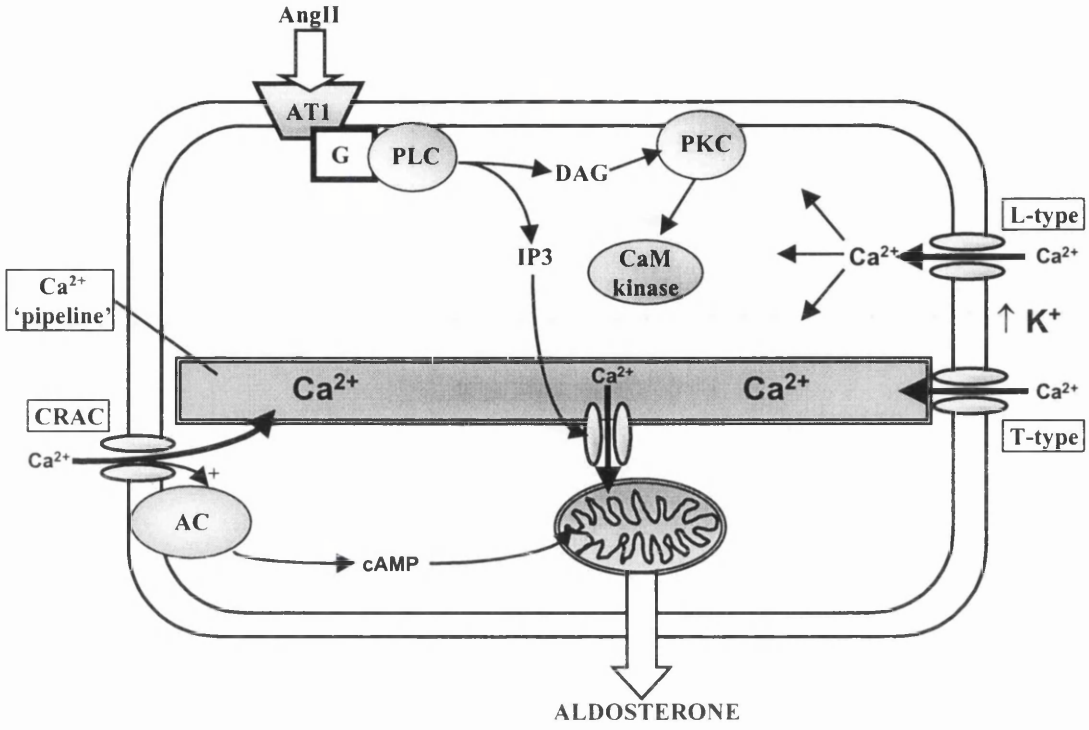


Figure 1.4c A model of intracellular calcium signalling (Rossier, 1997).

Intracellular stores are rapidly depleted but the initial rise in $[Ca^{2+}]_{ic}$ is sufficient to activate calcium release-activated calcium (CRAC) channels in the membrane. The CRAC channels carry more Ca^{2+} to the mitochondrion while simultaneously replenishing intracellular Ca^{2+} stores via the pipeline. CRAC channels also stimulate a specific adenylate cyclase to increase cAMP formation. DAG activates protein kinase C (PKC) which phosphorylates various proteins including the Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) (Rasmussen, 1989).

The actions of AngII – increased cAMP formation, Ca^{2+} influx and the phosphorylation of CaM kinases – are all of importance in aldosterone biosynthesis

and are able to interact in complex ways to regulate transcription. For example, AngII and ACTH (acting through the cAMP pathway) can exert antagonistic effects over the transcription of *fos* and *jun* genes which could account for the differential regulation of various steroidogenic genes by adrenocortical cells in response to these hormones (Viard *et al.* 1992). The cAMP and Ca^{2+} pathways can also converge to regulate CREB phosphorylation, cAMP acting through PKA (see **section 1.3.3**) and Ca^{2+} via the CaM kinases.

CaM kinases I, II and IV are able to interact with each other. CaM kinases I and IV are small enough to shuttle freely between the cytosol and nucleus. Their transcriptional activation is significantly upregulated by Ca^{2+}/calmodulin-dependent protein kinase (CaMK kinase). The CaMK kinases probably link Ca^{2+} signals and *CYP11B2* transcription (Clyne *et al.* 1997). CaM kinase II may also be involved in the regulatory phosphorylation of StAR (Arakane *et al.* 1997).

CaM kinases I and IV (but not CaM kinase II) can activate CREB and another protein of the same family, activating transcription factor-1 (ATF-1) (Sun *et al.* 1996). ATF-1 can heterodimerise with CREB and may, therefore, be able to mediate transcriptional activity through CRE sequences. ATF-1 is not responsive to PKA but is stimulated by raised $[Ca^{2+}]_{ic}$, suggesting that it may discriminate Ca^{2+} pathways from cAMP pathways (Heist and Schulman, 1998).

Studies of the human *CYP11B2* promoter show that two elements are required for effective Ca^{2+}-regulated transcription, one containing a CRE site and the other consisting of an SF-1 and COUP-TF site. These sites are also required for regulation of the same gene by cAMP, suggesting both mechanisms exploit the same site, although this does not explain the long-term differences in *CYP11B2* expression by these two pathways (Clyne *et al.* 1997).

Ca^{2+} is involved in so many intracellular pathways that one might wonder how the signal for a particular pathway is not engulfed by the signal for another. For this reason, the concept of compartmentalisation of the cell Ca^{2+} was postulated. For example, the mitochondrial pipeline concentration might rise without affecting the cytosolic concentration. There is evidence to support this idea. Nuclear $[Ca^{2+}]$ is significantly higher than that of the cytosol. High nuclear calcium is known to be necessary for CREB activation. Furthermore, the opening of CRAC channels, stimulated by the delivery of large amounts of Ca^{2+} to the mitochondrion, appears to be solely responsible for AngII's modulation of the ACTH-induced rise in cAMP.

1.4.3 Potassium

Extracellular potassium is maintained within narrow limits. Slight rises in extracellular K^+ concentration ($[K^+]_o$) stimulate aldosterone secretion and also raise the sensitivity of the cortex to AngII, possibly through up-regulation of AT1 receptors (Matsusaka and Ichikawa, 1997). Also, higher $[K^+]_o$ triggers action potentials in glomerulosa T-type and L-type Ca^{2+} channels (Pardo *et al.* 1992; Rossier, 1997) allowing calcium influx. It is striking that manipulation of adrenocortical cell membrane potential is a key process in response to agonists. This is, of course, a principal mechanism in brain neurotransmission, a function which is modulated by corticosteroids (see **section 1.7.4**). Therefore, it is also a possible factor in the control of neural corticosteroid synthesis (see **section 1.8**).

1.4.4 Neuroactive substances as agonists of adrenal function

ACTH, AngII and potassium are the most important regulators of corticosteroid secretion but other factors have effects.

Low concentrations of **serotonin** (5-hydroxytryptamine or 5-HT) stimulate aldosterone secretion *in vitro*, via serotonin type 2 receptors on zona glomerulosa cells. Serotonin also increases corticosterone secretion rates (Hinson *et al.* 1989). The physiological significance of this finding has been questioned, as serotonin in the blood would quickly be taken up by platelets. However, local release of serotonin may occur from the mast cells of the adrenal capsule (see earlier).

The adrenal cortex has **cholinergic** innervation. Cholinergic stimulation increases aldosterone secretion in bovine adrenal cortex, possibly through the calcium second messenger system (Kojima *et al.* 1986). However, ovine adrenal glands transplanted to the neck work normally, their function unimpaired by the severing of the adrenal nerve connections.

Nerve terminals supplying the adrenal capsule and zona glomerulosa contain **vasoactive intestinal peptide** (VIP). VIP infusions into rats can stimulate aldosterone secretion, although only when ACTH is also present (Nussdorfer and Mazzocchi, 1987).

Vasopressin is found within the adrenal cortex and has stimulatory effects on aldosterone secretion *in vivo*. It is thought to act through the phosphatidylinositol second messenger system (Woodcock *et al.* 1986).

Somatostatin is a 14 residue peptide released by the hypothalamus which can inhibit the AngII-stimulated release of aldosterone, probably by blocking AngII receptors. Somatostatin has no effect on basal or ACTH-stimulated steroidogenesis. Specific somatostatin receptors have been found in the zona glomerulosa of several species, including the rat, albeit only at very low concentrations (Srikant and Patel, 1985).

Catecholamines stimulate the glomerulosa, increasing cAMP production *in vitro* through β-adrenergic receptor binding (De Léan *et al.* 1984). Small amounts of catecholamines (adrenaline and noradrenaline) have been isolated from the adrenal cortex. These may derive from the adrenal medulla (Pratt *et al.* 1987).

Dopamine agonists inhibit aldosterone secretion, suggesting dopamine exerts some control over steroidogenesis in the zona glomerulosa (Carey *et al.* 1979). Dopamine antagonists such as metoclopramide increase aldosterone secretion. Dopamine receptors of the DA-1 and DA-2 subtypes have been identified in the zona glomerulosa, with DA-2 apparently inhibiting adenylate cyclase activity, thereby reducing aldosterone secretion (Missale *et al.* 1986). However, dopamine infusion studies performed on dogs suggest that the levels of dopamine required to affect aldosterone secretion by the zona glomerulosa would greatly exceed those encountered under physiological conditions (Ball *et al.* 1981).

In vitro studies suggest that type E **prostaglandins** stimulate aldosterone secretion while type F prostaglandins inhibit it or have no effect. Interpretation of studies *in vivo* is complicated because prostaglandins also influence renin secretion (Vinson *et al.* 1992).

In summary, the neuroactive substances which exert an effect on adrenal corticosteroid secretion are many and varied but their effects are small, often requiring high concentrations *in vitro* to elicit a response. These factors – and even elevated $[K^+]$ – are unable to sustain adrenocortical function in the absence of either ACTH or AngII. However, given the neuroactive nature of these substances, it is tempting to speculate that the true target of their steroidogenic actions is not the adrenal gland but the brain.

1.5 Corticosteroid Action And Effects

1.5.1 Classical corticosteroid action

The classical mechanism of corticosteroid action is summarised in **figure 1.5a**.

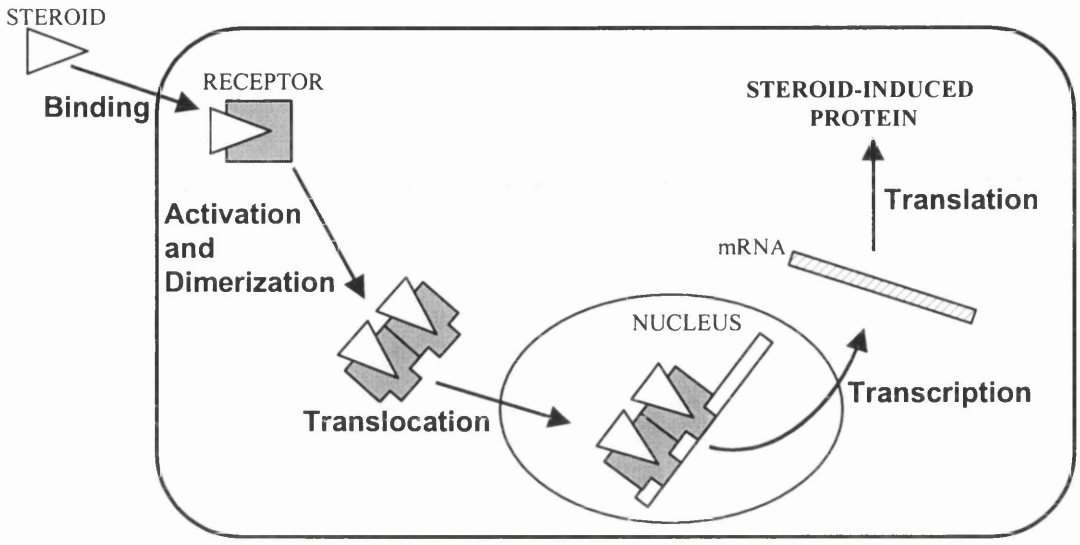


Figure 1.5a Classical steroid action

Corticosteroid receptors are intracellular. They are part of an oligomeric cluster comprising a number of other proteins such as heat shock proteins (see below), from which they dissociate on ligand binding. The corticosteroid receptors are ligand-dependent transcription factors. In the presence of ligand, they undergo structural changes which facilitate the transfer of the hormone-receptor complex to the nucleus where, as a homodimer, they bind chromatin at specific steroid response elements, modulating gene transcription. This modulation results in increased or decreased levels of certain mRNAs and, ultimately, proteins which account for the physiological effects of corticosteroids.

1.5.2 The mineralocorticoid and glucocorticoid receptors

The amino acid sequences of the major steroid receptor superfamily members have been deduced in recent years, including those of the glucocorticoid receptor and the mineralocorticoid receptor. Remarkable sequence homology is evident throughout the superfamily, most especially in the 70 amino acid DNA-binding domain. This domain forms two 'zinc fingers', each composed of four cysteines coordinating one zinc atom. It is this region which specifically recognises the steroid response elements adjacent to target genes. It has been suggested that the first finger is responsible for sequence specificity while the second stabilises the binding of the receptor to its response element. The C-terminal region of the receptors is also highly conserved and is required for high-affinity ligand binding. Conversely, the amino-terminal region is extremely variable and is thought to contribute to the diverse range of receptor specificities evident throughout the superfamily.

The glucocorticoid receptor (GR, also known as the type I receptor) is a monomer with a molecular weight of ~94,000. In target organs it has a concentration of around 25,000 molecules per cell. The human form of the mineralocorticoid receptor (MR or type II receptor) has a molecular weight of 107,000. The two share 57% amino acid identity in their ligand-binding domains and 94% identity in their DNA-binding domains (DBD) (Arriza *et al.* 1987). They share a similarly high DBD homology with the androgen receptors (AR) and progesterone receptors (PR). This is reflected in the fact that these receptors share a nuclear hormone response element (HRE) sequence, the consensus of which is AGAACAnnnTGTTCT. The fact that this sequence is an inverted palindrome means that members of the MR/GR/PR/AR subfamily can bind the HRE as homodimers or heterodimers (Funder, 1997).

The MR and GR ligand-binding domains possess slight but significant differences in their binding specificities (Vinson *et al.* 1992). The two may be characterised on the basis of the hierarchy of binding affinities they show for different steroids, as shown below:

MR: Aldosterone > DOC > Corticosterone > Cortisol > Dexamethasone

GR: Dexamethasone > Corticosterone > Cortisol = Aldosterone

The cascade of events that follow ligand binding of GR is more complicated than the above summary would suggest. The ligand-free form of GR has a molecular mass of 310kDa and consists of a single hormone-binding GR, two molecules of the 90kDa heat shock protein (Hsp90) and several other proteins and polyunsaturated fatty acids. The Hsp90 is thought to stabilise the complex in the cytosol. Once bound by ligand, the multiprotein complex dissociates to leave a 90kDa complex (see **figure 1.5b**) and the DBD is exposed. The receptor is also now able to homodimerise. The complex may then be translocated into the nucleus by specific transport through the nuclear pore. Inside, ligand-bound GR can bind glucocorticoid responsive elements (GREs) on the chromatin with its Zn-fingers. Once bound to this element, the homodimer can interact with other dimers or transcription factors. The nature of this interaction at a particular regulatory unit will ultimately determine how the GR modulates gene transcription. Although the activation of MR has not been investigated to the same extent as GR, it is likely to follow a similar activation mechanism.

In vitro systems have shown that GR and MR appear to have equivalent transcriptional activity when bound by an appropriate ligand. However, there are clear-cut differences in the actions of GR and MR. HREs do not modulate gene transcription in isolation. Steroid receptors must interact with other factors bound

nearby, and it is these interactions, unique to GR or MR, which are responsible for their specific effects (Robins *et al.* 1994).

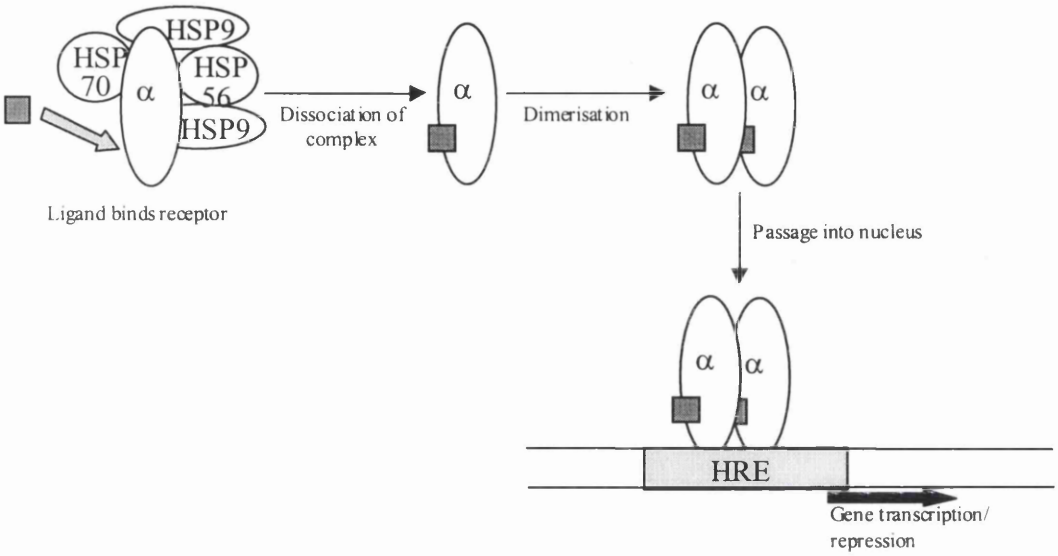


Figure 1.5b Activation of the glucocorticoid receptor

The GR is vital for life. Mice whose GR gene is knocked out die from respiratory failure shortly after birth as their lungs fail to mature in the absence of certain surfactant proteins. Surprisingly, GRs which cannot dimerise are not lethal. $GR^{dim/dim}$ mice subjected to a 'knock-in' mutation which prevents dimerisation survive. The GR monomer is unable to influence transcription either positively or negatively via its HRE sites but it is still capable of repressing the activity of AP-1 regulated promoters, either through some direct effect on AP-1 itself, or through an interaction with some other component of the transcription initiation complex. This interaction of the GR monomer, which does not bind DNA, is sufficient to permit the survival of $GR^{dim/dim}$ mice (Reichardt *et al.* 1998).

1.5.3 Classical mineralocorticoid effects

The net effect of mineralocorticoid action is to increase extracellular volume and blood pressure. Steroids with mineralocorticoid activity, of which aldosterone is the most important example, increase the reabsorption of sodium in the distal tubules and cortical collecting ducts of the kidney and in secretory epithelia. Unsurprisingly, there is a dense population of MR at these locations. Although reabsorption of less than 0.5% of the filtered sodium is aldosterone-dependent, this is essential for life; adrenalectomised animals must be given 0.9% NaCl as drinking fluid to compensate for these losses. The reabsorption of sodium by aldosterone-responsive epithelia is made possible by the asymmetric organisation of sodium pumps and amiloride-sensitive sodium channels in the cell membrane (see **fig 1.5c**).

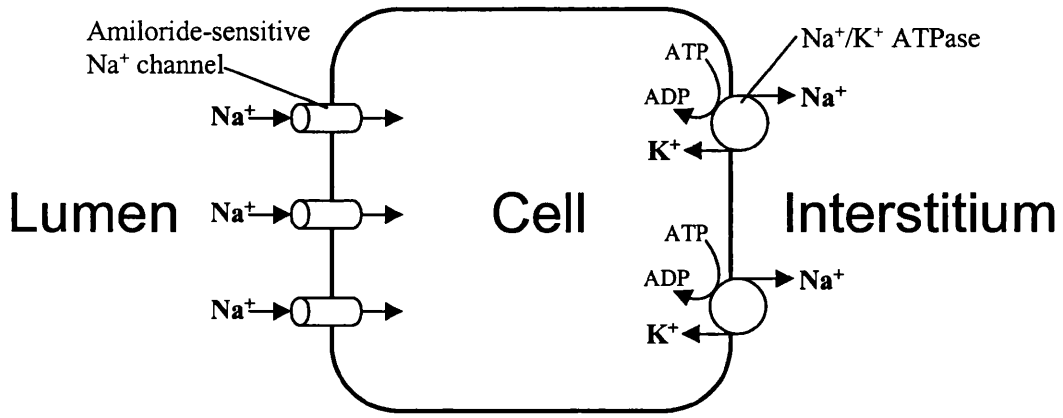


Figure 1.5c Sodium reabsorption by epithelial cells

Aldosterone drives this process by increasing the number of open sodium channels in the apical membrane. This could be achieved by increasing the proportion of time each channel is open, possibly by methylation, or by increasing the number of channels in the membrane (White, 1994). Chronic exposure of these cells to aldosterone increases the number of Na^+/K^+ ATPase molecules due to

increased Na^+ entering the cell (Petty *et al.* 1981). The amiloride-sensitive epithelial sodium channels (EnaC) is a hetero-oligomer. Increased Na^+ transport is due to EnaC activation. Aldosterone may activate existing channels and induce the synthesis of new channels. Increased synthesis of the mRNAs coding for these Na^+ pumps' α and β subunits has been detected (Verrey *et al.* 1987). However, activity is apparently unaffected in mice lacking MR. The specific role of aldosterone remains uncertain (Zennaro, 1998). Aldosterone also affects salt appetite in rats (see **section 1.7.9**).

Aldosterone is also of importance in the regulation of potassium metabolism, determining the level of potassium excretion following an acute load, possibly through increased K^+ channel activity at the apical membrane (Adam *et al.* 1987). The secretion of K^+ and H^+ are closely linked. Mineralocorticoid excess results in hypokalemic alkalosis.

Excess aldosterone, as in primary hyperaldosteronism or Conn's syndrome, results in hypokalaemia, high body sodium, increased plasma and extracellular fluid volumes and hypertension. Blood pressure is directly proportional to the exchangable sodium level (Fraser *et al.* 1989). In salt-sensitised rats, aldosterone increases blood pressure without necessarily increasing blood volume or cardiac output (Vinson *et al.* 1992) suggesting that mineralocorticoids might act in combination with Na^+ to increase vascular resistance by direct actions on the central nervous system (CNS) or on vascular smooth muscle.

1.5.4 Classical glucocorticoid effects

Glucocorticoids are involved in a much wider range of physiological processes than the mineralocorticoids (Vinson *et al.* 1992). They affect the metabolism, protecting against glucose deprivation by increasing protein catabolism,

glycogenesis, hepatic gluconeogenesis and lipolysis. They also affect the growth of skeletal muscle, kidney and liver tissue. Glucocorticoid action on bone can result in osteoporosis. They are anti-inflammatory and immunosuppressive, reducing lymphocyte numbers, inhibiting interleukin production and, at higher concentrations, suppressing antibody production. In the brain, glucocorticoids affect many aspects of behaviour, including mood and learning (see **sections 1.7.6** and **1.7.8**).

Finally, glucocorticoids affect blood pressure. 70–80% of individuals with glucocorticoid excess (i.e. Cushing's syndrome) are hypertensive. Unlike mineralocorticoid hypertension, this is not sodium dependent and can be blocked only by specific GR antagonists. Although various mechanisms, such as increases in vascular tone and responsiveness to AngII, are involved in the development of glucocorticoid-induced hypertension, their relative contributions are not known (Mantero and Boscaro, 1992).

1.5.5 The 11β-hydroxysteroid dehydrogenases

Although, as mentioned earlier, MR has ligand-binding properties which are different to those of GR, its affinities for cortisol, corticosterone and aldosterone *in vitro* are quite similar. Moreover, glucocorticoid concentrations are often approximately a thousand times those of mineralocorticoid. Therefore, the question arises of how some MR are able to selectively bind aldosterone *in vivo*? In some tissues, MR shows no preference for aldosterone over glucocorticoid – i.e. it is 'nonselective' – but in the distal nephron and other aldosterone target tissues there is very definite selectivity. This is achieved not by receptor specificity but by complete destruction of cortisol in the tissue.

These selective tissues possess abundant 11β-hydroxysteroid dehydrogenase (11β-HSD). It is able to confer aldosterone selectivity by rapidly converting cortisol to cortisone and corticosterone to 11-dehydrocorticosterone which cannot bind MR. Aldosterone's 11–18 hemiacetal bridge protects it from 11β-HSD's action. See **figure 1.5d**.

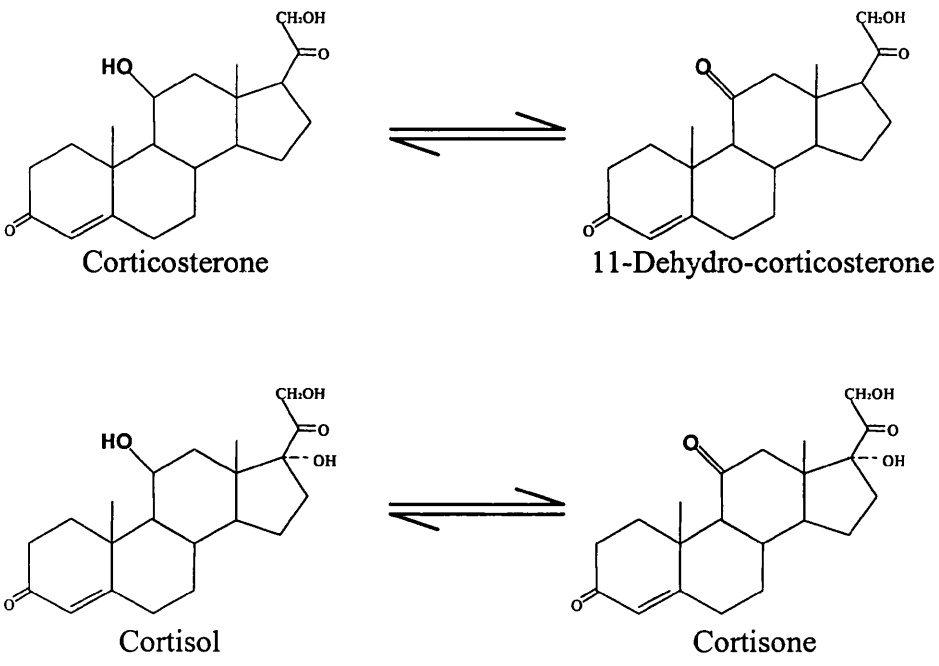


Figure 1.5d Actions of 11β-hydroxysteroid dehydrogenase

There are two distinct forms of 11β-HSD: type 1 (or 11β-HSD-1) and type 2 (11β-HSD-2) (Seckl, 1997). The two isozymes have been identified in a number of species including human, rat, sheep and rabbit. 11β-HSD-1 is widespread and can catalyse both oxidation and reduction of the 11-oxygen function. It probably functions predominantly as a reductase *in vivo*. It is NADP(H)-dependent, with a high K_m for glucocorticoids that renders it a poor protector of MR. Its activity is inhibited by several substances, including licorice and bile acids. It probably acts to

maintain high cortisol concentration in tissues such as the liver. 11β-HSD-2 has a much higher affinity for glucocorticoids and, unlike 11β-HSD-1, is exclusively a dehydrogenase, requiring NAD as a cofactor. It is this form of 11β-HSD that confers aldosterone specificity upon the MR of certain tissues. See **figure 1.5e**.

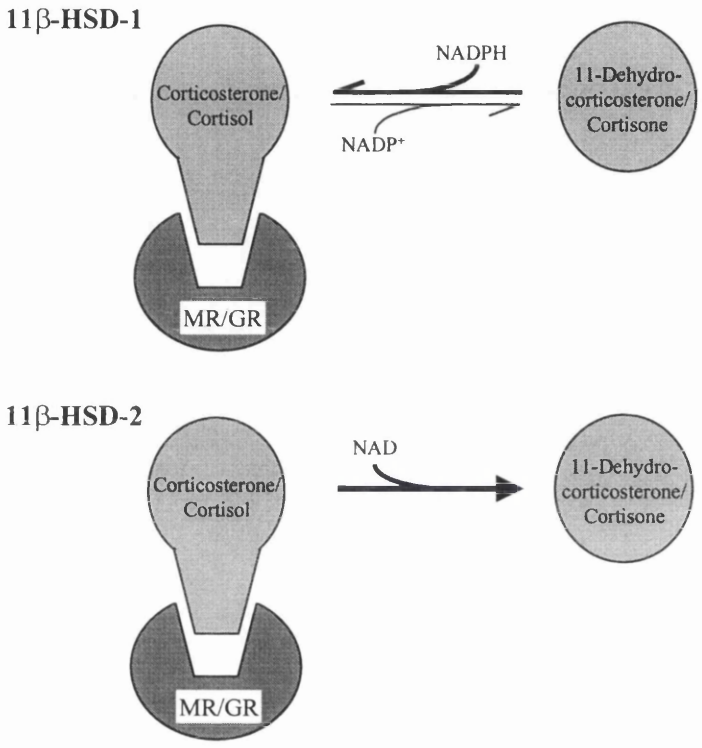


Figure 1.5e 11β-HSD isozymes (Seckl, 1997).

1.5.6 Nongenomic corticosteroid effects

Genomic steroid hormone action requires a time lag or latent period of 1-2 hours between steroid-receptor binding and the eventual alteration of protein levels within the target cell (Wehling, 1995). However, some responses occur between a few seconds and 2 minutes after steroid administration and cannot be blocked by inhibitors of DNA transcription or protein synthesis. These are therefore described as nongenomic. Some may occur through nonspecific interactions with proteins and/or

membrane lipids at high steroid concentrations. However, others appear to be specific and require relatively low levels of steroid (Wehling, 1997). For example, the generation of IP3 and DAG in rat vascular smooth cells (VSMC) is significantly stimulated by aldosterone within 30 seconds of its administration. Rapid increases in the activities of Na^+/H^+ countertransporters and Na^+/K^+ ATPases are also observed in these cells and in mononuclear leucocytes with very low aldosterone doses (Wehling, 1995).

Plasma membrane aldosterone binding sites of very high affinity have been identified in rat VSMC and human mononuclear leukocytes (HML). They have a K_d of ~0.04nM, significantly higher than the K_d of 1.4nM for MR. IP3 generation responds rapidly to 0.1nM aldosterone which is close to physiological levels of free aldosterone (Wehling, 1995). Unlabelled aldosterone (but not cortisol) inhibits the binding of ^{3}H-aldosterone, so they appear to be free of the nonspecificity encountered in MR and GR (Wehling, 1997).

These discoveries have led to the proposal of a two-step model for steroid action, whereby steroids exert a number of initial rapid effects which then give way to the more familiar genomic mechanisms. Importantly, these two actions could, after the latency period, occur simultaneously within the cell, comodulating a range of cellular steroid effects. Second messengers are known to be capable of modulating steroid-induced transcription (Moyer *et al.* 1993). Thus, steroids could control their own genomic effects through their nongenomic actions (Wehling, 1997).

Rapid steroid effects are not restricted to aldosterone, nor are they confined to VSMC or HML cells. In fact, their most important role may be in the modulation of neurons.

1.6 Extra-Adrenal Corticosteroid Synthesis.

Until relatively recently, the production of corticosteroids was assumed to be solely a function of the adrenal cortex. This view was challenged by the discovery of corticosteroidogenic enzymes in several other tissues. Considered together with the discovery that corticosteroids are also now known to affect the metabolism of 'nonclassical' target tissues – the effect of aldosterone on leucocytes (Wehling, 1995) is an example – this raised the possibility of a novel paracrine or autocrine role for these hormones. To sustain the hypothesis that corticosteroids have such a role, it is essential to establish that they have special local effects, that the biosynthetic apparatus (see **section 1.2**) is present and active and that the tissue also possesses the specific receptors necessary to exert their effects. Moreover, since systemic corticosteroids are lipophilic and therefore have unimpeded access to all cells, local synthesis must either produce significantly higher local concentrations or be controlled locally and possibly differently from that of the adrenal cortex. The next section summarises the evidence for ectopic production in the heart. This is followed by a more detailed review of the action, synthesis and roles of corticosteroids in the brain.

1.6.1 Production of adrenal corticosteroids in the cardiovascular system

Aldosterone rapidly alters the membrane permeability of vascular smooth muscle cells to Ca^{2+} through changes in channel and transporter expression (see **section 1.5.6**). This permeability increases smooth muscle tone and responsiveness to various vasoconstrictive agents (Morano, 1992; Hatakeyama *et al.* 1994). Over 50 years ago, Selye found that DOC and aldosterone promoted collagen deposition, thus enhancing vascular repair. He termed this effect *remodelling* and saw it as an

adaptation to arterial hypertension (Slight *et al.* 1999). Since then, chronic aldosterone administration has been shown to stimulate collagen deposition by cardiac fibroblasts in the rat myocardium, thus increasing myocardial stiffness (Brilla and Weber, 1992; Funder, 1995). For fibrosis to develop, both increased sodium intake and chronic aldosterone administration are necessary. This aldosterone-salt induced fibrosis can be prevented by low doses of the mineralocorticoid receptor antagonist spironolactone, suggesting that collagen deposition results from a direct action on MRs, which are known to be present in myocardium and blood vessels (Kornel, 1993). However, higher doses of spironolactone are required to prevent the associated hypertension and LVH (Funder *et al.* 1988; Brilla *et al.* 1993). The action on MRs by aldosterone would require the presence of 11β-HSD-2 within the heart; both 11β-HSD-1 and 11β-HSD-2 have been detected (Slight *et al.* 1996).

The induction of cardiac fibrosis may involve AngII acting through AT1 receptors. The density of AngII receptors in the tissue is increased by aldosterone, an effect which is blocked by spironolactone (Robert *et al.* 1999). AngII has growth-promoting properties and could be responsible for the proliferation of myofibroblasts observed in fibrosis.

Evidence of local synthesis has been obtained by demonstrating gene transcription – i.e. increased mRNA levels – and by specific identification of the compounds themselves. Furthermore, this synthesis may be controlled locally but by conventional means. Several corticosteroidogenic enzyme genes are expressed within the cardiovascular system. *CYP11B2* mRNA has been detected in endothelial and smooth muscle cells isolated from human pulmonary artery (Hatakeyama *et al.* 1994). Using RT-PCR, expression in the same cells of the genes encoding MR, type I and II 3β-HSD, P450c21, P450c18, but not P450scc or P45011β was also established

(Takeda *et al.* 1995; Hatakeyama *et al.* 1996). Human vascular endothelial cells (HVECs) derived from umbilical veins produce both *CYP11B1* and *CYP11B2* mRNAs. In the same cell type, AngII and K^+ increase *CYP11B2* but not *CYP11B1* mRNA, while ACTH raises *CYP11B1* mRNA but leaves *CYP11B2* levels unaffected (Takeda *et al.* 1996). It is relevant here that biochemical and molecular biological techniques show that all components of the renin-angiotensin system are expressed in blood vessels and in the heart, undergoing regulation at an autocrine-paracrine level (Samani, 1994)

Perfusion of Wistar-Kyoto (WKY) rat mesenteric arteries yields detectable amounts of corticosterone and aldosterone, albeit at much lower levels than in the adrenal cortex or plasma (Takeda *et al.* 1995; Takeda *et al.* 1995). Aldosterone production in the rat mesenteric vessels is significantly reduced by quinapril (an ACE inhibitor), and significantly increased by elevated AngII or K^+ levels. Corticosterone secretion is unaffected by quinapril.

3β-HSD activity was identified in the heart by Zhao *et al.* (1991). Using quantitative RT-PCR, Silvestre *et al.* (1998) were able to detect *CYP11B1* and *CYP11B2* mRNA in the four cardiac chambers of the adult rat heart. *CYP11B3* mRNA was also present in 21-day-old but not adult rat hearts. Aldosterone and corticosterone were detectable in homogenised and in perfused rat hearts and their levels were increased by treating rats with AngII, ACTH, or a low sodium diet for a week. Similarly, ventricular *CYP11B1* mRNA was increased by AngII or ACTH, while *CYP11B2* mRNA was raised by AngII or low sodium diets (Silvestre *et al.* 1998). These increases were not directly related to changes in plasma levels and therefore suggest independent regulatory mechanisms. However, studies of the

chronic regulation of the cardiac steroidogenic system conclude that it is controlled by pathways similar to those described in **section 1.4** (Delcayre and Silvestre, 1999).

The physiological and clinical significance of local synthesis is unknown. The importance of aldosterone levels in hypertension and cardiac disease is unambiguous (Pitt *et al.* 1999; Davies *et al.* 1999). However, there is as yet no direct evidence that local gene expression has a significant effect on the development of cardiac fibrosis. Nevertheless, rat myocardial aldosterone was found to have a concentration of 16nM, a level 17 times that found in the plasma. This difference may be due to slower aldosterone degradation in the heart than in the plasma, intracellular segregation or to local delivery of aldosterone to extracellular spaces rather than into the bloodstream (Delcayre and Silvestre, 1999). Regardless of the mechanism involved, this relatively high local aldosterone concentration in the heart suggests that it has some independent paracrine or autocrine action. A more general role in the etiology of hypertension is also possible. Stroke-prone spontaneously hypertensive rats (SHRSP) are a genetic model for human hypertension, although the precise cause of their hypertension is unknown. Aldosterone production and CYP11B2 levels were found to be significantly higher in the mesenteric arteries of young (2 week old) SHRSP rats compared to WKYs of the same age but these differences did not persist into later life (Takeda *et al.* 1997). However, the mRNA coding for the α_1 subunit of Na^+/K^+ ATPase, an aldosterone-responsive activity, was significantly higher in the mesenteric arteries of the SHRSP strain at all ages that were examined.

1.7 Corticosteroid Action In The Central Nervous System

The brain is a complex endocrine gland, producing unique hormones and controlling the production of others. It is also a target tissue for many hormones. Adrenocortical activity is modulated by the brain. The lipophilic properties of steroids ensure that their entry into the brain is not impeded by the blood-brain barrier and the brain is amply supplied with MR and GR. Abnormal brain function alters the rate and pattern of adrenocortical activity; abnormal corticosteroid secretion profoundly affects mental processes. What effects do corticosteroids have on brain metabolism and how and where do they act? Are they synthesised locally? These questions are addressed in the following sections.

1.7.1 Structure and general functions of the brain.

The principal components of the rat brain are illustrated in **figure 1.7a**. The central nervous system (CNS) is composed of the brain and the spinal cord. The neurons consist of a cell body composing the grey matter and axons and dendrites, the former of which are enclosed in myelin sheaths and constitute the white matter. The neurons are interspersed with glial cells which, among other things, assist in feeding the neurons. The ventricles contain cerebrospinal fluid, a lymph-like filtrate of blood.

Regions of the brain can often be related to particular functions; the more complex functions require the integration of the functions of several regions. For example, the cerebellum coordinates movement and balance. Two region of particular relevance to corticosteroid action are the hypothalamus and the hippocampus. The hypothalamus, whose role in adrenal function has already been described (see **section 1.4.1**), is involved in the regulation of homeostatic processes,

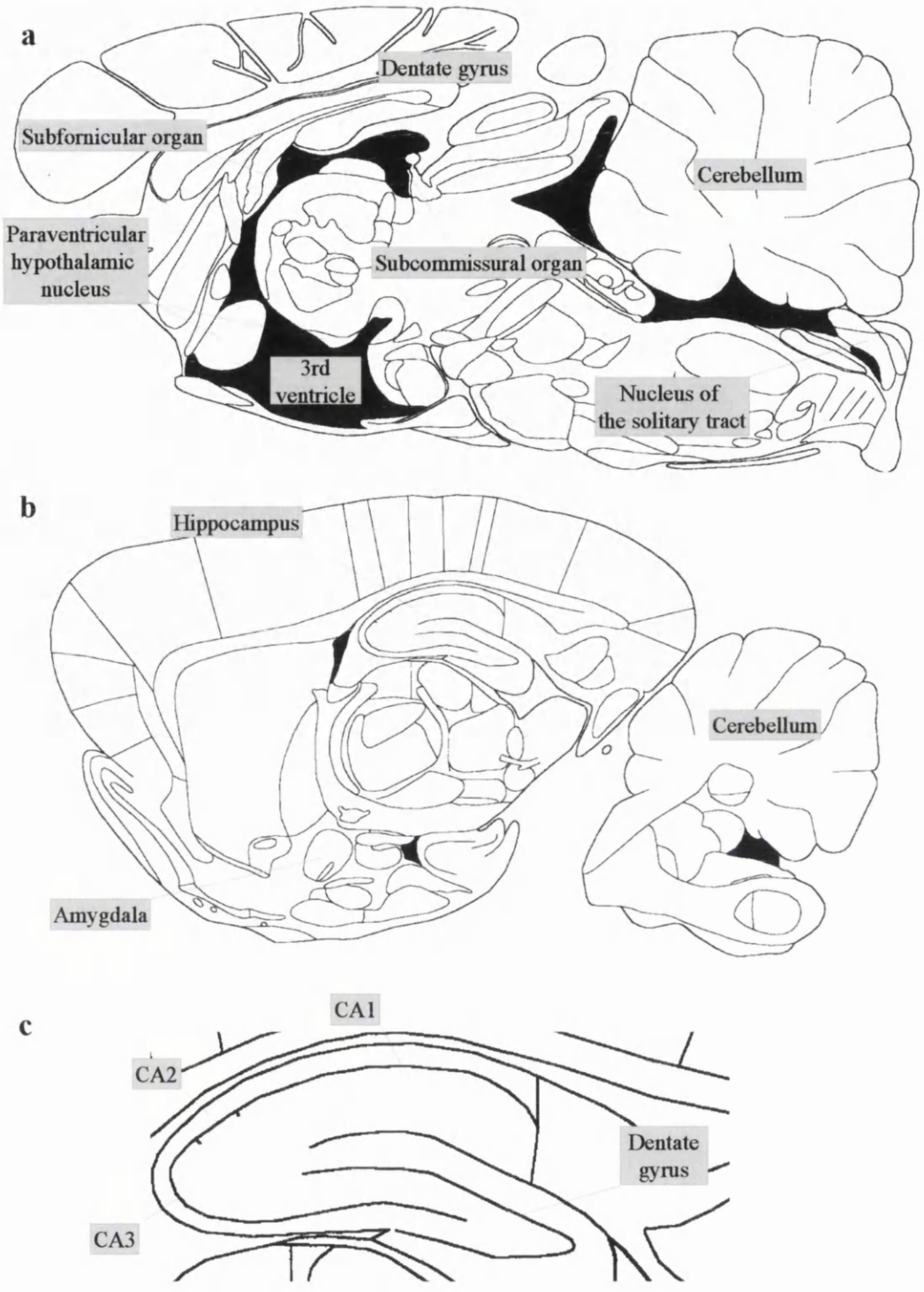


Figure 1.7a Diagrams of rat brain sections. a; sagittal brain section at –0.1mm from the midline; b: sagittal brain section at 3.4mm from the midline; c: detail of the CA1–3 cells of Ammon's horn and the dentate gyrus within the hippocampus of a sagittal section at 3.4mm from the midline (Paxinos and Watson, 1998).

modulating body temperature, hunger and thirst and is involved in behavioural responses such as the 'fight or flight' reaction. The limbic system comprises the amygdala and hippocampus and, in collaboration with the cerebral cortex, is associated with emotion and memory.

Other regions are important in the regulation of blood pressure. The subcommissural organ (SCO), dorsal to the third ventricle, responds to aldosterone introduced to the cerebrospinal fluid, by increasing sodium excretion; this is the converse of its renal action (Dundore *et al.* 1984). The AV3V area, comprising the anterior hypothalamic periventricular nuclei (PVN), the periventricular and median preoptic nuclei and the anterior wall of the third ventricle, integrates information on blood volume, thirst, sodium appetite and AVP secretion. Mineralocorticoid hypertension and the hypertensive response to salt in the Dahl salt-sensitive rat can be attenuated or prevented by destruction of this area (Gómez-Sánchez, 1997).

1.7.2 Central mineralocorticoid and glucocorticoid receptors

In the 1960s, it was observed that tritiated corticosterone administered to adrenalectomised (ADX) rats was retained in the hippocampus and other regions of the limbic forebrain (McEwen *et al.* 1968). The same was true of other species, such as dogs and primates which secrete cortisol instead of corticosterone. The distribution of corticosteroid receptors within the brain has now been investigated in greater detail using radioligand binding, immunohistochemistry and *in situ* hybridisation (Reul and De Kloet, 1986; Agarwal *et al.* 1993; Roland *et al.* 1995). Such studies showed that GR is widely distributed but that it is especially abundant in the hippocampus, lateral septum, cerebral cortex, amygdala and the nucleus tractus solitarii. All cells within the CNS probably possess GR. However, in most regions

there are fewer MR than GR, with high concentration limited to the hippocampus, the septum and the granular cells of the cerebellum (Reul and De Kloet, 1986; Agarwal *et al.* 1993). Although GR and MR colocalise in the hippocampus, MR is distributed evenly throughout the region, while GR is most concentrated in the granule cells of the dentate gyrus (DG) and pyramidal cells of CA1/2, decreasing in CA3/4. See **figure 1.7a**.

The rates of GR and MR gene expression within the hippocampus are dependent on corticosteroid levels (O'Donnell and Meaney, 1994). However, such effects are short-lived. In the rat, hippocampal GR gene expression follows a diurnal rhythm, with its mRNA falling during the dark, active phase, when corticosterone is at its peak. Tests for a diurnal rhythm of hippocampal MR expression have tended to be inconclusive or contradictory. However, stress increases MR and GR concentrations in the hippocampus, although such changes are dependent on the type and duration of stress imposed. Whether this relates to steroid levels or to other stress-induced neuroactive substances is not known; the latter would appear more likely since, as noted above, GR mRNA is at its lowest when corticosterone levels peak. Monoamines such as methamphetamine reduce MR and GR mRNA levels. Antidepressant drugs, which potentiate monoaminergic transmission, increase MR and GR concentrations in the hippocampus (Seckl, 1996). Splice variants of MR (MRα, -β and -γ) occur in the brain; all can be found within the rat hippocampus. MRα is most concentrated in the CA1 and dentate gyrus while MRβ and -γ are found in the pyramidal layer. MRα, but not MRβ, is upregulated after adrenalectomy and this effect can be reversed by steroid administration. As both splice variants result in identical proteins, the significance of this finding is unclear, although differences in

their 5'-untranslated regions may result in different translation efficiencies or transcript stabilities (Kwak *et al.* 1993).

Confocal microscopy revealed that GR and MR are concentrated in approximately 1000 discrete domains within the nuclei of rat hippocampal CA1 neurons. Some domains consist exclusively of GR or MR but a significant amount contain both (van Steensel *et al.* 1996). These heterogenous clusters present a possible way for MR and GR to interact and regulate gene expression in a coordinated manner. However, in other cell types, these receptor clusters may not colocalise with RNA polymerase II and are therefore unlikely to be involved in transcriptional activation (van Steensel *et al.* 1995). Nevertheless, these clusters have added significance to the discovery that GR and MR are capable of forming heterodimers *in vitro* (Trapp *et al.* 1994) which has implications for the regulation of gene transcription in muscle and kidney cells and in certain regions of the brain, including the hippocampus, where MR and GR colocalise (Trapp and Holsboer, 1996).

1.7.3 11β-Hydroxysteroid dehydrogenases in the brain

The structure and mechanism of action of the GR and MR have been explained (see **section 1.5.2**) as has the the importance of MR colocalisation with 11β-HSD-2 in maintaining specificity. In the absence of this crucial enzyme, the MR will be occupied by glucocorticoid. Indeed, the MR has a higher affinity for glucocorticoid than the GR. This is important in some regions of the brain. 11β-HSD-1 (see **section 1.5.5**) may reduce or oxidise the 11-oxygen function although in tissues such as liver, it acts principally as a reductase.

MR in the brain has been reported on several occasions to bind corticosterone and aldosterone *in vivo* with equal affinity, suggesting an absence of 11β-HSD-2. However, individual regions differ. The hippocampus has low enzyme activity so one would expect its MR to be nonselective. The anterior hypothalamus and parts of the brain stem are selective and have been found to possess 11β-HSD-2 transcripts (Zhou *et al.* 1995). 11β-HSD activity has been demonstrated in homogenates of rat cerebellum and hippocampus at levels of 10–30% of those found in the kidney and liver (Moisan *et al.* 1990; Seckl, 1997). The lower levels of 11β-HSD-2 in certain regions of the thalamus may modulate the access of glucocorticoids to receptors, perhaps affecting sensory processing, but such low levels of 11β-HSD-2 activity are difficult to detect against the more intense background of 11β-HSD-1 activity (Robson *et al.* 1998).

11β-HSD-2 is present in some regions of the neonatal rat brain, particularly the thalamus and cerebellum. In the early weeks of life, these levels fall sharply as the enzyme becomes localised to fewer cells which also tend to produce less enzyme. In the adult rat brain, high 11β-HSD-2 expression is found only in the SCO, the ventromedial nucleus of the hypothalalmus (VMN), scattered cells within the amygdala and the nucleus of the solitary tract (NTS) (Robson *et al.* 1998). The high levels of 11β-HSD-2 in the neonatal cerebellum and thalamus are thought to attenuate the neurotoxic effects of excess glucocorticoid rather than to confer MR selectivity on these regions, which possess little or no MR anyway.

The reversible form of the enzyme, 11β-HSD-1 is also widespread in brain cells, more so in neurons than glia. Immunostaining has detected it in axons and dendrites, as well as within the cell body (Sakai *et al.* 1992). There has been much debate over whether brain 11β-HSD-1 acts predominantly as a dehydrogenase or a

reductase *in vivo*. High glucocorticoid levels predispose to neuronal death and, indeed, may be part of the mechanism of degeneration following hippocampal damage. Recent experiments used kainic acid, a nonspecific neurotoxin, which was administered to the rat hippocampus (Ajilore and Sapolsky, 1999). If reductase activity was predominant, the higher local corticosterone concentrations would be expected to exacerbate the neurotoxicity of kainic acid whereas dehydrogenase activity would tend to be protective. The authors concluded that reductase activity was predominant in the hippocampus. Its effect could be blocked by carbenoxolone.

1.7.4 Corticosteroids and brain function

In vivo studies of corticosteroid action in the brain have followed one of two approaches. In intact animals, it can be assumed that the high-affinity MR is already substantially saturated by endogenous corticosteroids and that exogenous corticosteroids activate the GR. In adrenalectomised (ADX) animals, receptors will be unoccupied and MR- or GR-selective ligands can be used to distinguish GR- and MR-mediated physiological actions (Joëls, 1997). Other *in vitro* studies have tested the neuronal activity in brain slices from animals pretreated with steroids by measuring ion fluxes and electrical activity.

Neuronal excitability

Like most cells, the neuron has a high intracellular K^+ concentration and a low Na^+ concentration, generated and sustained by the Na^+-K^+ ATPase pump. The axonal membrane is therefore polarised with the intracellular surface being negatively charged compared with the extracellular surface. Thus, in the quiescent axon, there exists a potential difference – the resting potential – of –60mV. A nerve impulse or action potential is generated by a progressive reversal of polarity – i.e. depolarisation

– along the axon (see **figure 1.7b**). This is followed rapidly by a Na^+-K^+ATPase-facilitated repolarisation. Factors which lower the resting potential will sensitise the neuron to produce an action potential; those which increase it (i.e. hyperpolarise the neuron) will desensitise it.

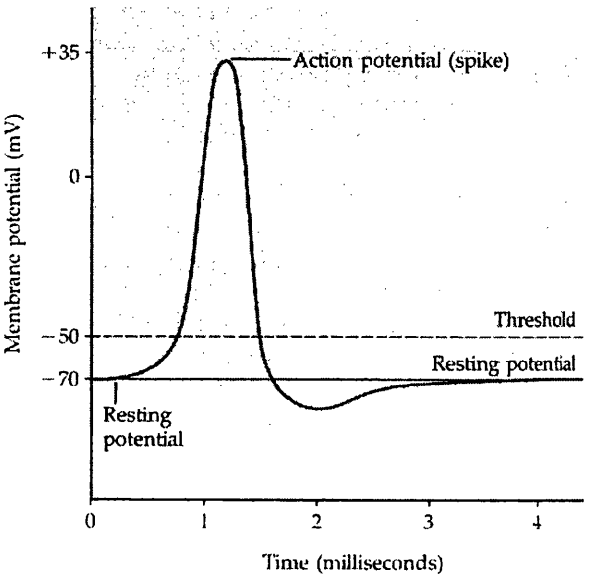


Figure 1.7b Depolarisation of an axon membrane resulting in an action potential.

Transmembrane ion fluxes are controlled by voltage-dependent sodium and potassium channels (VDSC and VDKC respectively). On arrival at the terminal synapse, the action potential operates voltage-dependent (VDCC) calcium channels which couple excitation to neurotransmitter release. Studies of the adult rat CA1 hippocampal neurons show that changes in protein synthesis caused by MR- or GR-mediated transcription alter the membrane properties of neurons, affecting ion channel function and receptor-mediated neurotransmitter responses.

Studies of corticosteroid effects on neuronal excitability have been performed in the spinal cord, hypothalamus and hippocampus. Only the hippocampus shows

any effects with the distinctive time-lag that can be attributed to classical GR or MR action (Joëls, 1997). Corticosteroids do not alter rat CA1 hippocampal resting membrane potentials and membrane resistances under basal conditions but only when the cells are hyperpolarised or depolarised. This suggests that corticosteroids have a homeostatic role in the brain (De Kloet *et al.* 1998).

Corticosteroids affect the inwardly-rectifying potassium current, I_Q, in adult rat CA1 neurons, which is a device activated by very negative membrane potentials to prevent strong hyperpolarisation. The current is small when recorded 1–3 hours after predominant MR activation but increases when GRs are also occupied. Activation of GRs alone does not induce the large response, showing that cooperativity between MR and GR is necessary. This effect is known to require protein synthesis (Karst *et al.* 1993).

Depolarisation of hippocampal neurons induces a short burst of action potentials, during which there is considerable calcium influx. After prolonged stimulation, the cell ceases firing due to the activation of calcium-dependent K^+ channels and consequent K^+ efflux. This is called accommodation, and prevents the transmission of further excitatory signals. When the depolarising stimulus ceases, this K conductance (I_{AHP}) is slowly deactivated leading to a brief lowering of membrane potential termed the afterhyperpolarisation (AHP) (see **figure 1.7c**).

The predominant occupation of MRs (i.e. at low glucocorticoid levels) reduces accommodation and the amplitude of AHP, indicating that calcium-dependent K^+ conductance is suppressed. When GR is also occupied (i.e. at higher agonist concentrations), accommodation is enhanced as well as the amplitude and duration of AHP; calcium current amplitudes (I_{Ca}) are increased considerably (Joëls and De Kloet, 1989; Joëls and De Kloet, 1990). ADX animals have elevated I_{Ca} and AHP

responses intermediate to those found in MR and MR+GR occupation (see **figure 1.7d**) (Karst *et al.* 1994). These effects take at least an hour to develop and can be prevented by the inhibition of protein synthesis (Karst and Joëls, 1991). The mechanism of action, which has yet to be resolved, may involve a direct effect on calcium-dependent K^+ channels, or an alteration in the calcium availability within the cell through factors such as Ca-influx, local intracellular buffering and calcium efflux from the cell.

To summarise, hippocampal output is maintained at a relatively high tone when MRs are predominantly activated, and is reduced when GRs are also activated.

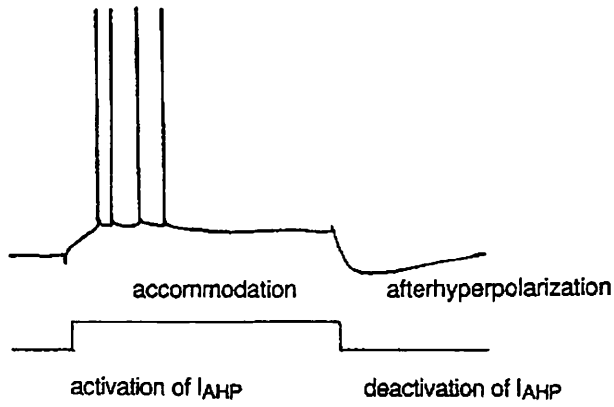


Figure 1.7c Accommodation and afterhyperpolarisation in a rat CA1 hippocampal neuron (Joëls, 1997).

Neurotransmitters

Neurotransmitters are released, usually from granules in the neuron terminus, into the synaptic spaces where they interact with specific postsynaptic receptors to propagate the nerve impulse or to institute processes such as secretion or muscle contraction. Corticosteroids affect the components of these systems also. The

principal neurotransmitter systems are discussed individually below and their effects in combination with corticosteroids are summarised in **table 1.7**.

Occupied Receptors	Transmitter	Action	Consequence
MR	mACh	Less depolarisation	Less modulatory input
	$5HT_{1A}$	Less binding/hyperpolarisation	
	EPSP/FP	Stable with repeated stimulation	Stable amino acid transmission
	IPSP	Stable with repeated stimulation	
	AHP	Less accommodation/AHP	Neuroprotection
	Ca	Less influx	
MR + GR	mACh	More depolarisation	Initial reduction in excitability
	$5HT_{1A}$	More synthesis and turnover; hyperpolarisation restored	
	NA	Less cAMP; more accommodation	
	EPSP/FP	Attenuated with repeated stimulation; more free glutamate	
	sIPSP	Decreased	
	AHP	More accommodation/AHP	
	Ca	More influx, less extrusion	Delayed increase in vulnerability to neurodegeneration
	Glucose	Less uptake/breakdown	

Table 1.7 Effects of predominant MR or MR+GR occupation in combination with neurotransmitters in hippocampal subfields. MR activation stabilises excitability in the CA1 area and protects neuronal integrity in the dentate gyrus. In the short-term, GR-mediated actions reduce local excitability but become damaging over a longer timescale (Joëls and De Kloet, 1994).

Glutamate Glutamate has three types of receptor, each named after their preferred agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) and kainate (KA). They are ionotropic i.e. they contain a cation channel. Glutamate causes the channel to open, thus depolarising the cell

towards its action potential-firing threshold. Therefore, glutamate is an excitatory peptide.

Treatment of ADX rats with high corticosterone doses increases brain basal and stimulated extracellular glutamate levels (Stein-Behrens *et al.* 1992). This could be due to increased release of glutamate or to decreased uptake by neurons and glia. In contrast to skeletal muscle (Max *et al.* 1988), glutamate synthetase synthesis is not regulated by corticosteroids in the brain (Tombaugh and Sapolsky, 1990).

Extracellular recording of excitatory postsynaptic potentials (EPSPs) can be performed on a group of cells, where the number of cells generating an action potential determines the amplitude of the so-called population spike. Predominant MR activation (and GR antagonism) is associated with larger population spikes. Occupation of GRs as well as MRs sharply reduces the population spike upon repeated stimulation. This effect is enhanced by elevated extracellular calcium concentrations, indicating that it is at least partly due to increased Ca-influx. In ADX animals, this population spike response is also attenuated, indicating that the dose-response relationship for corticosterone-mediated effects on glutamate transmission follows an inverted U-shape (Joëls and De Kloet, 1994). Response to GR agonists usually occurs within 20 minutes which is rapid for a gene-mediated event. However, rapid responses to steroid which require mRNA and protein synthesis that occur within 30 minutes have been described (Wehling, 1995). The effects persist for less than an hour, suggesting rapid turnover.

γ-Aminobutyric acid (GABA) This transmitter system is inhibitory. Synaptic stimulation of the CA1 hippocampal area induces an EPSP followed by a sequence of fast inhibitory postsynaptic potentials (fIPSPs) and slow inhibitory postsynaptic potentials (sIPSPs). GABA mediates fast inhibition through its ionotropic $GABA_A$

and $GABA_C$ receptors which admit negative ions – mainly Cl^- – but not positive ones, thus holding the membrane potential at a resting or even hyperpolarised value and making depolarisation of the cell more difficult. $GABA_A$ receptors are to be found on neurons and glial cells. They also bind drugs such as benzodiazepines, barbiturates and certain steroids which compete with GABA and reduce the proportion of time that its ion channel is open. $GABA_A$ receptors mediate fast inhibitory postsynaptic potentials (fIPSPs), which are not greatly affected by changes in MR/GR occupation, although very high corticosterone concentrations depress inhibitory responses to GABA (De Kloet *et al.* 1998). Slow inhibitory postsynaptic potentials (sIPSPs) are mediated through $GABA_B$ receptors which are affected by steroids. Unlike $GABA_A$ and $GABA_C$, $GABA_B$ is linked, through a G-protein, to potassium and calcium channels. Repeated stimulation of rat hippocampal CA1 pyramidal neurons results in a slow decline in the $GABA_B$ receptor-mediated IPSP. In ADX rats and at low agonist doses where predominantly MR are occupied, the amplitude of the sIPSP remained more stable. At higher doses where MR and GR are both occupied, there is a gradual decline in sIPSP. This mirrors the U-shaped dose-response observed with glutamate-mediated EPSPs. The difference between steroid modulation of fIPSP and sIPSP would appear to rule out a steroid-mediated reduction in GABA release, except possibly at very high steroid concentrations.

To summarise, where MRs are predominantly activated, both excitatory and inhibitory outputs are stable. When GRs also become occupied, excitatory transmission, and therefore CA1 hippocampal output, is reduced. At higher steroid concentrations, inhibitory mechanisms are also impaired. Therefore, ionic conductances and the responsiveness of transmitters may vary throughout the day

and after stress due to the changing levels of circulating corticosteroid (Joëls *et al.* 1994).

Noradrenaline (NA) NA can cause increased excitability within the CA1 hippocampal area. This effect is achieved via β-adrenergic receptors and cAMP, resulting in the blockage of the slow calcium-dependent potassium conductance and thus suppressing AHP and accommodation. The occupation of GRs (high corticosterone levels) reduces this effect, after an hour's delay (Joëls and De Kloet, 1989). The effect of NA is inversely related to the initial plasma corticosterone concentration (Joëls *et al.* 1991). The accommodation-suppressing effect of NA is even more pronounced in ADX animals (Joëls and De Kloet, 1994). In addition, mild glucocorticoid excess has been reported to increase pressor sensitivity to noradrenaline in the rat (Russo *et al.* 1990) and in man (Whitworth *et al.* 1986).

Dopamine (DA) Corticosteroids increase DA turnover observed in mouse and rat brain but these effects are transient. Adrenalectomy causes decreases in the number of DA receptors in certain regions of the brain and this effect can be reversed by the administration of dexamethasone (Biron *et al.* 1992).

Acetylcholine (ACh) Two classes of membrane receptor are triggered by acetylcholine, the nicotinic (nACh) and the muscarinic ACh receptors (mACh). High chronic corticosterone treatment has been reported to decrease nACh binding strength in mouse brain, while chronic stress increases mACh binding strength in cell preparations from rat hippocampus. In CA1 neurons, small postsynaptic depolarisations induced by the cholinergic analogue carbachol (CCh) acting through mAch are observed during predominant MR activation; additional GR activation significantly increases this CCh-induced depolarisation. However, these steroid effects are probably of little consequence when compared with other CCh actions in

the CA1, such as the evocation of EPSPs and IPSPs, which are unaffected by steroids (Joëls and De Kloet, 1994; Hesen and Joëls, 1996).

Serotonin (5HT) The effects of corticosteroids upon the serotonergic system are more pronounced. Tryptophan is actively transported into the brain and converted by tryptophan hydroxylase (TPH) into 5-hydroxytryptophan, the rate-limiting step in serotonin formation. It is then converted to 5-hydroxytryptamine (5HT or serotonin) by amino acid decarboxylase and stored in vesicles at nerve terminals. Serotonin transporters located on serotonergic nerve terminals remove free 5HT from the synapse, catabolising and excreting it. Most 5HT-containing cell bodies are found in the two raphe nuclei of the brainstem. The effects on the raphe-hippocampal 5HT system, which sends ascending projections from the median raphe into the hippocampus, septum, hypothalamus and amygdala will be described here. Fourteen mammalian 5HT receptor subtypes have been identified thus far, comprising ligand-gated ion channels, neurotransmitter transporters and G protein-coupled receptors. Each subtype has its own distinctive distribution and corticosteroids regulate their expression. The $5HT_{1A}$ receptor is the best characterised and is distributed throughout the brain, with particularly high levels in the hippocampus, hypothalamus, septum, amygdala and dorsal raphe. This distribution correlates well with that of GR (Reul and De Kloet, 1986; McKittrick and McEwen, 1996). The $5HT_{1B}$ receptor is far less abundant and is found mainly in the hippocampus, striatum, cerebellum and raphe. The $5HT_1$ receptors are coupled to G proteins, sharing an ability to inhibit adenylate cyclase and a very high affinity for 5HT. $5HT_{1A}$ and $5HT_{1B}$ are both upregulated within the dentate gyrus following adrenalectomy; $5HT_{1A}$ also increases in CA2–4 regions of the hippocampus.

Physiological doses of corticosterone reverse this effect (Mendelson and McEwen, 1992).

Steroids also affect 5HT synthesis through TPH. They do not affect enzyme quantity but have a permissive effect on TPH activity, with ADX reducing and corticosterone enhancing it (Azmitia and McEwen, 1969). Dexamethasone administered to ADX rats, on the other hand, reduces 5HT turnover. This difference between corticosterone and dexamethasone treatments suggests that MR and GR may have different effects, with MR stimulating and GR inhibiting 5HT synthesis. This would result in an inverted U-shaped dose-response to corticosterone like that observed in glutamate transmission (Joëls and De Kloet, 1994).

The $5HT_{1A}$ receptor-mediated hyperpolarisation of the membrane is the principal effect of 5HT in CA1 hippocampal neurons. Predominant MR occupation suppresses this hyperpolarisation by comparison with ADX or adrenally intact animals. Additional GR occupation increases the amplitude of the hyperpolarisation, causing marked suppression of excitatory transmission in the CA1 region after a delay of approximately 2 hours (Joëls and De Kloet, 1992). The timescale, together with the fact that corticosteroids do not have this effect in the presence of protein synthesis inhibitors, supports a genomic mechanism of steroid action (Karst and Joëls, 1991).

Thus, in general, predominant MR activation tends to evoke small effects on neurotransmitter function while simultaneous MR+GR activation usually results in large effects. Only the effect on noradrenaline action, which is suppressed by high occupation of GR, defies this general trend. The range of corticosteroid receptor occupancies, from predominant MR to MR+GR, represents the range encountered due to circadian rhythmicity and stress induction of corticosteroids. In more extreme

cases, as represented by adrenalectomised animals or, at the other end of the scale, application of very high amounts of corticosteroids to normal animals (or therapeutic doses in man), responses are explicable by MR+GR occupation, thus resulting in the distinctive inverted U-shape referred to previously. This is illustrated in **figure 1.7d**.

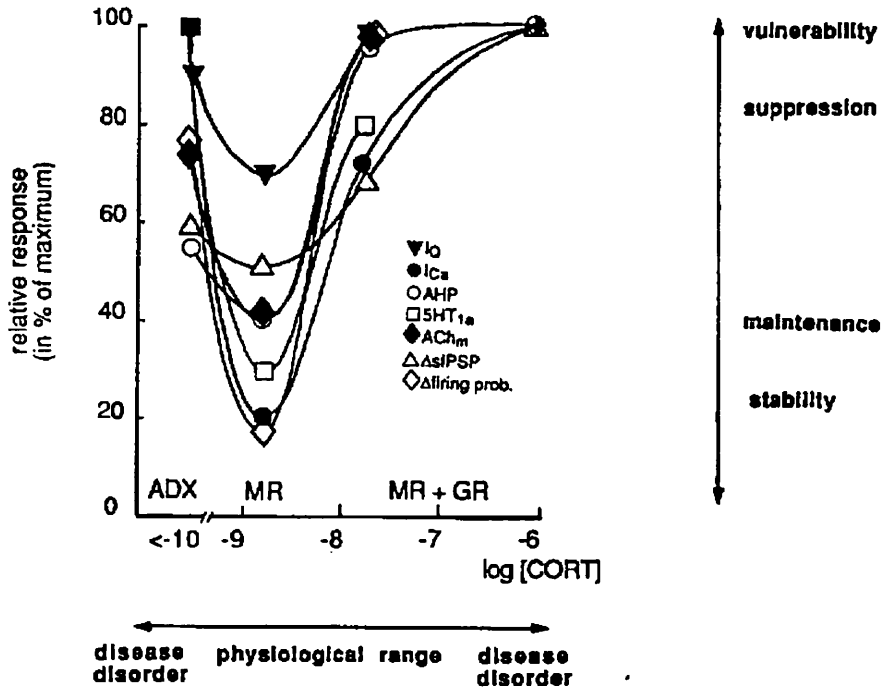


Figure 1.7d Plot of various CA1 neuron responses *in vitro* under varying conditions of MR and GR occupation, resulting in a U-shaped dose-response relationship (Joëls and De Kloet, 1994).

1.7.5 Central effects of corticosteroid deficiency and excess

Predominant MR activation, which would occur during the circadian trough of corticosteroid levels, tends to promote excitability within the CA1, thus resulting in excitatory hippocampal output from steady inputs. The GR-mediated actions reduce excitablility within the CA1 region. This may be of importance in returning the brain

to normal after a stressful experience. Sustained high levels of corticosteroids can be harmful.

Corticosteroid excess and deficiency both result in structural changes to hippocampal neurons. Within three days of adrenalectomy, the degeneration of mature granule cells in the adult rat dentate gyrus – but not the other hippocampal fields – can be seen (Gould *et al.* 1990). This degranulation can be prevented by exogenous corticosterone or aldosterone indicating that MR occupation alone is sufficient to protect the granule cells but that basal secretion of corticosterone normally performs this function. The granule cells are, along with the olfactory bulb, the only region of the adult brain to continually replace neurons in a behaviorally-regulated manner. Adrenalectomy not only increases the rate of degeneration within the dentate gyrus, but also the density of new neurons and glia (Gould *et al.* 1992).

It now appears that NMDA glutamate receptors (see **section 1.7.4**) and corticosteroids interact to regulate neuronal death and neurogenesis in the adult dentate gyrus. By enhancing excitatory input through the hippocampus, MR activation may maintain the balance between cell birth and death in the region. Occupation of MRs may not, therefore, directly affect neurons, but stimulate the excitatory transmission required by the cells for their survival. This theory is supported by the fact that new neurons which appear in the dentate gyrus after adrenalectomy do not possess MR or GR (De Kloet *et al.* 1998).

Chronically high corticosteroid levels also lead to hippocampal neurodegeneration or suppression of neurogenesis, most particularly in the CA3 region, where the apical dendrites of CA3 pyramidal neurons atrophy. The CA1 and dentate fields are also vulnerable (Arbel *et al.* 1994). These effects can be mimicked by subjecting rats to restraint stress. Similar effects have been observed in some

studies of primates and man (Reagan and McEwen, 1997) but not in others (Leverenz *et al.* 1999).

These neurodegenerative effects may be mediated by the NMDA receptors. Blockage of the NMDA receptor prevents dendritic atrophy (Magarinos and McEwen, 1995). Also, the gene for glutamine synthetase contains a HRE and is upregulated by chronically high corticosterone levels, thus depleting the cell of the glutamate required for synaptic transmission.

Short-term GR activation decreases responsiveness to glutamate and reduces excitability by the activation of Ca-dependent K conductances (see **section 1.7.4**), which may produce a beneficial damping effect in stressful situations. Chronic activation may, however, substantially raise intracellular Ca levels, thus increasing responsiveness. CA3 neurons are thought to lack the calcium-buffering capabilities of other hippocampal neurons due to the absence of calcium-binding proteins such as calbindin-D28k and parvalbumin (Sloviter *et al.* 1993).

The turnover of hippocampal neurons in adult life is due to the involvement of hippocampal function in spatial learning and memory. The more an animal requires a knowledge and memory of large spaces, the greater is its hippocampal volume. In mice, an enriched environment enlarges the dentate gyrus volume by stimulating neuronal survival but leaving neurogenesis unaffected. In subsequent tests, those mice with greater dentate gyrus volume performed better at certain spatial learning tasks (Kempermann *et al.* 1997). Similarly, chronically-stressed tree shrews have impaired spatial learning and memory, although whether this is due to dendritic atrophy of CA3 or to reduced dentate gyrus neurogenesis is unclear (McEwen, 1999). Regardless of this, the balance between neuronal apoptosis and neurogenesis, in which corticosteroids play a part, is clearly of importance.

1.7.6 Long-term potentiation and memory

Stressful experiences and steroid hormones produce short-term deficits in episodic and spatial memory, both in animal models and in man. Corticosteroids have been implicated in these effects which are consistent with the modulation of long-term potentiation (LTP), where the same information is repeatedly passed down a particular neuronal pathway. It has often been linked to memory formation. NMDA receptors and calcium influx are required for its induction. Corticosterone potentiation within the CA1 region is also found to follow an inverted-U dose-response relationship, with potentiation strongest in animals with intermediate levels of corticosterone, corresponding to predominant MR occupation. A similar effect occurs in the dentate gyrus. LTP is attenuated in the CA1 region of animals subjected to novel environments or psychological stress, which would increase corticosterone levels (Joëls, 1997). However, no link could be seen between LTP and diurnal corticosterone rhythm in intact rats.

The neuronal cell adhesion molecules, PSA-NCAM, are associated with the maturation of newborn granule cells and with LTP. PSA-NCAM expression is increased within the hippocampus of ADX rats and can be suppressed by corticosterone. Thus, PSA-NCAM could contribute to the stress- and age-related effects of corticosteroids on the hippocampus (Rodriguez *et al.* 1998).

1.7.7 Central corticosteroids and the hypothalamic-pituitary-adrenal axis

The actions of corticosteroids on the hypothalamic-pituitary-adrenal (HPA) axis are summarised in **figure 1.7e**.

Corticosteroids exert a negative feedback effect on the HPA axis (see **section 1.4.1**) via GR in the neurons of the hypothalamic paraventricular nucleus (PVN) and

the anterior pituitary gland. In addition, stress and corticosteroids can convey excitatory inputs to the PVN via GR in the brain stem. The PVN also receives excitatory and inhibitory information from the suprachiasmatic nucleus (SCN), an effect which is modulated by GR in the SCN according to a circadian rhythm. Finally, GABA-ergic neurons exert an influence over the PVN through both MR and GR in the hippocampus and the amygdala (De Kloet *et al.* 1998).

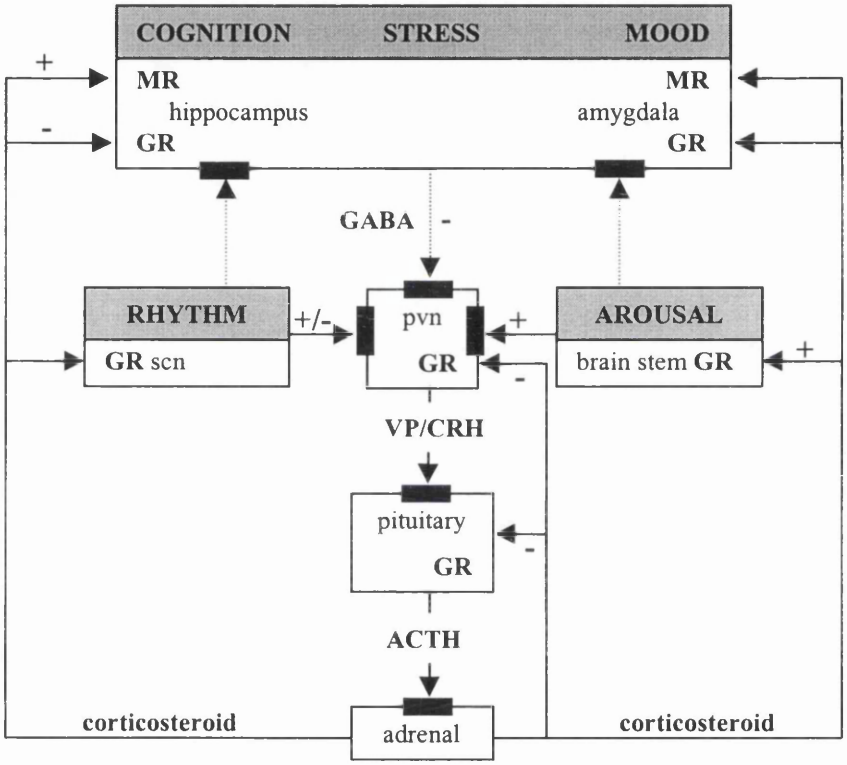


Figure 1.7e Corticosteroid modulation of the HPA axis (De Kloet *et al.* 1998).

The amygdala, which is involved in anxiety and fear responses, secretes CRH. In contrast to the PVN, this is stimulated by corticosteroids. The overall effect of the amygdala appears to be stimulatory.

The hippocampus exerts an inhibitory influence on the activity of the HPA axis, with dorsal hippocampectomy increasing basal activity, particularly at the

circadian trough (Herman *et al.* 1989). Administration of small doses of corticosterone to the dorsal hippocampus suppresses ADX-induced rises in ACTH via MRs which allow corticosterone to maintain the basal HPA activity (Kovács and Makara, 1988) and maintain hippocampal excitability. However, at higher doses of corticosterone, GRs are activated and suppress this output. This GR effect disinhibits the GABA-ergic influence of the hippocampus over PVN neurons, stimulating the release of CRH and ACTH. Therefore, HPA regulation is critically regulated by the balance of MR- and GR-mediated effects (De Kloet *et al.* 1998).

1.7.8 Stress, psychological disorders and ageing

Stress

The HPA axis of animals subjected to chronic stress can become adapted to these conditions, altering adrenal sensitivity to ACTH. This is important, as it demonstrates that emotion can affect the physiological stress response systems. When animals are behaviourally adapted to stress, then the negative feedback actions override the excitatory influences on the HPA axis from the various extrahypothalamic sources. An inability to react to stress results in an imbalance of signals to the PVN. Altered CRH and ACTH responses will then occur.

The set points of HPA regulation and of MR and GR balance are genetically determined but can be reset by interactions between mother and infant, with the effects persisting into adulthood. A pup separated periodically from its mother to be handled will have blunted emotional responses and adrenocortical reactivity in adulthood. The severity of these effects is dependent on the duration, frequency and age of the pup at the time of separation. Moreover, changes in brain GRs can be detected. Sex differences are also apparent; handling decreases hippocampal GR

concentration in males but increases it in females. Wistar rats that have been handled during development have more effective glucocorticoid feedback mechanisms and, in their old age, have lost fewer neurons, possibly suffering less from chronically raised corticosteroid levels. (see **section 1.7.5**) (Meaney *et al.* 1988). While impairing mother-pup interactions has profound effects on the HPA axis, other mild psychological and physiological stressors do not. The important factor in the separation of mother and pup appears to be the loss of physical contact; stress effects can be avoided simply by stroking the animal for 45 seconds. This also increases – but does not completely restore – ACTH secretion (van Oers *et al.* 1998; van Oers *et al.* 1999).

Psychological disorders

Maternal deprivation is a possible predisposing factor to psychological disorders in later life, given its effects upon the HPA axis. In man, about 50% of patients suffering from depression have a hyperactive HPA system and raised cortisol levels. Correction of hypercortisolism by administration of dexamethasone decreases the psychopathology significantly (Dorn *et al.* 1997). Healthy subjects at a genetic risk of depression have HPA feedback resistance and mild hypercortisolism (Holsboer *et al.* 1995). Beneficial behavioral effects are obtained in chronically stressed rats by the application of antiglucocorticoids to the dentate gyrus (De Kloet *et al.* 1988). The suppression of 5HT turnover in the hippocampus by high glucocorticoid levels (see **section 1.7.4**) is similar to that seen during depression.

The balance between MR and GR expression in the brain may also be important to the development of depression. The tricyclic antidepressants restore 5HT and noradrenergic transmission and increase GR and MR levels within the brain, normalising the HPA tone (Seckl, 1996). Administration of

antimineralocorticoids impairs the efficacy of these antidepressants, further implicating MRs in vulnerability to depression (De Kloet *et al.* 1998).

In contrast, enhanced feedback to the HPA axis and reduced cortisol production is characteristic of post-traumatic stress disorder and chronic fatigue syndrome. Post-traumatic stress disorder may involve hippocampal MRs. Chronic fatigue syndrome, on the other hand, appears to be caused by impaired CRH secretion, resulting in reduced adrenocortical output (Demitrack *et al.* 1991).

Ageing

In old animals, the response of the HPA to stress is both more sensitive and more prolonged, leading to relative increases in ACTH and corticosterone levels. Older animals also possess fewer MR and GR in the hippocampus and PVN than younger ones. This would predispose the animal to dendritic atrophy and an increased rate of cell death (see **section 1.7.5**). Aged animals lose the ability to produce hippocampal granule neurons. Adrenalectomy restores this ability (Cameron and McKay, 1999). Higher corticosteroid levels and decreased neurogenesis may, therefore, provide an explanation for age-related memory deficits.

Individuals with Alzheimer's disease have increased cortisol levels which correlate with mental deterioration and decreased hippocampal volume. The neurodegeneration encountered in Alzheimer's disease may, however, result from a resistance to corticosteroids that causes an inability to suppress the immunological defence reaction against deposits of β-amyloid from glial cells, a reaction which would also result in neurodegeneration (De Kloet *et al.* 1998).

1.7.9 Central effects of corticosteroids on blood pressure

As noted previously, the SCO is one of the few brain areas to maintain high 11β-HSD2 expression into adult life. The SCO is located dorsal to the third cerebral ventricle. Infusion of 5ng/h aldosterone into the roof of the third ventricle, close to the SCO, results in increased urinary sodium excretion and adrenal (but not plasma) corticosterone concentrations (Dundore *et al.* 1987). In the Dahl Salt-Sensitive (SS) rat, mineralocorticoid hypertension and salt-induced hypertension can be attenuated or prevented by the destruction of tissue anteroventral to the third cerebral ventricle, the AV3V area, which comprises the anterior hypothalamic periventricular nuclei (PVN), the periventricular and median preoptic nuclei and the anterior wall of the third ventricle. This area integrates information concerning blood volume, thirst, sodium appetite and AVP secretion. However, it is probable that the hypertension of the SS rat involves several genes. For example, it possesses polymorphic alleles of *CYP11B2* which affect mineralocorticoid synthesis.

In order to define the role of mineralocorticoids in the brain, the group of Elise Gómez-Sánchez compared the blood pressures of rats subjected to varying subcutaneous sc and icv doses of GR and MR agonists and antagonists. Continuous icv infusion of doses of aldosterone as low as 5ng/hour into rats resulted in a significant increase in blood pressure. Similar doses had no effect on blood pressure when administered sc. The effects were blocked by simultaneous infusion of the MR antagonist, prorenone (Gómez-Sánchez, 1986). Furthermore, if aldosterone was administered sc at doses which do increase blood pressure, then the administration of the MR antagonist RU28318 blocked this if administered icv but not sc (Gómez-Sánchez *et al.* 1990). However, prevention of hypertension through the icv infusion of RU28318 does not prevent the development of cardiac hypertrophy and fibrosis

caused by systemic mineralocorticoid excess (see **section 1.6.1**). Also, increases in cardiac mass can only be caused by systemic excess and not by icv administration.

Thus the pressor effects of aldosterone are distinct from those on fluid and electrolyte balance, salt appetite and the trophic effects on the vasculature and the heart. Moreover, the icv dose of RU28318 required to block the mineralocorticoid hypertension is less than that required to inhibit the increased salt appetite that is also associated with mineralocorticoid excess. A dose of 10ng/h of aldosterone administered icv induced the same pressor response to 1μg/h sc. The icv dose of 10ng/hour was insufficient to increase circulating aldosterone concentration. It is now known that the increases in Na^+ retention, K^+ excretion, salt appetite and drinking produced by systemic mineralocorticoid excess do not occur after 10ng/h, or even 15ng/h, of aldosterone administered icv although blood pressure does rise (Gómez-Sánchez, 1997). However, the icv infusion of the higher dose of 45ng/h of aldosterone does stimulate salt appetite. The effects of MR antagonists and antisense oligonucleotides support the conclusion that the MR of the amygdala mediate the salt appetite response to mineralocorticoids (Sakai *et al.* 1996). AngII also stimulates salt appetite, although neither AngII nor aldosterone is essential to changes in appetite (Johnson and Thunhorst, 1997).

The mechanism of the steroid action is unclear. The timescale of the effect is consistent with a genomic route. Two possibilties are that they modulate the activity of the local renin-angiotensin system and that they alter neuronal electrolyte metabolism.

Components of the renin-angiotensin system are distributed throughout the brain. AT1 and AT2 AngII receptor subtypes are found in several regions of the brain and both glucocorticoids and mineralocorticoids can alter their binding

properties. GR and MR agonists increase AT1 binding in the hypothalamic PVN and the subfornicular organ (SFO) which projects along the AV3V region (see **figure 1.7a**). Animals treated with both agonists had a greater salt appetite than those treated with either separately. There is also evidence that GR agonists increase angiotensinogen expression in the preoptic region of the adult rat brain. No MR-mediated effect was apparent (Riftina *et al.* 1995).

Mineralocorticoids enhance Na^+ transport in the brain. They increase the number of open amiloride-sensitive Na^+-channels in the luminal face of target epithelial cells (see **section 1.5.3**). The amiloride analogue benzamil, when administered icv at systemically-insignificant doses, prevents the hypertensive response to aldosterone administered icv or sc in rats. Again, salt appetite is not affected (Gómez-Sánchez and Gómez-Sánchez, 1994). It seems likely that, by altering their membrane potential and excitability, mineralocorticoid-induced increases in neuronal $[Na^+]_{ic}$ modulate the activity of the cardiovascular system, (Gómez-Sánchez, 1997).

The central hypertensive effect of aldosterone is mediated by MR (see above) which are protected from glucocorticoids. The 11β-HSD inhibitor carbenoxolone is able to induce hypertension when infused icv at systemically insignificant doses. This would suggest that glucocorticoids are being inactivated by 11β-HSD in regions of the brain involved in blood pressure control. However, this is certainly an oversimplification. Icv infusion of corticosterone at the same time as aldosterone attenuates the blood pressure increase in a dose-dependent manner (Gómez-Sánchez *et al.* 1990). This suggests that glucocorticoids and mineralocorticoids interact at the MR to mediate pressor/depressor responses. This was confirmed by the injection of GR and MR agonist or antagonist pellets into the lateral ventricles of normotensive

rats at high levels. The opposing actions of GR and MR have different latency periods and durations; glucocorticoid agonists and antagonists affected blood pressure 24 hours after administration whereas mineralocorticoids acted earlier (Van den Berg *et al.* 1990).

Corticosteroids injected into the rostral ventrolateral medulla (RVLM) rapidly increase heart rate and blood pressure. This region is important in the control of cardiovascular function. Corticosterone has an excitatory effect on the cardiovascular neurons of the RVLM, such as the bulbospinal pre-sympathetic neurons, which coincides with a rise in blood pressure. Whether corticosterone exerts this effect through putative membrane receptors (see **section 1.5.6**) or through interactions with other neurotransmitters is as yet unknown (Rong *et al.* 1999).

This section shows the important effects of corticosteroids on neural function via their modulation of neurotransmitter efficacy. The main theme of this thesis, however, is the synthesis of corticosteroids within the brain by specific neurons. Clearly, the relationship here must be reversed. Neurotransmitters would be expected to modulate corticosteroid synthesis. What little is known of this relationship is detailed in the following section.

1.8 Corticosteroid Production in the Central Nervous System

From the previous section, it is beyond question that corticosteroids exert profound effects on brain metabolism and consequently on behaviour and many aspects of systemic metabolism. The importance of the brain as a source of these hormones is now examined.

The term *neurosteroids* was devised to describe steroids synthesised within the central nervous system from cholesterol or similar early precursors; pregnenolone and dehydroepiandrosterone (DHEA) synthesised from cholesterol within the rat brain were early examples (Baulieu and Robel, 1990). Local synthesis obviously requires local enzyme activity. The evidence supporting the CNS expression of the genes of each enzyme in the steroid pathway from cholesterol to aldosterone and corticosterone (see **section 1.2.4**) will be described.

StAR: The presence of StAR transcripts in the CNS of the adult rat has been detected by RNase protection assay, RT-PCR and *in situ* hybridisation (Furukawa *et al.* 1998). They are abundant in the cerebral cortex, the pyramidal cell layers of the hippocampus and dentate gyrus, and the olfactory bulb. The cerebellum has the highest levels of all. StAR mRNA levels in the brain are approximately two to three orders of magnitude lower than those in the adrenal gland. No sex differences are apparent.

P450scc: The initial discovery of P450scc within the white matter of the rat brain is widely regarded as one of the most significant findings in neurosteroid research (Mensah-Nyagan *et al.* 1999). Using an antibody raised against bovine P-450scc, the rat form of the enzyme could be detected in tracts of myelinated fibres within the olfactory bulb and throughout the white matter. Cell bodies were also immunolabelled, with the staining restricted to the cytoplasm; it was unclear whether

these cells were neuronal or glial (Le Goascogne *et al.* 1987). Later studies showed immunohistochemical P450scc staining to be confined to glial cells (Iwahashi *et al.* 1990).

CYP11A1 transcripts have been detected in the rat brain by RT-PCR, always at lower levels than those found in adrenal tissue (Mellon and Deschepper, 1993; Sanne and Krueger, 1995; Strömstedt and Waterman, 1995; Furukawa *et al.* 1998). Indeed, brain transcripts cannot be detected by the ribonuclease (RNase) protection assay (Mellon *et al.* 1991; Mellon and Deschepper, 1993; Furukawa *et al.* 1998). There appear to be no significant sex differences in the distribution of brain *CYP11A1* transcripts. Expression is highest in the cerebral cortex but it is also detectable in the amygdala, hippocampus, midbrain and the granule layer of the cerebellum (Mellon and Deschepper, 1993; Sanne and Krueger, 1995). Using primary cell cultures, *CYP11A1* transcription was shown to occur in glial cells (Mellon and Deschepper, 1993; Sanne and Krueger, 1995). Such cells can convert cholesterol to pregnenolone *in vitro* (Jung-Testas *et al.* 1989), and the presence of P450scc within them has also been confirmed by immunoblotting, using two different types of P450scc-specific antibodies (Mellon and Deschepper, 1993). However, P450scc is not confined to glial cells and has recently been found within the Purkinje cells of the cerebellum (Ukena *et al.* 1998).

CYP11A1 transcripts have also now been identified in the human brains of children and adults. Positive results were obtained using hippocampal and frontal lobe specimens, with levels in adults tending to be higher than those in children. Women tended to have higher *CYP11A1* mRNA levels in the frontal lobe than men, although Watzka *et al.* attribute this finding to shortcomings in their RT-PCR

technique rather than to real differences in transcript concentrations (Watzka *et al.* 1999).

Adrenodoxin: The mRNA of adrenodoxin has been found in virtually all the rat tissues that have been examined (Mellon *et al.* 1991; Strömstedt and Waterman, 1995). It is present at a higher level than *CYP11A1* transcripts as it is detectable by RNase protection assay, Northern blotting and RT-PCR. The latter method does not require the sensitivity of Southern blotting to visualise the transcripts; they can be seen directly on ethidium-bromide stained gels.

NADPH-cytochrome P450 reductase: Like adrenodoxin, NADPH-cytochrome P450 reductase has been detected in all tissues that have been tested and at sufficient levels to render Southern blotting unnecessary (Strömstedt and Waterman, 1995).

3β-Hydroxysteroid dehydrogenase: The rat isoenzymes of 3β-HSD, types I–IV, are expressed in a tissue-specific manner. In the brain, transcripts for types I, II and IV can be detected by restriction of RT-PCR products (Sanne and Krueger, 1995). Interestingly, the absent type III has a completely different activity to the other isoenzymes, possessing a 3-ketosteroid reductase activity (Mensah-Nyagan *et al.* 1999). *In situ* hybridisation localised these transcripts to the cerebral cortex, hippocampus, cerebellum, thalamus, hypothalamus and olfactory bulb. The highest levels of 3β-HSD expression within the hippocampus are found within the CA1–2 fields and the dentate gyrus while, in the cerebellum, expression is strongest in the Purkinje cells, granule cells and stellate/basket cells (Guennon *et al.* 1995; Furukawa *et al.* 1998; Ukena *et al.* 1999). In the rat CNS, 3β-HSD mRNAs have only been detected in neurons. However, cultured astrocytes and oligodendrocytes have been

found to express 3β-HSD, suggesting that glial cells may acquire this ability *in vitro* (Jung-Testas *et al.* 1989; Mensah-Nyagan *et al.* 1999).

17α-Hydroxylase: The evidence for 17α-hydroxylase expression in the adult rat brain is conflicting. *CYP17* transcripts have been detected by RT-PCR and Southern blotting in four different brain homogenates – brain stem, cerebellum, cerebral cortex and the remainder of the brain – by Strömstedt & Waterman (1995) but other groups failed to detect transcripts in whole brain homogenates or primary glial cultures by the same method (Mellon and Deschepper, 1993). The fact that expression was not detected in whole brain homogenates may have been due to the confinement of *CYP17* transcripts to a few brain cells. When homogenised with the rest of the brain, these transcripts may become so diluted as to be undetectable.

21-Hydroxylase: Failure to detect *CYP21* transcripts in the rat brain by RNase protection assay led to claims that extraadrenal 21-hydroxylation was mediated by another enzyme (Mellon and Miller, 1989). However, this method assumed that human and rat forms of *CYP21* are relatively similar. RNase protection assays had already failed to pick up brain *CYP11A1* (see above). Unfortunately, the rat *CYP21* sequence is unknown and RT-PCR analysis is therefore not possible. Brain *CYP21* transcription has been demonstrated by RT-PCR in the mouse, where the sequence is known, but the rat gene is sufficiently different to disqualify the use of these same amplifying primers in the rat (Strömstedt and Waterman, 1995).

11β-Hydroxylase: A paper describing the immunohistochemical detection of 11β-hydroxylase in rat brain tissue was published in 1991 (Ozaki *et al.* 1991). As the existence of the highly homologous aldosterone synthase enzyme was not known at the time, the authors understandably made no attempt to ensure that their polyclonal antibodies, raised against bovine 11β-hydroxylase, did not cross-react with the rat

aldosterone synthase enzyme. The fact that immunohistochemistry performed with these antibodies on rat adrenal gland sections produced positive staining in all three adrenocortical regions suggests that their findings are flawed. Nevertheless, the 11β-hydroxylase/aldosterone synthase antibodies still provide interesting data. Immunoblots of rat adrenal and brain homogenates yield a single band with a molecular weight of approximately 45,000. From sequence information, 11β-hydroxylase and aldosterone synthase would be expected to have very similar molecular weights. Immunohistochemical localisation within rat brain tissue require amplification by the avidin-biotin complex (ABC) method. In this way, staining can be identified within the white matter of the brain. Upon closer investigation, it is restricted to the tracts of myelinated fibres in such regions as the cerebellum and the olfactory bulb. There are no age or sex differences and antibody binding can be inhibited by preincubating the antibodies with bovine 11β-hydroxylase.

Since the cloning of *CYP11B1* and *CYP11B2*, most work on 11β-hydroxylase and aldosterone synthase expression has been performed at the transcript level. Several groups have detected *CYP11B1* mRNA in rat brain tissue homogenates using RT-PCR (Mellon and Deschepper, 1993; Strömstedt and Waterman, 1995; Gómez-Sánchez *et al.* 1996), RNase protection assay (Mellon and Deschepper, 1993) and Northern blotting (Erdmann *et al.* 1996). Transcripts can be detected in whole brain samples, as well as cerebellum, cerebral cortex, amygdala, hippocampus and hypothalamus homogenates. According to RT-PCR studies, female rats have consistently higher *CYP11B1* transcript levels in the hippocampus than males (Mellon and Deschepper, 1993). The strongest *in situ* hybridisation signal is in the cerebral cortex. A weak signal is also seen in the hippocampus but this is probably a non-specific interaction as it also appears in control sections exposed only to a sense

probe (Erdmann *et al.* 1996). The positive signal cannot be seen in the white matter, and is apparently confined to nerve cells. *CYP11B1* mRNA cannot be detected in primary cultures of glial cells (Mellon and Deschepper, 1993). Brain minces are able to convert DOC to corticosterone, proving that 11β-hydroxylase activity is present in the rat brain (Gómez-Sánchez *et al.* 1996).

Aldosterone synthase: Apart from immunohistochemical detection of aldosterone synthase due to the cross-reacting bovine 11β-hydroxylase polyclonal antibodies (see above), the occurrence of aldosterone synthase in the brain has been investigated at the transcript level. One group failed to find *CYP11B2* transcripts in brain tissue by RNase protection assay or RT-PCR, nor could they be detected in primary cultures of glial cells (Mellon and Deschepper, 1993). However, others have since used RT-PCR to detect *CYP11B2* mRNA in whole brain and in cortex, cerebellum, brain stem, hippocampus, hypothalamus and amygdala homogenates (Strömstedt and Waterman, 1995; Gómez-Sánchez *et al.* 1997). Brain minces converted DOC to aldosterone, corticosterone, 18-OH-DOC and, in the greatest amounts, to 11-dehydrocorticosterone.

Evidence of specific functions for locally-synthesised corticosteroids is sparse. 19-Ethynyldeoxycorticosterone irreversibly inactivates aldosterone synthase (Johnston *et al.* 1995). When administered sc to the Dahl SS/jr rat, it decreases salt-induced blood pressure. The icv infusion of 19-ethynyldeoxycorticosterone in the Dahl SS/jr rat is able to achieve this effect at far lower doses (Gómez-Sánchez *et al.* 1997). Thus, aldosterone produced within the brain may be capable of exerting real physiological effects.

There is preliminary evidence, therefore, that *CYP11B1* and *-B2* are expressed in the brain but, because of the similarity of their transcripts and protein products,

ambiguity remains. This is exacerbated by the very low levels of expression. The following experimental work, using a variety of biochemical and cell and molecular biological methods, is designed to demonstrate specific transcription, translation and synthetic activity and to identify more precisely those regions of the brain where this occurs.

Chapter 2

Materials And Methods

Unless otherwise stated, all standard chemicals and reagents were purchased from Sigma (Sigma-Aldrich Chemical Co. Ltd., Poole, Dorset, U.K.). For further details of buffers, reagents, etc. see **appendix 3**.

2.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) And Southern Blotting Of Steroidogenic Gene Transcripts

Due to the instability of RNA, extensive precautions were taken to prevent the contamination of RNA solutions and the various reagents with RNases. Water treated with diethyl pyrocarbonate (DEPC), an inhibitor of RNase activity, was used for the preparation of RNA solutions and RT reactions. All consumables were baked or autoclaved, as appropriate, before use.

2.1.1 RNA isolation

Two-month-old female Wistar-Kyoto rats fed on normal diets (Labsure Diets, Cambridgeshire, U.K.; 113 mmol Na^+/kg and 187 mmol K^+/kg) were killed by lethal overdose of halothane when approximately two months old. Tissue was removed and immediately immersed in liquid nitrogen, then stored at -70°C until required.

To isolate RNA, 4ml ice-cold RNAzol B (Biogenesis, Poole, U.K.) was placed in a 14ml polypropylene Falcon tube (Becton Dickinson U.K. Ltd., Oxford, U.K.). ~1cm^3 of tissue was added to the tube and homogenised at full speed for approximately 15 seconds using a Polytron homogeniser (Kinematica AG, Littau-Lucerne, Switzerland). 400µl ice-cold chloroform was added to the homogenate

before being mixed and placed on ice for 5 minutes. The tube was then centrifuged at 12,000g for 15 minutes at 4°C. The upper layer was retained and transferred to autoclaved 15ml Corex tubes. An equal volume of isopropanol was added to the tube, mixed by inversion and left on ice for 15 minutes. The tube was centrifuged at 12,000g for 15 minutes at 4°C to give an RNA pellet. (If the pellet was especially large due to the presence of glycogen, it was resuspended in 2ml of 4M lithium chloride and centrifuged at 12,000g for 15 minutes at 4°C before proceeding with the rest of the protocol.) The supernatant was removed and the pellet resuspended in 2ml of 70% ethanol before being centrifuged at 7,500g for 10 minutes at 4°C. Using a sterile Pasteur pipette, as much of the ethanol as possible was removed and the pellet left on ice to dry. The pellet was resuspended in 100µl of cold DEPC-treated H_20. The RNA solution was stored at -70°C.

2.1.2 Analysis of isolated RNA

Agarose gel electrophoresis: A 1% (w/v) agarose gel was prepared as described in section 1.1.6. A mixture of 2µl RNA solution, 3µl DEPC H_2O and 1µl 6×loading dye was loaded per well. The gel was electrophoresed at 60V for an hour and then visualised under UV light. Distinct rRNA bands indicated good RNA samples whereas poor or degraded RNA appeared as a smear on the gel.

Spectrophotometry: 5µl of RNA solution was added to 995µl of H_2O in a quartz cuvette (i.e. a 1:200 dilution) and its optical density at 260nm (OD_{260}) measured using a spectrophotometer. An OD_{260} of 1 corresponds to 40µg/ml RNA in an undiluted sample. Therefore, for the diluted sample:

$$x = (OD_{260} \times 40 \times 200)\ \mu g\ ssRNA\ in\ 1{,}000\mu l$$

where x is the RNA concentration of the unknown sample in µg/ml.

OD_{280} of the sample was also measured. Pure preparations of RNA have an OD_{260}/OD_{280} ratio of 2.0. Ratios of 1.8 to 2.3 indicate a good level of purity.

Aliquots of the stock RNA solutions were diluted with DEPC-H_2O to give working solutions with a concentration of 1μg/ml.

2.1.3 Reverse transcription

RT-PCR reactions were performed using the GeneAmp RNA-PCR kit (Perkin Elmer, Warrington, U.K.). The reverse transcription reaction mixture was assembled in GeneAmp thin-walled reaction tubes (Perkin Elmer) on ice:

Solution	Volume
RNA (1μg/μl)	1.0μl
$MgCl_2$ (25μM)	4.0μl
10× PCR buffer II	2.0μl
dATP (10μM)	2.0μl
dCTP (10μM)	2.0μl
dGTP (10μM)	2.0μl
dTTP (10μM)	2.0μl
RNase inhibitor (20U/μl)	1.0μl
MuLV reverse transcriptase (50U/μl)	1.0μl
Oligo-$(dT)_{16}$ primers (25μM)	1.0μl
DEPC-treated H_2O	2.0μl

Control reactions omitting either reverse transcriptase (-RT control) or RNA (water blank) were also prepared. Reaction constituents were mixed briefly and then overlaid with a drop of mineral oil before being incubated on a 480 DNA thermal cycler (Perkin Elmer) under the following conditions:

15 minutes	42°C
5 minutes	99°C
5 minutes	5°C

The resulting first strand cDNA products were stored at -20°C until required for PCR.

2.1.4 Oligonucleotide synthesis

Synthetic oligonucleotide primers were designed from published sequences and obtained, in HPLC-purified form, from a commercial source (Oswel DNA Service, Southampton, UK). Primers were designed to be free of hairpin structures and, where possible, to consist of approximately 50% guanine and cytosine nucleotides with a melting temperature (T_m) of around 65°C. For a list of primers, see **appendix 2**.

2.1.5 PCR of first strand cDNA

Solutions from the GeneAmp RNA PCR kit were combined with the RT reaction product from **section 2.1.3** in GeneAmp thin-walled reaction tubes:

Solution	Volume
RT reaction product	20.0µl
10× PCR buffer II	8.0µl
Sense primer	1.0µl
Antisense primer	1.0µl
$MgCl_2$ (25mM)	4.0µl
AmpliTaq DNA polymerase	0.5µl
H_2O	65.5µl

The reactions were mixed and microcentrifuged briefly before being overlaid with a drop of mineral oil and placed on a Perkin Elmer 480 DNA thermal cycler. The sense and antisense primers relevant to each reaction are listed in **appendix 2** together with their specific annealing temperatures.

The basic template for all PCR incubations was:

1.	Denaturation	94°C for 3 minutes	
2.	Denaturation	94°C for 1 minute	
3.	Annealing	*x*°C for 1 minute	35 cycles
4.	Extension	72°C for 2 minutes	
5.	Extension	72°C for 7 minutes	

Steps 2 to 4 were repeated for 35 cycles before proceeding to step 5. The resulting PCR reactions were stored at 4°C until required for subsequent electrophoresis and Southern blotting.

2.1.6 Agarose gel preparation and electrophoresis

A 1% (w/v) agarose gel was prepared by adding 1g agarose (Life Technologies, Paisley, U.K.) to 100ml 1×TAE buffer. This was heated for 2 minutes at full power in a 750W microwave oven, stirring at 1 minute. 1µl of 10mg/ml ethidium bromide (EtBr) solution was added to the agarose before being poured into a gel tray with a comb and left to set. The comb was removed and the gel placed in an electrophoresis tank (Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K.) containing 1×TAE buffer. 10µl of loading dye was added to the PCR reactions from section 2.1.5 and mixed. 30µl of this was loaded per well. 10µl of the relevant marker (e.g. HaeIII ϕX174 digest or HindIII λ digest (New England Biolabs (U.K.)

Ltd, Hitchin, U.K.)) was loaded in the appropriate wells. The gel tank was connected to a power source and the gel was run for ~1 hour at 100V. The gel was visualised under UV light to ensure that the bands were well separated.

2.1.7 Southern blotting

The agarose gel resulting from the previous section was immersed in 0.25M HCl with gentle shaking, causing the loading dyes in the gel to change colour. The gel was left in the HCl for a further 10 minutes, rinsed in distilled water and then immersed in denaturing buffer for 30 minutes, again with gentle shaking. After this, the gel was rinsed in distilled water and immersed in neutralising buffer for 15 minutes with gentle shaking; this step was repeated.

A capillary blot was constructed. A platform was placed in a container filled with 20×SSC buffer. Three strips of 3MM chromatography paper (Whatman Inc., Maidstone, U.K.) were placed on the platform, dipping into the buffer to act as a wick. On top of this was placed the gel, then a sheet of Hybond-N+ nylon membrane (Amersham Pharmacia Biotech U.K. Ltd., Little Chalfont, U.K.) and a thick wad of paper towels. The membrane and towels were cut to the same size as the gel to avoid short-circuiting of the buffer and care was taken to avoid the formation of air bubbles between the layers. Weights were placed on top of the paper towels, and the whole apparatus was covered with clingfilm and left overnight.

The next day, the membrane was washed briefly in 2×SSC. DNA on the blotted membrane was crosslinked using a Stratalinker 1800 (Stratagene Europe, Amsterdam, The Netherlands) . The blot was wrapped in clingfilm, ensuring that it was kept moist until required for hybridisation.

2.1.8 Radioactive labelling of probes

Primers required for hybridisation to a Southern blot were radioactively labelled with γ^{32}P adenosine triphosphate (γ^{32}P-ATP) (Amersham Pharmacia Biotech U.K. Ltd.) using T4 polynucleotide kinase (New England Biolabs (U.K.) Ltd.) by assembling the following in a sterile Eppendorf tube:

Components	Volume
Primer (10pmol/μl)	5.0μl
H_2O	34.0μl
Polynucleotide kinase (10U/μl)	1.0μl
P.K. buffer (New England Biolabs)	5.0μl
γ^{32}P-ATP	5.0μl

The reaction tube was placed in a radioactive shield box and incubated at 37°C for 1 hour. Addition of the γ^{32}P-ATP was performed behind a perspex shield, and basic precautions against radioactive contamination were observed throughout.

2.1.9 Hybridisation and washes

The blot was placed in a hybridising oven and rotated in hybridising solution for 2 hours at 40°C. The hybridising solution was replaced by sufficient fresh hybridizing solution to cover the blot, and the 50μl of radioactively-labelled primer from **section 2.1.8** was also added. This was left to hybridise overnight at 40°C.

The next day, the hybridising solution was disposed of and the blot incubated with 6×SSC for 15 minutes, at a temperature appropriate to the hybridised primer (see **appendix 2**). The radioactivity of the blot was monitored with a Geiger/Muller tube and, if still uniformly high, the blot was washed for a further 15 minutes with 2×SSC at the same temperature. This was repeated until the backgorund level of

radioactivity had diminished to <30 counts per second. At this point, the blot was wrapped in clingfilm and the radioactive bands detected using either a Fluor-S MultiImager (Bio-Rad Laboratories Ltd.) or X-ray film (Amersham Pharmacia Biotech U.K. Ltd.) with subsequent development in a Kodak X-Omat..

2.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) And Immunoblotting

Preparation of immunoblots was performed under both denaturing/reducing conditions and native (i.e. non-denaturing/reducing) conditions. The basic protocol for denaturing/reducing immunoblotting is given in **sections 2.2.2** to **2.2.8**. Variations to this protocol for native conditions are given in **section 2.2.9**.

2.2.1 Antibody production

The antibodies raised against the rat forms of aldosterone synthase and 11β-hydroxylase were a gift from Professor Celso E. Gómez-Sánchez. They were monoclonal antibodies of the IgG1 subclass and were prepared in mice using synthetic peptides corresponding to hydrophilic areas of aldosterone synthase and 11β-hydroxylase that exhibited minimal homology (Research Genetics Inc., Huntsville, AL, U.S.A.).

The aldosterone synthase monoclonal antibody was generated by immunising CD-1 mice with the multiple antigenic peptide MAP–KVRQNARGSLTMDVQQ corresponding to residues 175-190 (Tam, 1988). The 11β-hydroxylase antibody was generated in Swiss-Webster mice immunised with the peptide KNVYRELAEGRQQS corresponding to residues 272-285 and conjugated to chicken serum albumin. The spleens of the mice with the highest titre were fused to an SP-2 myeloma cell line and the clones selected for their ability to bind

immobilised sonicated zona glomerulosa mitochondria from rats on a low sodium diet. Both antibodies were of the IgG1 type.

Three aldosterone synthase antibodies were supplied by Professor Gómez-Sánchez, all derived from the same mouse fusion but resulting from different clones. These were named AS IG5 HC3, AS IE6 and AS IC11. The 11β-hydroxylase antibody was named 11BIA4.

2.2.2 Tissue homogenisation

Total tissue protein: Two-month-old female Wistar-Kyoto rats that had been fed on normal diets (see **section 2.1.1**) were killed by lethal overdose of halothane. Tissue was removed and placed in ice-cold homogenisation buffer for transport to the laboratory where homogenisation was performed immediately. ~1cm^3 lump of tissue was placed in a 14ml polypropylene Falcon tube (Becton Dickinson U.K. Ltd., Oxford, U.K.) with ~4ml homogenisation buffer. The tissue was homogenised at full speed for approximately 15 seconds using a Polytron homogeniser (Kinematica AG, Littau-Lucerne, Switzerland), until no large lumps of tissue were visible. The Polytron head was changed between different tissue types to prevent cross-contamination of samples. The homogenates were aliquotted and stored at -70°C until required for use.

Mitochondrial protein: Two-month-old female Wistar-Kyoto rats that had been fed on normal diets (see **section 2.1.1**) were killed by lethal overdose of halothane. Tissue was removed and placed in ice-cold isolation buffer for transport to the laboratory where homogenisation in isolation buffer using a Polytron was performed, as above. The homogenate was transferred to polypropylene Falcon tubes and centrifuged at 800g for 7 minutes at 4°C to sediment nuclei, cell debris and red blood

cells The supernatant was decanted to fresh tubes; this centrifugation step was repeated. A crude mitochondrial pellet was formed by centrifuging the supernatant at 6,500g for 15 minutes at 4°C. A single pellet was resuspended in a small volume of isolation buffer, and divided between two fresh tubes which were then filled to capacity with isolation buffer and centrifuged at 6,500g for 7 minutes at 4°C. These pellets were resuspended, combined in a single tube filled to capacity with isolation buffer and recentrifuged at 6,500g for 7 minutes at 4°C. The resulting pellet was resuspended in a small volume of isolation buffer and stored at -70°C until required.

2.2.3 Determination of protein concentration

The protein concentration of a given homogenized sample was calculated using either of the following methods.

Lowry assay: Bovine serum albumin (Fraction V) standards in the range 0 – 100mg/ml were prepared in duplicate, and 1:10 dilutions of samples in triplicate. These were made up to 1ml with distilled H_2O. 100µl 0.15% sodium deoxycholate was added to each and left for 10 minutes at room temperature. 100µl 72% trichloroacetic acid was then added and the standards and samples mixed. The standards and samples were centrifuged at 2500g for 15 minutes at room temperature. The supernatant was discarded and the pellets resuspended in 1ml distilled H_2O. 1ml of reagent A was added to each, mixed and left to stand for 10 minutes at room temperature, after which 0.5ml reagent B was added, mixed and left to stand for a further 30 minutes. Light absorption of the samples at 750nm was measured for each standard and sample. A standard curve was derived from the standards and used to determine the protein concentration of the samples.

Bio-Rad protein assay: Protein concentration was also measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd.). Bovine serum albumin standard solutions (100µl, 0.2-1.4mg/ml) were prepared in duplicate. Bio-Rad reagent concentrate was diluted 1:5 and filtered through Whatman No.1 filter paper (Whatman Inc.). 5ml diluted dye reagent was added to each standard and to 100µl volumes of protein sample. These were mixed, then incubated at room temperature for 30 minutes. Light absorption of the samples at 595nm was measured for each standard and sample. A standard curve was derived from the standards and used to determine the protein concentration of the samples. The Bio-Rad protein assay has been shown to yield results comparable to the Lowry assay under most circumstances.

2.2.4 Polyacrylamide gel preparation

The gel-facing sides of glass plates measuring 20cm × 20cm were cleaned with ethanol and the gel-casting apparatus assembled with 1.5mm spacers. A 10% resolving gel was assembled from the following solutions:

Solution	Volume
Buffer 1	8.5ml
H_2O	11.6ml
30% Acrylamide solution/0.8% N,N'-methylene-bis-acrylamide (Life Technologies, Paisley, U.K.)	11.3ml
50% Glycerol	2.3ml
10% Ammonium persulphate	127.0µl
TEMED	11.5µl

The reagents were mixed well and poured between the gel plates using a pipette. 0.1% SDS was layered over the top of the gel to prevent oxidation and it was left to set for 2 hours. A 3% stacking gel was then prepared as follows:

Solution	Volume
Buffer 2	5.6ml
H_2O	14.4ml
30% Acrylamide solution/	
0.8% N,N'-methylene-bis-acrylamide	2.2ml
10% Ammonium persulphate	225.0µl
TEMED	12.0µl

The 0.1% SDS was poured away from the resolving gel and the stacking gel poured on top, with a comb in position at the top of the plates. The gel was left to set for exactly one hour and then clipped into the gel tank, gaps sealed with vacuum grease and the wells of the tank filled with tank buffer. Air bubbles around the gel were flushed out with a syringe and hypodermic needle.

2.2.5 Precipitation of protein for electrophoresis

A volume of homogenate containing the desired amount of protein (up to a maximum of 200µg) was added to an Eppendorf tube, together with sufficient H_2O to bring the total volume up to 150µl. The following solutions were added, in order:

Solution	Volume
2% Sodium deoxycholate	6.3µl
H_2O	750.0µl
24% Trichloroacetic acid	250.0µl

The contents of the tube were mixed and then centrifuged at 7,000g in a microcentrifuge for 20 minutes at room temperature. The supernatant was poured away and the protein pellet left to dry. The pellet was resuspended in 20µl each of 1M Tris base and Laemmli buffer and the tubes were boiled for 3 minutes. Each protein sample was then loaded into a single well of the gel. 20µl of Amersham full range Rainbow markers (Amersham Pharmacia Biotech U.K. Ltd.) or Bio-Rad Kaleidoscope prestained standards (Bio-Rad Laboratories Ltd.) was also loaded directly on to the gel for size comparison. The gel tank was then connected to a power source and left to run at ~50V until the dye front ran off the bottom of the gel.

2.2.6 Protein transfer

The gel was unclipped from the tank, the plates prised apart and the stacking gel cut away from the resolving gel with a scalpel. The blotting cassette was built up in a container filled with blotting buffer, the resolving gel placed next to a layer of Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech U.K. Ltd.). These were sandwiched by filter paper and sponge, then placed in a blotting cassette. Care was taken to avoid the formation of air bubbles between the gel and the membrane. The cassette was placed into a blotting tank filled with blotting buffer, ensuring polarity was correct. The tank was transferred to a cold room and connected to a power pack. Transfer was performed at 100V for 1 hour 30 minutes at 4°C.

After transfer, the membrane was blocked (see **section 2.2.7**), while the gel was immersed in Coomassie brilliant blue G stain for 2 hours and then in destain overnight in order to confirm the efficiency of protein transfer from the gel to the membrane.

2.2.7 Blocking and antibody incubation

The membrane was washed in distilled water for 10 minutes and then blocked for 2 hours in 100ml of blocking solution at room temperature, with gentle shaking. The membrane was removed from the blocking solution and split into several sections. Each section could be incubated in different concentrations of antibody. One section was incubated without any primary antibody; this served as a control, showing which of the bands detected on the blot could be attributed to primary antibody binding and which to secondary. Antibodies were diluted in antibody diluent (Dako Ltd., Ely, Cambridge, U.K.). Sections of membrane were left in primary antibody solution overnight at 4°C with gentle shaking. The blot was removed from the primary antibody solution and subjected to five vigorous 10-minute washes in generous amounts of TBS/0.4% Tween 20. Horseradish peroxidase-labelled sheep anti-mouse IgG secondary antibodies were used (Scottish Antibody Production Unit, Carluke, U.K. or Amersham Pharmacia Biotech U.K. Ltd.). The membrane was incubated in the secondary antibody solution for 2 hours at 37°C with gentle shaking and then washed vigorously in five 10-minute washes with TBS/0.4% Tween.

2.2.8 Signal detection

Antibody binding to the membrane was detected by enhanced chemiluminescent (ECL) reactions catalysed by the horseradish peroxidase enzymes bound to the secondary antibody. Three different types of ECL system were used: ECL Plus (Amersham Pharmacia Biotech U.K. Ltd.), Supersignal chemiluminescent substrate for Western blotting and Supersignal ULTRA chemiluminescent substrate

(both Pierce & Warriner, Chester, U.K.). All systems, used according to the manufacturer's instructions, involved immersion of the membrane in chemiluminescent substrate for 1–10 minutes. The membrane was then drained of substrate, wrapped in clingfilm and exposed to Hyperfilm-ECL film (Amersham Pharmacia Biotech U.K. Ltd., Little Chalfont, U.K.) for a period of 30 seconds to several hours, depending on the intensity of the signal. The film was then developed in a Kodak X-Omat.

2.2.9 Native PAGE and immunoblotting

Immunoblotting was also performed under non-denaturing conditions. The basic procedure was the same as for the SDS-PAGE and immunoblotting described in **sections 2.2.3** to **2.2.8**, with the following changes:

1) Buffer 1 is replaced by an equal amount of 1.5M Tris (pH 8.8) in the 10% resolving gel mixture.
2) Buffer 2 is replaced by an equal amount of 0.5M Tris (pH 6.8) in the 3% stacking gel mixture.
3) The required amount of protein homogenate is mixed with native loading buffer and loaded directly on to the gel. No pelleting or resuspension is necessary.
4) SDS is omitted from the tank buffer.

2.3 Preparation And Immunostaining Of Cryosections

2.3.1 Preparation of cryosections

Two-month-old female Wistar-Kyoto rats that had been fed on normal diets (see **section 2.1.1**) were killed by lethal overdose of halothane. Tissue was removed

and immediately flash-frozen in liquid nitrogen. Sections with a thickness of 5μm were cut using a cryostat and mounted on 3-aminopropyltriethoxysilane (APES) coated slides by Mr Niall Whyte of the Department of Pathology, Western Infirmary, Glasgow, U.K. Sections were stored at -70°C.

2.3.2 Immunostaining of cryosections (ABC protocol)

All subsequent steps were performed at room temperature; slides were kept in a humidified chamber during incubation steps to prevent drying of samples. Sections were thawed for 10 minutes, fixed in 100% acetone for 10 minutes and washed in PBS for 5 minutes, all at room temperature. A wax ring was drawn round the tissue with an immunohistochemistry pen (Dako Ltd.). Peroxidase activity was removed from the tissue by immersion in 0.5% H_2O_2 in methanol for 30 minutes. Slides were then washed thoroughly in PBS for 10 minutes; this step was repeated. Samples were blocked with 20% rabbit serum (Scottish Antibody Production Unit) in PBS for 30 minutes. The serum was tapped off the sections and replaced with primary antibody diluted in antibody diluent (Dako Ltd.). Sections were left to incubate overnight at room temperature.

The next day, slides were subjected to two 5-minute washes in PBS and then incubated for 30 minutes with biotinylated rabbit anti-mouse secondary antibody (Dako Ltd.) at a 1:200 dilution in 2% rabbit serum and 5% rat serum (both Scottish Antibody Production Unit). The slides were given another two 5-minute washes in PBS and then incubated for 30 minutes with streptavidin-HRP (Dako Ltd.) diluted 1:400 in PBS. Sections were washed twice more in PBS, followed by incubation in 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining solution for 10 minutes in the dark. The slides were washed in PBS for 5 minutes, followed by running tap

water for a further 10 minutes. Sections were counterstained in filtered Harris hematoxylin solution for 1 minute and then washed under running tap water again for several minutes. Sections were dehydrated through alcohols in the sequence 50% ethanol, 70% ethanol, 95% ethanol twice, 100% ethanol twice and, finally, 100% xylene twice. Sections were mounted in DPX mountant under glass coverslips (both BDH, Poole, Dorset, U.K.).

2.4 Preparation And Immunostaining Of Paraffin-Embedded Tissue Sections

2.4.1 Preparation of paraffin-embedded tissue sections

Adult female Wistar-Kyoto rats fed on normal diets were killed by lethal overdose. The tissue was removed and fixed in formalin or 4% (w/v) paraformaldehyde solution for 24 hours. Mr Niall Whyte dehydrated the tissue through a series of alcohols before embedding it in paraffin wax. Sections with a thickness of 5μm were mounted on to APES-coated slides and air-dried.

2.4.2 Deparaffinisation and rehydration of tissue sections

All subsequent steps were performed at room temperature; slides were kept in a humidified chamber during incubation steps to prevent drying of samples. Sections were placed in an oven at 60°C for 30 minutes and then deparaffinised and rehydrated by passing through 100% xylene for 5 minutes twice, 100% ethanol for 5 minutes twice, 95% ethanol for 5 minutes twice and distilled water for 5 minutes. Pretreatments were performed on the tissue at this point (see **section 2.4.3**).

2.4.3 Antigen-retrieval pretreatments of paraffin-embedded tissue sections

The deparaffinised and rehydrated sections were subjected to one or both of these pretreatments before proceeding with the immunohistochemistry protocols described in section **2.4.4** or **2.4.5**.

Microwave oven heating: Slides were immersed in 200ml of target retrieval solution (Dako Ltd.) within a non-metallic container. Empty spaces within the slide rack were filled with blank slides to ensure uniform heating throughout. Optimal conditions for these tissues involved heating the container on full power for two 7-minute periods in a 750 watt microwave oven with turntable; the level of liquid within the container was topped up with distilled water between heatings. The sections were left in the container for 20 minutes to cool at room temperature before proceeding with the immunostaining protocol.

Proteolytic pretreatment: 300ml of 0.01% calcium chloride solution was heated to 37°C in a water bath. 30mg of type XXVII protease was dissolved in this solution immediately before the slides were to be treated. Slides were immersed in the 0.01% protease/0.01% $CaCl_2$ solution for 30 seconds and then washed thoroughly in cold running water.

2.4.4 Immunostaining of paraffin-embedded tissue sections (ABC protocol)

A wax ring was drawn round the tissue with an immunohistochemistry pen and the slides were left in PBS for 5 minutes. Peroxidase activity was removed from the slides by immersion in 0.5% H_2O_2 in methanol for 30 minutes. Slides were then washed thoroughly in PBS for 10 minutes; this step was repeated. Samples were blocked with 20% rabbit serum in PBS for 30 minutes. This was removed and the sections were then subjected to endogenous avidin blocking treatment. This involved

incubation with avidin D blocking solution (Vector Laboratories, Peterborough, U.K.) for 15 minutes, followed by a 5 minute wash in incubation and a 15 minute incubation with biotin blocking solution (Vector Laboratories). The blocking solution was tapped off and replaced with primary antibody in antibody diluent (Dako Ltd.). From this point on, the method was identical to that used for the immunostaining of cryosections, as given in **section 2.3.2**.

2.4.5 Immunostaining of paraffin-embedded tissue sections (CSA kit protocol)

A wax ring was drawn round the tissue with an immunohistochemistry pen and the slides were left in TBS for 5 minutes. Subsequent steps used components from the CSA, peroxidase kit (Dako Ltd.) supplied in bottles labelled 1–12. Endogenous peroxidase activity was removed from the sections by covering them with hydrogen peroxide (bottle 1) for 5 minutes. Slides were rinsed gently in 0.1% Tween 20 in TBS (TBST) from a wash bottle and then immersed in TBS for 5 minutes. Excess buffer was tapped off the slides and the section covered in protein block (bottle 2) for 5 minutes. The blocking solution was tapped off and the section covered in avidin D blocking solution (Dako Ltd.) for 15 minutes. This was removed and replaced by biotin blocking solution (Dako Ltd.) for 15 minutes. The blocking solution was tapped off and primary antibody diluted in antibody diluent was applied and left to incubate for 2 hours at room temperature.

Slides were rinsed with TBST from a wash bottle then immersed in fresh TBST for 5 minutes. The sections were incubated with link antibody (bottle 4) for 15 minutes and then washed in TBST as before. Streptavidin-biotin complex was applied to the sections. Streptavidin-biotin complex was prepared 30 minutes prior to use by mixing thoroughly 40µl each of streptavidin-biotin complex reagent A (bottle

5) and streptavidin-biotin complex reagent B (bottle 6) with 1ml of streptavidin-biotin complex diluent (bottle 7). The streptavidin-biotin complex was removed after a 15 minute incubation and the tissues were again washed with TBST. Amplification reagent (bottle 8) was applied to the section for 15 minutes and then washed away with TBST in the usual manner. Streptavidin-peroxidase (bottle 9) was applied to the slides for 15 minutes and then washed away with TBST. Buffer was tapped from the slides and any excess liquid removed with a tissue before the application of substrate-chromogen solution. Substrate chromogen solution was prepared by dissolving a substrate tablet (bottle 10) in 10ml distilled water mixed with 10 drops of tris buffer concentrate (bottle 11). 200µl of substrate hydrogen peroxide (bottle 12) was added to the solution immediately before use. Substrate-chromogen solution was removed from the slides after 5 minutes. They were then rinsed in distilled water from a wash bottle and counterstained in filtered Harris hematoxylin solution for 5 minutes. The sections were dipped quickly into 37mM ammonia 10 times and rinsed in distilled water. Sections were dehydrated through alcohols in the sequence 50% ethanol, 70% ethanol, 95% ethanol twice, 100% ethanol twice and, finally, 100% xylene twice. Sections were mounted in DPX mountant under glass coverslips.

2.4.6 Quenching of antibody binding by antigenic peptides

Peptides matching the sequences against which the anti-11β-hydroxylase and anti-aldosterone synthase antibodies had been raised were synthesised by Alta Bioscience, Birmingham, U.K. The peptide sequences were KNVYRELAEGRQQS for the anti-11β-hydroxylase antibody and KVRQNARGSLTMDVQQ for the anti-aldosterone synthase antibodies (see **appendix 1**). The peptides were dissolved in TBS and a series of ten-fold dilutions were made. These were mixed with a fixed

amount of primary antibody in antibody diluent (Dako Ltd.) and incubated overnight at 4°C. The peptide/antibody mixture was then incubated with tissue at the primary antibody incubation stage of the immunostaining protocol (see **section 2.4.5**).

2.5 Preparation And Immunostaining Of Fetal Hippocampal Neurons

2.5.1 Preparation of fetal hippocampal neurons for immunostaining

Primary hippocampal neuronal cultures were prepared from embryonic day 18 Wistar rats. Briefly, hippocampi were dissected out according to the methods detailed by Goslin and Banker (Banker G and Goslin K, 1991). Tissue was washed three times in Hanks balanced salt solution (Life Technologies) containing HEPES buffer, pH 7.4, and was then trypsinised (1mg/ml) for 10 minutes at 37°C and dissociated by trituration through a 25-gauge sterile needle. Cells were seeded at a density of 4×10^5 cells per 8 well poly-L-lysine-coated culture slide (Becton Dickinson) in Neurobasal medium (GibcoBRL, Paisley, U.K.) containing B27 growth supplement and 0.5mM L-glutamine and grown in a humidified incubator at 37°C/5% CO_2. The next day, cytosine arabinoside (5mM) was added to the medium to inhibit glial proliferation. Cells were left to grow for 7 days before experiments were carried out. Cultures typically contained less than 5% glia as determined by immunohistochemical staining with glial fibrillary associated protein (GFAP) antibody.

Cells were fixed for immunostaining by immersion in acetone for 10 minutes.

2.5.2 Immunostaining of fetal hippocampal neurons

Fetal hippocampal neurons were immunostained using the CSA peroxidase kit (Dako Ltd.) as described in **section 2.4.5**. No pretreatments were used.

2.6 *In Vitro* Conversion Of ^{3}H-DOC and DOC To Aldosterone And Corticosterone By Fetal Hippocampal Neurons

2.6.1 Preparation of fetal hippocampal neurons for steroid conversion studies

Cells were prepared for conversion studies using the same basic protocol given in **section 2.5.1**. The only difference occurred after dissociation, where cells were seeded at a density of 2×10^{6} cells per 6 well poly-L-lysine-coated tissue culture plate (Becton Dickinson). Cells were then grown as normal.

24 hours prior to the incubation experiment, the composition of the cell medium was altered, with N2 supplement being substituted for B27. This was due to the small levels of corticosterone present in B27.

2.6.2 DNase treatment of RNA

DNase reactions were performed using the RQ1 DNase (Promega, Southampton, U.K.). The DNase reaction mixture was assembled in GeneAmp thin-walled reaction tubes (Perkin Elmer) on ice:

Solution	Volume
RNA (200ng/µl)	50.0µl
$MgCl_2$ (25mM)	20.0µl
10× PCR buffer (Mg^{2+}-free)	10.0µl
RQ1 DNase (1U/µl)	20.0µl

Tubes were incubated at 37°C for 1 hour. The reaction was terminated by the addition of 100µl phenol/chloroform. Tubes were vortexed for 10 seconds then spun at 7,000g for 5 minutes in a microfuge kept at 4°C. The aqueous upper layer was removed and placed in a clean tube with 3 volumes of 100% ethanol and 1/10th volume of DEPC-treated 3M sodium acetate (pH 5.5) to precipitate the RNA. This

was spun at 4°C again at 7,000g for 30 minutes. The supernatant was discarded and the pellet air-dried on ice for 15 minutes. The pellet was then resuspended in 10μL of DEPC-treated water. RNA concentration and purity was determined as described in **section 2.1.2**. RNA was stored at –70°C.

2.6.3 Steroid conversion experiments

Each well of a 6-well plate was incubated with 2mL of 10μM DOC in Neurobasal medium (GibcoBRL, Paisley, U.K.), 1% N-2 supplement and 0.1% gentomycin. Control medium omitted DOC. Medium was removed after 24 hours and stored at –20°C. Cells were suspended in PBS and retained for subsequent protein measurement using the Bio-Rad protein assay kit (see **section 2.2.3**).

2.6.4 Measurement of tritiated steroid products

The medium was extracted with freshly distilled dichloromethane and the extract was evaporated to dryness under nitrogen at 30°C. Unlabelled aldosterone (100μg) and corticosterone (100μg) were added and the residue subjected to paper chromatography (system B5; (Bush, 1952)). The steroid regions were located by both ultraviolet light (220-240nm) and by scanning for ^{3}H (Bioscan System 200 Imaging Scanner) and then eluted in methanol (5ml). The solution was evaporated to dryness and the residue rechromatographed. Finally, the steroids were again located, eluted and their ^{3}H content measured by liquid scintillation spectrometry using Ecoscint scintillant (National Diagnostics, Atlanta, GA, U.S.A.). Pure ^{3}H-standards were subjected to the same procedure to allow correction for recovery. The ^{3}H-standards' specific activities were 50 Ci/mmol for 3H-DOC (ICN Pharmaceuticals Ltd., Basingstoke, U.K.), 50 Ci/mmol for ^{3}H-aldosterone (Amersham Pharmacia) and 49

Ci/mmol for ^{3}H-corticosterone (NEN, Boston, MA, U.S.A.). Recoveries for ^{3}H-aldosterone and ^{3}H-corticosterone were 17.3% and 29.4% respectively. A 2ml aliquot of incubation medium was extracted with dichloromethane, the residue dissolved in methanol (100µl) and ^{3}H content measured. Efficiency for ^{3}H was 63%.

2.6.5 Measurement of nonradioactive steroid products

^{3}H-Corticosterone (25,000cpm) or ^{3}H-aldosterone (25,000cpm) was added to aliquots of incubation medium removed from replicate samples of two cell batches. The samples were extracted with freshly distilled dichloromethane and the extract washed once with water and evaporated to dryness with at 37°C under nitrogen. The residues were chromatographed on paper (system B5 for aldosterone, system B1 for corticosterone) and the appropriate steroid regions located by isotope scanning and eluted in methanol (5ml). Aldosterone samples were evaporated to dryness at 37°C under nitrogen and the residue redissolved in radioimmunoassay diluent (Diagnostic Products Ltd., Gwynedd, U.K.). Aliquots were removed for measurement of ^{3}H content in order to assess recovery. Aldosterone was measured by direct radioimmunoassay (Diagnostic Products Ltd.) and corticosterone by the method of Fraser *et al.* (1973).

2.6.6 GCMS identification of aldosterone

Aldosterone standards were derivatised as follows. 100µg of aldosterone was dissolved in 100µg methanol to which 0.5ml of 0.5M periodic acid (HIO_4) in 1% pyridine (aq) was then added. This was incubated in the dark at room temperature for 1.5 hours to form γ-lactone. 1ml of water was added and steroid extracted by the addition of 6ml of freshly distilled dichloromethane followed by the removal of the

aqueous layer. The extract was washed in 1ml of water, the aqueous layer was again removed and the remainder evaporated to dryness under nitrogen. To the residue was added 60µl of heptafluorobutyric anhydride (HFBA) and 60µl toluene. This was incubated for 30 minutes at 60°C and then evaporated to dryness under nitrogen. The residue was dissolved in 1ml of hexane. 1µl of this was injected on to the gas chromatography mass spectrometer (GCMS) (Varian Saturn GCMS System, Varian, Walnut Creek, California, U.S.A.). The derivatisation is summarised in **figure 2.6a.**

Aldosterone from cell medium samples was prepared by extracting 1ml of the cell medium with 6ml of freshly distilled dichloromethane, which was then washed with 1ml of water and evaporated to dryness under nitrogen. This was partially purified by paper chromatography using system B5 (Bush, 1952). Aldosterone standards were run alongside and used to detect the samples on a UV scanner (Bioscan System 200 Imaging Scanner). Samples were eluted in 5ml of methanol, evaporated to dryness under nitrogen and then derivatised in the same way as the aldosterone standards (see above).

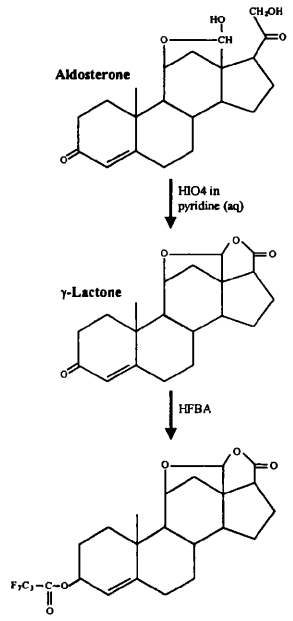


Figure 2.6a Derivatisation of aldosterone for GCMS

Chapter 3

Results: Reverse Transcription-Polymerase Chain Reaction (RT-PCR) And Southern Blotting Of Steroidogenic Gene Transcripts

3.1 Introduction

Total RNA from a range of rat tissues was analysed for the presence of *CYP11B1*, *CYP11B2*, *CYP11B3*, *CYP11A1* and adrenodoxin gene transcripts using the reverse transcription-polymerase chain reaction (RT-PCR). *CYP11B1* and *CYP11B2* are of obvious importance, their products being the terminal enzymes in the cortisol and aldosterone pathways without which the corticosteroids cannot be produced. *CYP11B3* transcription was also investigated in these tissues as comparatively little is known about where and when this occurs. *CYP11A1* transcription was examined due to its position at the start of the biosynthetic pathway, performing the initial conversion step from cholesterol to pregnenolone. Its presence in extraadrenal tissues would give an indication of whether corticosteroid synthesis occurs *de novo* (i.e. from cholesterol), or whether synthesis of aldosterone or cortcosterone relies on the tissue converting intermediary metabolites such as progesterone or DOC. Finally, adrenodoxin is a vital cofactor for aldosterone synthase, 11β-hydroxylase and the side-chain cleavage enzyme; the expression of these enzymes' genes would serve no purpose without the co-expression of adrenodoxin.

The tissues selected for analysis were whole brain, kidney, liver, heart, mesenteric artery and spleen, the last anticipated to act as a negative control. Previous work by other groups had singled out the hypothalamus as a brain region of particular interest. This was also dissected out and subjected to RT-PCR separately.

3.2 Methods

The general RT-PCR, Southern blotting and probing methods for all genes is described in **section 2.1**. The RT-PCR protocols were adapted from previously published work (Nomura *et al.* 1993; Strömstedt and Waterman, 1995).The full sequences of the rat *CYP11B1* and *CYP11B2* genes, together with the corresponding positions of relevant oligonucleotides, are given in **appendix 1**. The sequences of all oligonucleotide primers are listed in **appendix 2**.

Adrenal tissue was used as a positive control throughout. The integrity of isolated RNA was tested by spectrophotometry and agarose gel electrophoresis; the distinctive bands formed by the three ribosomal RNA (rRNA) species (28S, 18S and 5S) which account for 85% of mammalian RNA are not visible in degraded samples. An example of this is given in **figure 3.2a**. RNA integrity was also confirmed through the RT-PCR of transcripts from the constitutively-expressed 'housekeeping' gene for glyceraldehyde phosphate dehydrogenase (GAPDH).

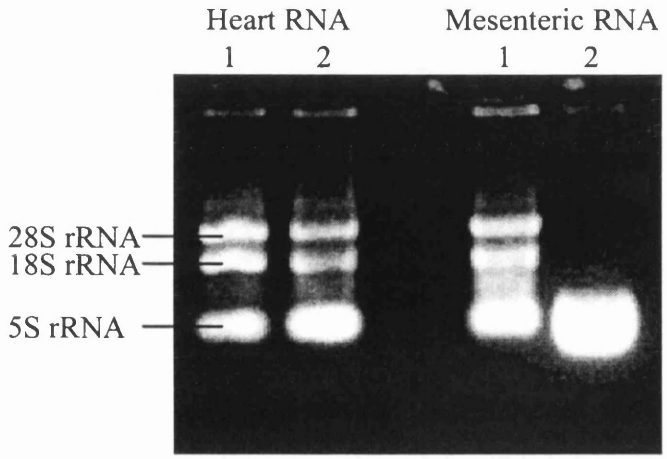


Figure 3.2a RNA isolated from heart and mesenteric artery separated on a 1% agarose gel stained with ethidium bromide and visualised under ultraviolet light. The samples display the distinctive bands of rRNA except for mesenteric RNA sample 2 which has degraded during the isolation process.

The design of oligonucleotide primers in experiments such as these is crucial to the specificity of the reactions. Primers were used which spanned the exon-intron boundaries of the relevant genes. Therefore, if there was any genomic DNA contamination of the RNA sample, it resulted in a larger band size which could easily be distinguished from those caused by the amplification of transcripts. Such genomic bands would also be visible in control samples that had not been treated with reverse transcriptase.

3.3 Results

3.3.1 Adrenodoxin

The RNA from all the tissues tested was found to contain adrenodoxin gene transcripts (see **figure 3.3a**). Indeed, the distinctive 289 base pair band was intense enough to be seen on ethidium bromide-stained agarose gels under ultraviolet light, rendering Southern blotting unnecessary. Control reactions performed in the absence of reverse transcriptase, or with water substituted for RNA, were all negative.

3.3.2 *CYP11A1*

The RT-PCR products were separated on 1% agarose gels and Southern blotted to nylon membranes before probing with the *CYP11A1*-specific oligonucleotide V8822. After exposure to film, this revealed specific bands within the adrenal, brain, kidney, heart and hypothalamus samples at positions on the blot that corresponded to the expected 583bp band size on the original agarose gels (see **figure 3.3b**). A slightly smaller band was also visible in these lanes. No bands were visible in liver, spleen or mesenteric artery RNA reactions, nor in those control reactions performed in the absence of reverse transcriptase or with water instead of RNA.

3.3.3 *CYP11B1*

As with *CYP11A1*, the RT-PCR products were separated on 1% agarose gels and Southern blotted to nylon membranes. The blots were probed with the *CYP11B1*-specific oligonucleotide P5815. Exposure to film revealed bands only in the adrenal, brain and hypothalamus samples (see **figure 3.3c**). These bands corresponded to the expected 342bp band size on the original agarose gels. No bands were visible in the kidney, liver, spleen, heart or mesenteric artery reverse transcriptase reactions. Control reactions were also negative.

3.3.4 *CYP11B2*

RT-PCR products subjected to conditions identical to the previous *CYP11B1* samples were separated on 1% agarose gels and Southern blotted to nylon membranes. The *CYP11B2*-specific oligonucleotide P5814 was used to probe the blots, which were then washed and exposed to film. Bands corresponding to the expected 342bp product size were identified in adrenal, brain, spleen and hypothalamus samples (see **figure 3.3d**). Kidney, liver and heart samples were negative, as were the mesenteric artery sample and the control reactions.

3.3.5 *CYP11B3*

RT-PCR products subjected to the same conditions as those of *CYP11B1* and *CYP11B2* were run on 1% agarose gels, Southern blotted and the resulting membrane probed with the *CYP11B3*-specific oligonucleotide P5816. Despite lengthy exposures to film, no bands, specific or otherwise, could be identified on the blot, even in the adrenal sample.

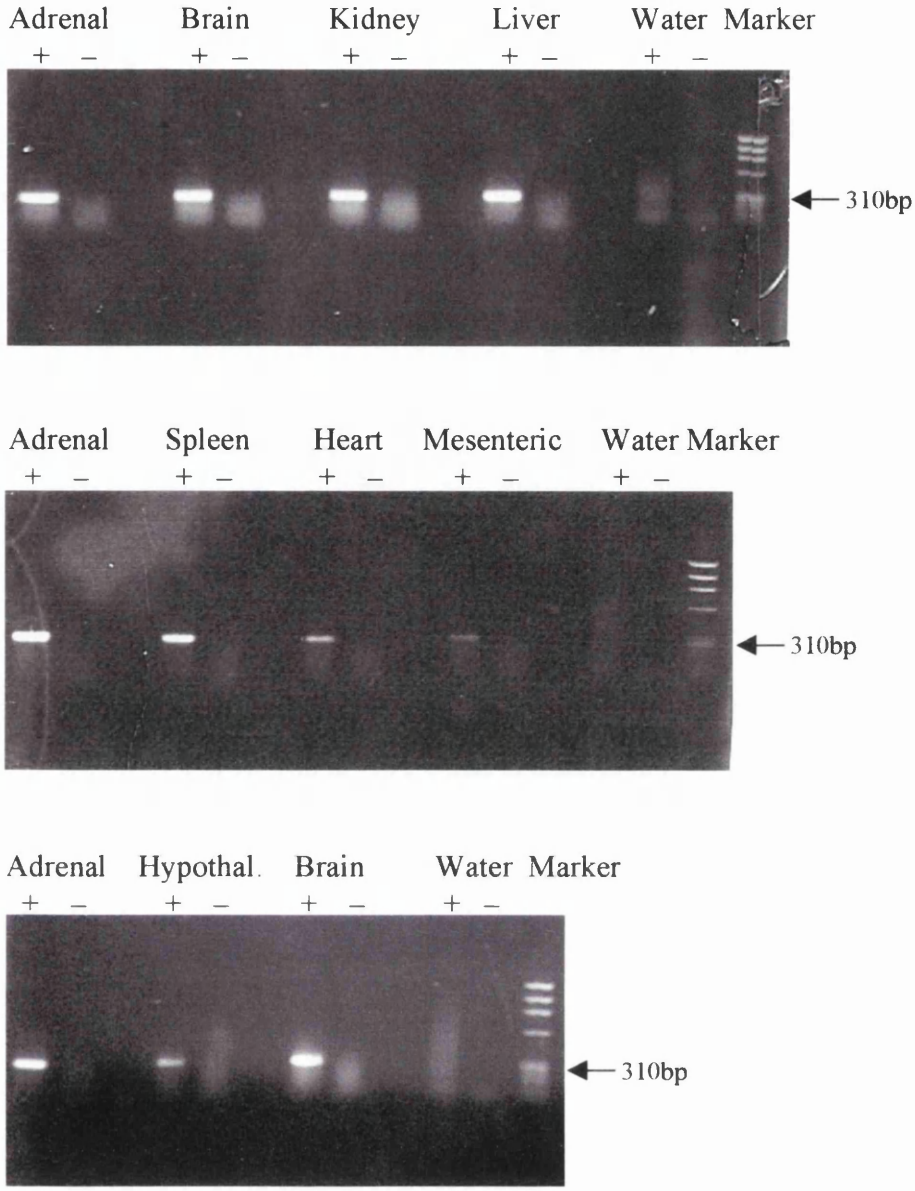


Figure 3.3a RT-PCR of RNAs from various tissues using primers specific for adrenodoxin in the presence (+) and absence (–) of reverse transcriptase. RT-PCR products were separated on 1% agarose gels and detected by ethidium bromide staining visualised under ultraviolet light. RT-PCR products were run alongside HaeIII ϕX174 digest marker; the position of the 310bp marker band is indicated.

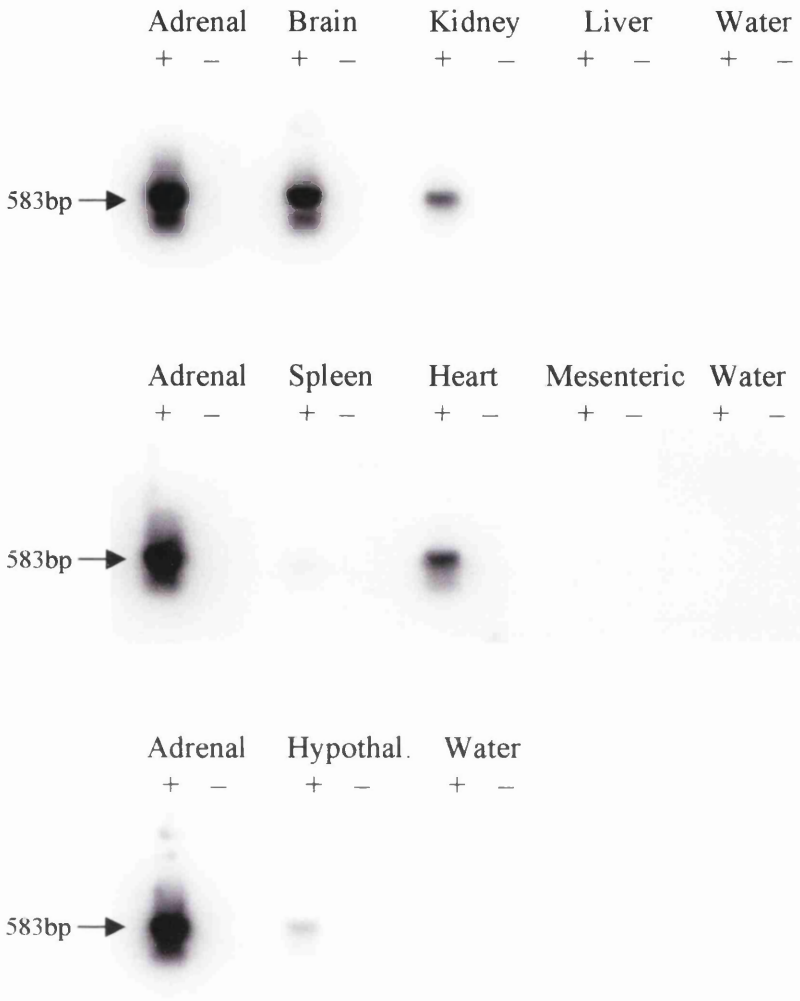


Figure 3.3b RT-PCR of RNAs from various tissues using primers specific for *CYP11A1* in the presence (+) and absence (–) of reverse transcriptase. RT-PCR products were separated on 1% agarose gels and Southern blotted to nylon membranes, then probed with the radiolabelled *CYP11A1*-specific oligonucleotide V8822. The blots were washed and exposed to film. Bands corresponding to the expected size of 583bp are indicated.

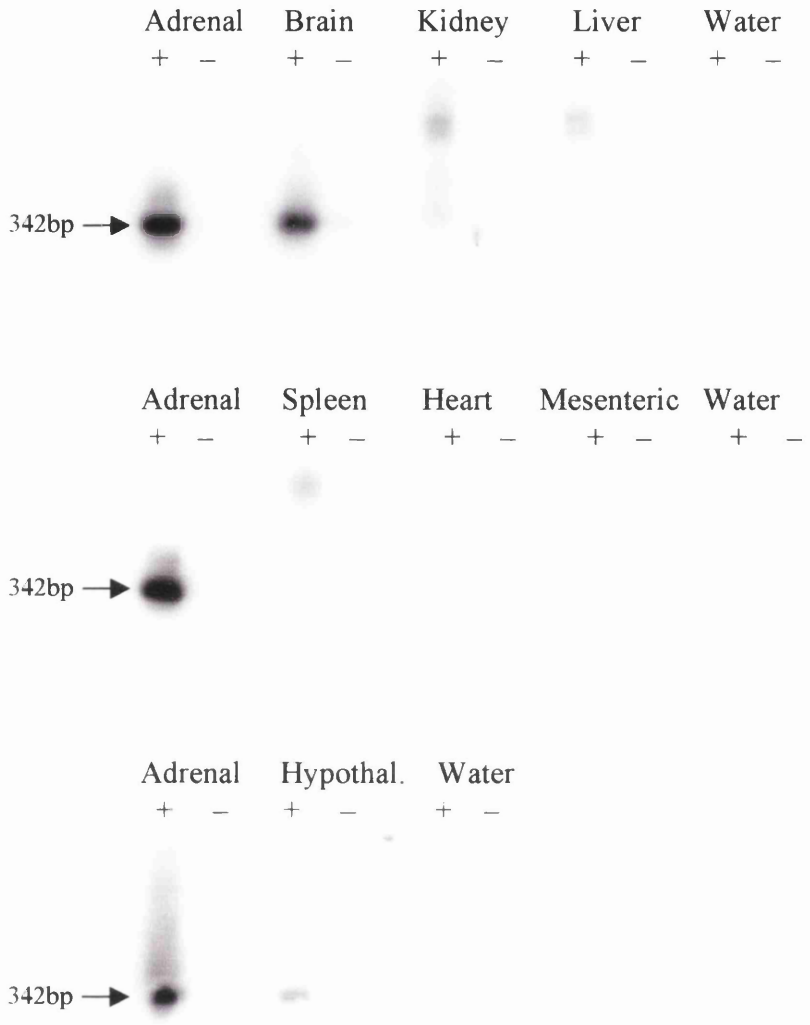


Figure 3.3c RT-PCR of RNAs from various tissues using primers specific for *CYP11B* in the presence (+) and absence (–) of reverse transcriptase. RT-PCR products were separated on 1% agarose gels and Southern blotted to nylon membranes, then probed with the radiolabelled *CYP11B1*-specific oligonucleotide P5815. The blots were washed and exposed to film. Bands corresponding to the expected size of 342bp are indicated.

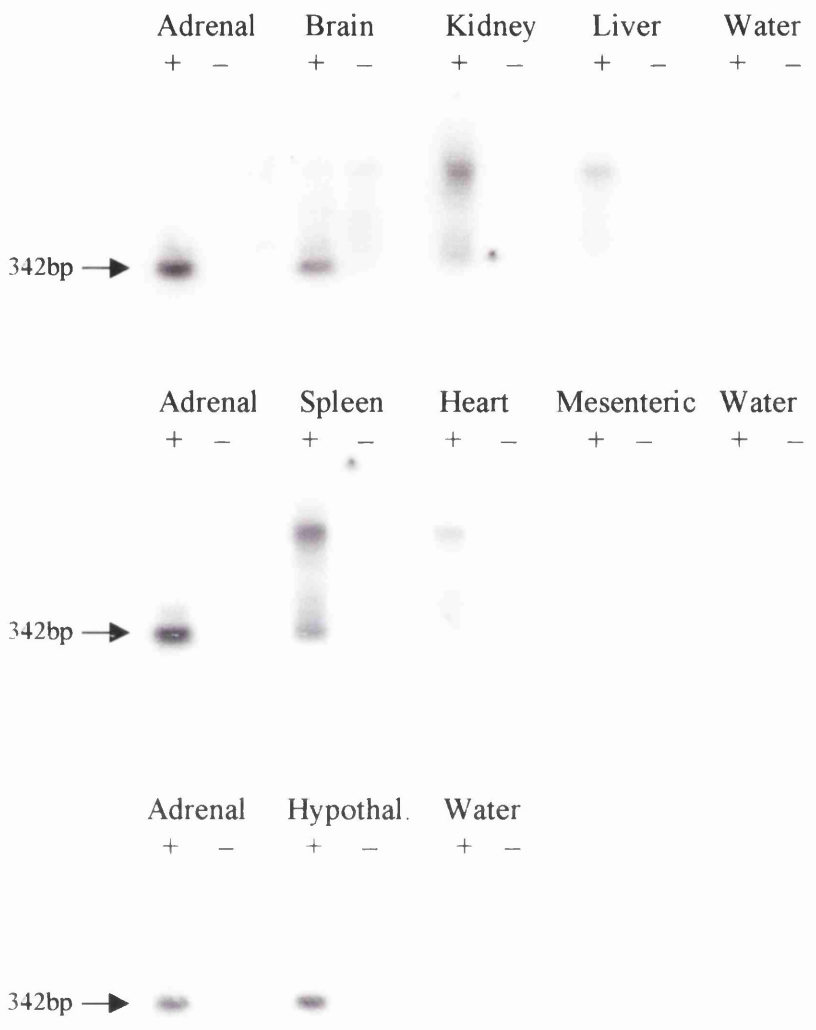


Figure 3.3d RT-PCR of RNAs from various tissues using primers specific for *CYP11B* in the presence (+) and absence (–) of reverse transcriptase. RT-PCR products were separated on 1% agarose gels and Southern blotted to nylon membranes, then probed with the radiolabelled *CYP11B2*-specific oligonucleotide P5814. The blots were washed and exposed to film. Bands corresponding to the expected size of 342bp are indicated.

3.4 Discussion

In recent years, and due in no small part to the increased sensitivity of molecular biological techniques, evidence has accumulated to suggest that corticosteroid production may occur in tissues other than the adrenal cortex. The reverse transcription-polymerase chain reaction (RT-PCR) allows gene transcription to be analysed with much greater sensitivity than was previously possible. Using this technique, isolated RNAs from a variety of different tissues can be screened for the presence of a particular mRNA species. RT-PCR, therefore, commends itself to the detection of steroidogenic enzyme mRNAs in extraadrenal tissues.

The sensitivity of RT-PCR is often such that the final reaction product can be separated and visualised directly on ethidium bromide-stained agarose gels under ultraviolet light, as was the case here with the adrenodoxin samples. Previous investigations into adrenodoxin expression concluded that it was transcribed in virtually all rat tissues and these results supported this. Strömstedt and Waterman (1995), using a protocol similar to the one employed here, were also able to detect bands directly on ethidium bromide-stained gels. Their work investigated RNA from the adrenal, liver, testis and various brain subregions. Mellon and Kushner (1991) achieved positive results in all the tissues they tested, apart from spleen, using an RNase protection assay. The detection of adrenodoxin transcripts in spleen here may be attributed to the greater sensitivity of RT-PCR. The apparent ubiquity of adrenodoxin expression suggests that mitochondrial cytochrome P450 enzymes such as side-chain cleavage enzyme, 11β-hydroxylase and aldosterone synthase would be able to function in any extraadrenal tissues where they happened to be expressed.

Where direct visualisation of PCR products on a gel is not possible, the further technique of Southern blotting is required; this also increases specificity. Products

are transferred from the agarose gel to a nylon membrane which can then be probed with specific radiolabelled oligonucleotides. This technique was used here for two reasons. First, the radiolabelled oligonucleotides are a more sensitive method of detection than visualisation of the gel under UV light; probing can identify previously invisible positive PCR bands. Second, the *CYP11B1*, *CYP11B2* and *CYP11B3* genes are so similar that it is difficult to find long regions of sufficient heterogeneity between them in order to construct specific PCR primers. Instead, common PCR primers were used for the *CYP11B* genes, the three being distinguished only by specific radiolabelled oligonucleotides after Southern blotting.

Strömstedt and Waterman (1995) also used RT-PCR and Southern blotting to demonstrate *CYP11A1* transcription in certain brain subregions (cerebellum and brain stem) but, in common with the findings here, could not do so in liver. They also encountered bands of a higher molecular weight; these could not be attributed to the amplification of genomic DNA as they were absent from the –RT controls. Non-specific bands were also found with the *CYP11A1* probe here, but in this case they were of a lower molecular weight. Although it is possible that they are the products of alternative splicing events, it is more probable that they result from non-specific interactions similar to those found by the other group.

The investigation of *CYP11A1* expression was designed to demonstrate extraadrenal tissues' potential for *de novo* corticosteroid production. While these results confirm that this is possible in tissues such as kidney, brain and heart, side-chain cleavage enzyme is required for numerous other steroid pathways besides that which leads to the corticosteroids. *CYP11A1* was also deemed worthy of examination due to its supposed role at the rate-limiting step of the corticosteroid pathway. However, the rate-limiting factor in the corticosteroid pathway is now known to be

the delivery of cholesterol by StAR and, in retrospect, it would have been advisable to investigate this protein's extraadrenal transcription too. Recent work by Furukawa *et al.* (1998) has used RT-PCR and RNase protection assays to show that the StAR gene and *CYP11A1* are transcribed in the rat brain. Their subsequent *in situ* hybridisation work showed a colocalisation of these genes' transcripts within the pyramidal cells of the hippocampus and the Purkinje cells of the cerebellum. Transcripts for 3β-HSD, the enzyme that follows CYP11A1 in the pathway, were also found in these regions. This suggests a real potential for brain tissue to produce corticosteroids from cholesterol.

CYP11A1 transcription was also found in heart, although work by Zhao *et al.* failed to find evidence of 3β-HSD transcription in this tissue thus undermining the possibility of *de novo* corticosteroid production. However, their analysis involved the less sensitive RNase protection assay, and also failed to find the brain 3β-HSD transcripts subsequently identified by Furukawa *et al.*

According to the findings here, *CYP11B1* and *CYP11B2* are both transcribed in brain tissue, with the isolated hypothalamus also giving a strong signal for both. This agrees with findings by several other groups (Mellon and Deschepper, 1993; Strömstedt and Waterman, 1995; Gómez-Sánchez *et al.* 1996; Erdmann *et al.* 1996). These groups performed their analysis on various subregions of the brain, leading to data that are often contradictory. RT-PCR is not an ideal technique to provide detailed information about localisation of expression; such data are better provided by *in situ* hybridisation or immunostaining of tissue sections (see **chapter 5**).

The strong positive signal for *CYP11B2* in the spleen is intriguing, given the negative results for *CYP11B1* and *CYP11A1* in the same tissue. The inability to detect either transcript in heart or mesentric artery was puzzling given the previous

success by others in this area. Takeda *et al.* (1997) found *CYP11B2* in the mesenteric arteries of SHRSP and WKY rats. Silvestre *et al.* (1998) were also able to show *CYP11B1* and *CYP11B2* transcription in the left ventricles of WKY animals. It may be that their technique – which employed digestion of the RT-PCR product rather than Southern blotting to distinguish between the amplified *CYP11B1* and *CYP11B2* – was more sensitive than the one used here. As with the *CYP11A1* RT-PCR, additional bands appeared on the final blots. As these were absent from the –RT controls, they cannot be attributed to genomic DNA amplification but are, again, most probably the result of non-specific interactions between the radiolabelled oligonucleotide primer and certain cDNA species within the RT-PCR product.

The final RT-PCR investigation involved the *CYP11B3* gene and proved to be entirely negative. This is not surprising as the gene is thought to be transcribed only for a short time in the neonatal adrenal gland (Mellon *et al.* 1995). Therefore, the usual positive control of adult adrenal RNA was not adequate. Similarly, Silvestre *et al.* (1998) showed the *CYP11B3* transcripts' presence in the 21-day-old rat heart but not the two-month-old. Given these data, it would have been better to use neonatal adrenal RNA as a positive control for these experiments to confirm whether the negative results were due to the absence of *CYP11B3* transcription or to a failure in the protocol.

Taken in combination, these results from various tissues suggest that the most promising candidate as an extraadrenal tissue capable of *de novo* corticosteroid synthesis is the brain. Together with the findings of Silvestre *et al.* (1998), the heart also appears to be a good prospect. Although other tissues were positive for certain transcripts – *CYP11A1* in the kidney, *CYP11B2* in the spleen – they did not have the multiple gene expression required for corticosteroid production.

Female rats were used throughout these experiments. This was due to the practical reason that they tend to have larger adrenal glands, thus yielding more RNA. As male rats were not tested, the possibility that they have different patterns of expression cannot be ruled out although similar studies have tended to find no sex differences (see **section 1.8**).

The RT-PCR technique employed here is not quantitative; semi-quantitative RT-PCR would require an additional PCR product within the same reaction derived from a sequence of known quantity which could be used as a reference point for comparison with the transcript of interest. Nevertheless, there can be little doubt that the levels of all transcripts, with the possible exception of adrenodoxin, occurs at lower levels within the extra-adrenal tissues than in the adrenal cortex. This may be due to less transcription within extra-adrenal tissues or to production of less stable transcripts that degrade more rapidly than their adrenal equivalents. Less transcription within the tissue does not necessarily mean that every cell is transcribing the same genes more slowly than those of the adrenal cortex. Instead, transcription may be occurring at the same rates – or even higher – but within fewer cells. Thus, for example, parts of the brain may transcribe *CYP11B1* at a very high rate but the concentration of these transcripts would decrease within the total brain RNA samples used for these RT-PCR reactions. Such local concentration of expression would also be reflected in the concentrations of steroids produced at such sites. For example, aldosterone in the rat myocardium has a concentration of 16nM which is almost 20 times the level found in plasma (Delcayre and Silvestre 1999). Given such differences between adrenal and extra-adrenal expression, the question arises of whether transcriptional regulation in extra-adrenal tissues occurs

independently of that in the adrenal cortex and, if so, what factors might influence this (see **section 1.4**).

Having established that the transcription of steroidogenic genes occurs in select extraadrenal tissues, it is necessary to show that these transcripts go on to form the relevant polypeptide products. This is the subject of **chapters 4** and **5**.

Chapter 4

Results: SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) And Immunoblotting

4.1 Introduction

The control of gene expression operates at several levels and, although the most common of these is the regulation of gene transcription, the presence of mRNA within a cell does not guarantee its translation into a functioning polypeptide product. Therefore, besides the transcriptional information provided by such techniques as RT-PCR and *in situ* hybridisation, studies of gene expression must show evidence of the protein's presence. This can be done by immunoblotting (also known as Western blotting) and/or immunostaining which require an antibody specific for the protein of interest. Given the range of proteins expressed by an organism and their variations from species to species, it is a common source of frustration that no specific antibody is readily available for the detection of a particular protein. It was fortunate, therefore, that antibodies specific for the rat forms of 11β-hydroxylase and aldosterone synthase were made available for this research through a kind gift from Professor Celso E. Gómez-Sánchez. This chapter deals with attempts to detect 11β-hydroxylase and aldosterone synthase by immunoblotting.

4.2 Methods

The techniques used in this chapter are described in **section 2.2**. A brief account of antibody preparation is given in **section 2.2.1**. The general SDS-PAGE protocol is detailed in **sections 2.2.2** to **2.2.8**. This was also performed in a modified,

non-denaturing, non-reducing (i.e. native) form and the changes to the standard protocol this entailed are given in **section 2.2.9**.

4.3 Results

Using SDS-PAGE, a band corresponding to the expected 52,000 molecular weight was identified using the anti-11β-hydroxylase 11BIA4 antibody in mitochondrial fractions prepared from adrenal tissue. See **figure 4.3a**.

The 11BIA4 antibody was raised against residues 272–285 of 11β-hydroxylase. The band could not be detected in mitochondrial fractions derived from brain tissue.

No specific bands corresponding to aldosterone synthase could be identified in adrenal or brain mitochondrial fractions using any of the three antibodies raised against a peptide from this enzyme (AS IG5 HC3, AS IE6 and AS IC11).

A single band corresponding to a molecular weight of 25,000 was present in all lanes, including those which had not been exposed to 1° antibody. For this reason, this band was attributed to a non-specific interaction with the horseradish peroxidase-labelled 2° antibody.

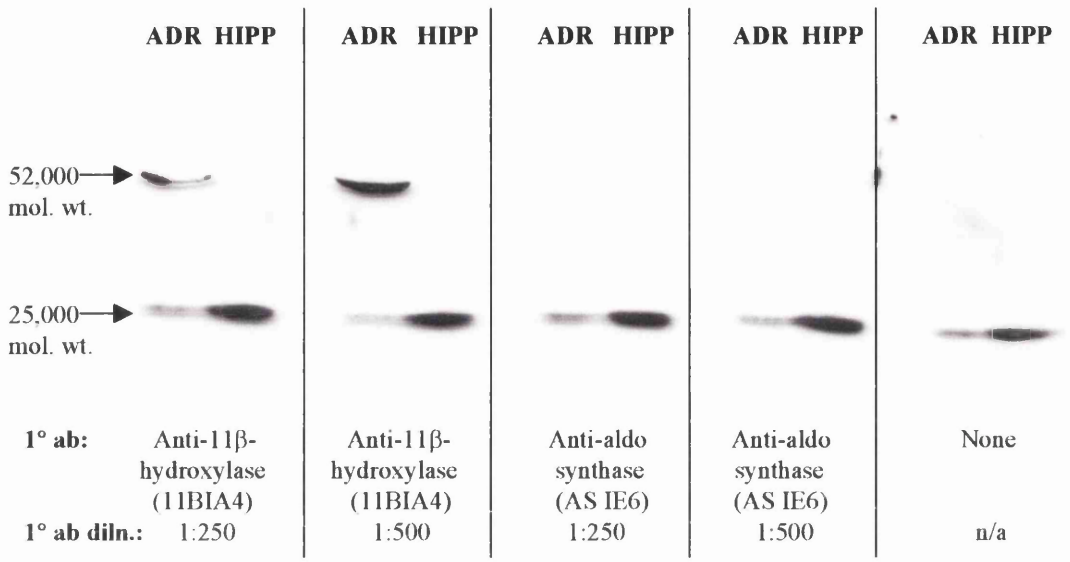


Figure 4.3a Immunoblot detecting 11β-hydroxylase in adrenal tissue. 200 μg of denatured, reduced adrenal or hippocampal mitochondrial protein samples were separated on a 10% SDS-polyacrylamide gel. The proteins were then transferred to Hybond-P membrane which was blocked, then split into five parts. Each part was subjected to different 1° antibody incubations at 37°C overnight, followed by incubation with HRP-labelled sheep anti-mouse 2° antibody at a 1:1,000 dilution for 2 hours at 37°C. Bands were detected using Supersignal ULTRA chemiluminescent substrate followed by exposure to film for 5 minutes. Size was determined by comparison with Rainbow markers (range 10,000–250,000) run alongside samples on the gel.

4.4 Discussion

As the primary antibodies were not obtained from a commercial source, it was necessary to determine the optimum conditions for their use in immunoblotting and a large number of variations to the SDS-PAGE protocol were attempted before the final form given in **section 2.2** was found to be the most effective. The various stages of the SDS-PAGE procedure and the modifications to the protocol at each of these stages are detailed below.

1. **Preparation of protein from Wistar-Kyoto rats.** Initially, whole adrenal gland and brain homogenates were used for the immunoblotting procedure. When this failed to yield positive results, mitochondrial fractions were prepared from fresh tissue in the hope that this would concentrate the mitochondrial cytochrome P450 enzymes under investigation and thus give a stronger signal.
2. **Preparation of polyacrylamide gel and protein for electrophoresis.** Most antibodies are raised against the protein in its denatured, reduced state and, for this reason, gels were prepared containing sodium dodecyl sulphate (SDS). Later, proteins were electrophoresed under non-denaturing, non-reducing (i.e. native) conditions, with SDS omitted from the gel and tank buffer to see if this favoured antibody binding but this was found to offer no advantage. Under denaturing conditions, proteins were loaded on to the gel in Laemmli buffer which contains SDS, urea and dithiothreitol (DTT). Samples were usually boiled for 3 minutes before loading but this step was omitted to see if this altered the outcome of the experiment. Immunoblots prepared under native conditions used a non-denaturing, non-reducing loading buffer.
3. **Blotting of proteins on to membrane.** Proteins were initially blotted on to nitrocellulose membranes. This was later changed to Hybond-P, a hydrophobic

polyvinylidene difluoride (PVDF) transfer membrane, which has a higher protein-binding capacity and gives a more intense, longer-lasting signal when used with certain ECL substrates (see step **8**).

4. **Blocking of membrane.** Various blocking times, from 30 minutes to 3 hours, were tried, before settling on a 2 hour period.
5. **Incubation of membrane with 1° antibody.** The optimum dilution of each antibody was resolved experimentally. The length and temperature of antibody incubations is often a compromise between the amount of antibody binding and its specificity; attempts to increase the intensity of a signal will often result in the emergence of additional, non-specific bands. Various incubation temperatures ranging up to 37°C (where one would expect antibody binding to be greatest but also least specific) were tried, with the best results being obtained from incubations at a low temperature of 4°C overnight.
6. **Washing of membrane.** The wash solution consisted of Tris-buffered saline (TBS) plus the detergent Tween 20. A low amount of Tween 20 may not wash off sufficient antibody to reduce background and non-specific signals, while too high a concentration can wash off specifically-bound antibody. A range of Tween 20 concentrations from 0.05% to 0.5% were used within this TBST mixture before settling on 0.4% Tween 20 in TBS.
7. **Incubation of membrane with 2° antibody.** The optimisation of experimental conditions that applied to the 1° antibody (see above) was also appropriate for the 2° antibody. A 2-hour incubation at 37°C was found to be best. Also, the supplier of HRP-labelled sheep anti-mouse IgG was changed from SAPU (Scottish Antibody Production Unit, Carluke, U.K.) to Amersham Pharmacia Biotech U.K. Ltd., as the latter product yielded fewer non-specific bands, possibly due to its

high-species specificity which would prevent cross-reaction with rat proteins on the membrane.

8. **Signal detection by enhanced chemiluminescence (ECL).** Chemiluminescent reagents such as the diacylhydrazide luminol undergo a reaction in the presence of horseradish peroxidase which results in the production of light (see **figure 4.4a**). This light can be detected by exposure to film making the technique a valuable method for the detection of bound antibody. Several types of chemiluminescent substrate were used during the course of the immunoblotting experiments. The use of ECL Plus (Amersham Pharmacia Biotech U.K. Ltd.) was stopped in favour of Supersignal and Supersignal ULTRA (both Pierce & Warriner, Chester, U.K.) upon a colleague's recommendation, and these were found to give more intense and sustained signals.

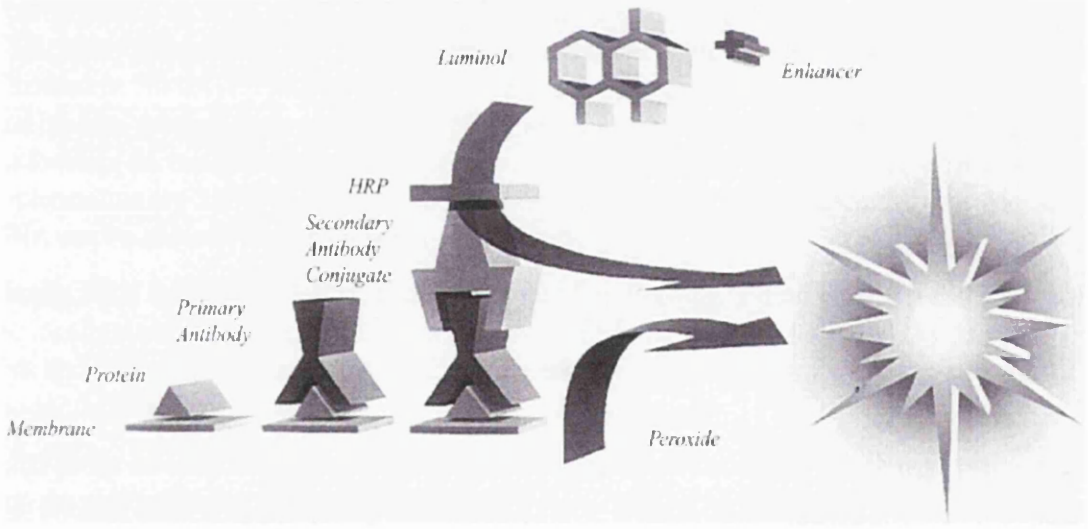


Figure 4.4a Schematic representation of a chemiluminescent reaction using enhanced luminols.

Despite these adjustments to the protocol, 11β-hydroxylase was not detected in extra-adrenal tissues and aldosterone synthase could not be found in any tissue,

including the adrenal gland. The failure to detect these enzymes was attributed to the insensitivity of the immunoblotting technique. One would expect adrenal 11β-hydroxylase to be the most abundant of the proteins that were sought, whereas adrenal aldosterone synthase levels would be much lower given the size of the zona glomerulosa in comparison to the zona fasciculata/reticularis; brain levels of either enzyme are likely to be lower still given the results of the RT-PCR experiments. The maximum amounts of protein (~ 200μg/well) had been loaded on to the gel, so this could not be increased, although it might have been possible to enrich the levels of CYP11B1 and/or CYP11B2 within the samples by treating the animals with, for example, a low sodium diet. LeHoux *et al.* (1996) were able to increase dramatically the levels of CYP11B2 in hamster adrenal tissue in this way.

Attempts were made to increase the sensitivity of the immunoblotting technique using a commercially available avidin biotin kit (Vectastain ABC, Vector Laboratories Ltd., Peterborough, U.K.) to amplify weak signals. Unfortunately, this technique resulted in the appearance of numerous non-specific bands as well as a significant increase in background signals; any positive bands that might have been present were masked by these non-specific signals (**see figure 4.4b**). A compromise between the lack of signal and complete overload by background could not be established.

Personal communications with Professor Gómez-Sánchez established that he had had some success in his immunoblotting experiments with these antibodies. With the anti-11β-hydroxylase antibody, he identified a distinct band of unspecified size in adrenal tissue but, additionally, found a band in the brain with a molecular weight of approximately 35,000. Using one of the three aldosterone synthase antibodies, he was also able to detect a distinct band of unspecified size in adrenal samples as well

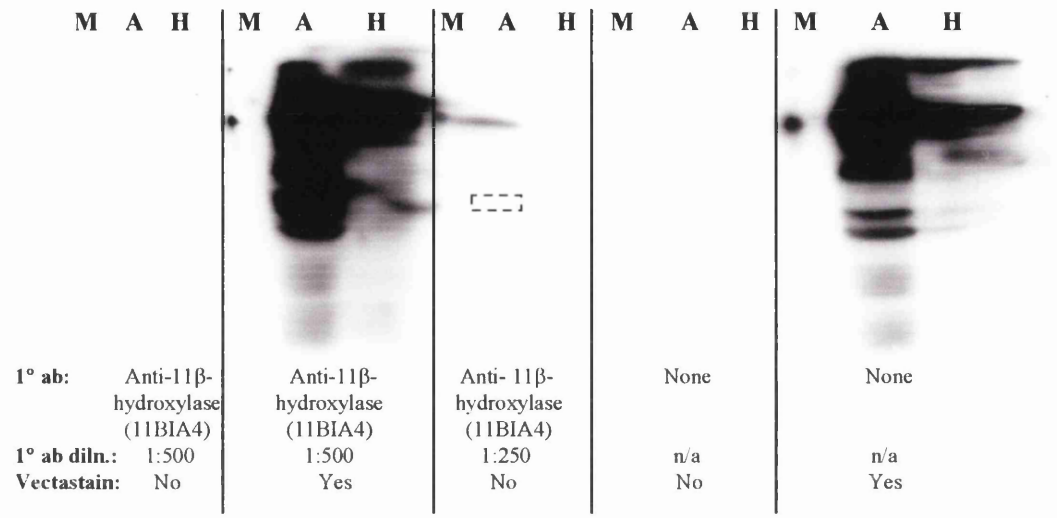


Figure 4.4b Immunoblot prepared using Vectastain ABC kit. 200 μg of denatured, reduced adrenal (A) or hippocampal (H) mitochondrial protein samples were separated on a 10% SDS-polyacrylamide gel alongside Rainbow markers (M) of molecular weight 10,000 to 250,000. The proteins were then transferred to Hybond-P membrane which was blocked and then split into five parts. These were subjected to differing 1° antibody incubations at 37°C overnight. Membrane sections prepared without the use of Vectastain were then incubated with HRP-labelled sheep anti-mouse 2° antibody at a 1:1,000 dilution for 4 hours at 37°C. Other sections were incubated with biotinylated rabbit anti-mouse 2° antibody at a 1:1,000 dilution for 2 hours at 37°C and then Vectastain ABC for 1 hour at room temperature. Bands were detected using Supersignal ULTRA chemiluminescent substrate followed by exposure to film for 5 seconds. A faint positive band for 11β-hydroxylase in adrenal tissue is indicated by the dotted line.

as a larger band of molecular weight 60,000–70,000 in brain. Despite emulating some of his techniques, attempts to reproduce these unpublished results proved unsuccessful and this must be attributed to some unidentified technical difference.

From their amino acid sequences, rat 11β-hydroxylase and aldosterone synthase should have identical molecular weights. This is also the case with the human forms of the enzymes. However, when the human adrenal forms of aldosterone synthase and 11β-hydroxylase underwent electrophoresis on SDS-polyacrylamide gels, they were found to have molecular weights of 49,000 and 51,000 respectively (White *et al.* 1994). This may be related to post-translational modifications to these proteins, although nothing concerning the phosphorylation or glycosylation of the enzymes is known.

The differing rigours that proteins must undergo during preparation for immunoblotting and immunostaining mean that a target antigen may adopt slightly different conformations. Therefore, it is possible for an antibody to be better suited for one or other of these techniques, depending on which of them presents the epitope in the form best recognised by the antibody. For this reason, it was decided to proceed with immunostaining experiments using these same primary antibodies in spite of the disappointing results of immunoblotting.

Chapter 5

Results: Detection Of 11β-Hydroxylase And Aldosterone Synthase In Adrenal And Extra-Adrenal Tissue By Immunostaining

5.1 Introduction

In the previous two chapters, homogenised tissue was assayed for the presence of particular mRNAs or polypeptides. While extremely valuable, such methods are limited in the information they convey about the localisation of expression. This is of particular importance when looking at brain tissue, where gene expression is by no means homogenous even within a single region such as the cerebellum or hypothalamus. Detailed localisation studies require that whole sections of tissue be stained specifically for particular enzymes. This chapter describes the staining of rat tissue sections using specific antibodies in order to establish the distribution of 11β-hydroxylase and aldosterone synthase within adrenal and extra-adrenal tissue.

5.2 Methods

The protocol used for the preparation and immunostaining of cryosections is described in **section 2.3**. The protocols for preparation, pretreatment and immunostaining of paraffin-embedded tissue sections are given in **section 2.4**. Briefly, an avidin biotin enzyme complex (ABC) technique was used for the immunostaining of cryosections and initially for paraffin-embedded sections also. This was later changed to the DAKO CSA kit method (see **section 2.4.5**). This technique is capable of greater signal amplification than the ABC method.

Control sections were incubated with DAKO Mouse IgG1 Negative Control. This is a mouse monoclonal antibody with a specificity for *Aspergillus niger* glucose

oxidase, an enzyme absent from mammalian tissues. As an additional control, primary antibodies were preincubated with their respective immunogenic peptides (see **section 2.4.6**). This quenches specific antibody binding and, therefore, positive staining thereby confirming the specificity of the primary antibodies.

The preparation of the primary antibodies, which are identical to those used in **chapter 4** for immunoblotting, is described in **section 2.2.1**.

5.3 Results

3,3'-Diaminobenzidine tetrahydrochloride (DAB) was the chromogen used in all immunostaining reactions, resulting in a brown colour in positively-stained areas. The counterstain, Harris hematoxylin solution, gave a purple/blue colour.

Initial immunostaining experiments were performed on adrenal cryosections using an ABC method (see **figure 5.3a**). Immunostaining using the anti-11β-hydroxylase antibody 11BIA4 was localised to the zona fasciculata/reticularis of the adrenal cortex and was absent from the zona glomerulosa. Incubation with the anti-aldosterone synthase antibodies resulted in no staining. Control sections were also negative.

A similar protocol was used on adrenal paraffin-embedded tissue that had been fixed for 24 hours in formalin solution (40% w/v formaldehyde). Protease and microwave pretreatments were performed on the sections before immunostaining and a biotin blocking step was added to the protocol that had been used on cryosections (see **figure 5.3b**). Again, the 11BIA4 antibody stained the zona fasciculata/reticularis, albeit rather faintly. Use of the AS IG5 anti-aldosterone synthase antibody was also able to produce staining. This localised to the zona glomerulosa, forming a thin, broken ring round the outside of the adrenal cortex.

Control sections were negative. No staining could be produced in brain tissue using this method.

A CSA kit protocol was adopted to increase staining intensity. The tissue used throughout was fixed in 4% w/v paraformaldehyde for 24 hours prior to embedding in paraffin and sectioning. Microwave pretreatments were used. The anti-11β-hydroxylase antibody 11BIA4 and the anti-aldosterone synthase antibody AS IE6 were used throughout. This method was found to intensify the staining in adrenal tissue which followed the same zonal patterns observed using the previous methods (see **figures 5.3c** and **5.3d**). In particular, the outer zona fasciculata was intensely stained, with a more diffuse or banded appearance in the inner zona fasciculata. Little staining was apparent in the zona reticularis. In addition, however, the staining produced by 11BIA4 extended into the adrenal medulla (see **figure 5.3e**).

Immunostaining was also observed in brain sections and, unlike the adrenal gland, staining for 11β-hydroxylase and aldosterone synthase was found to colocalise. Two particular regions of the brain produced distinct, reproducible staining for both enzymes. One was the cerebellum (see **figure 5.3f**). Staining was heavier in the molecular layer, lighter in the granular layer and was almost absent from the white matter. However, the Purkinje cells which line the boundary of the molecular and granular layers were found to be stained most intensely of all (see **figure 5.3g**).

The other region of the brain to show strong staining for both enzymes was the hippocampus (see **figure 5.3h**). The pyramidal cells of this region were stained intensely throughout the dentate gyrus and CA1–3 cells of Ammon's horn. The cells of the CA3 region are shown with higher magnification in **figure 5.3i**.

The specificity of staining produced by this method was confirmed by two types of control. The first employed the Mouse IgG1 Negative Control described previously. The second involved the preincubation of a constant amount of monoclonal antibody together with differing concentrations of the peptide against which it was raised. At higher concentrations of peptide, specific staining is quenched as all antibody is bound by its peptide and it is therefore unable to bind to the tissue. As the peptide concentration decreases the antibody-tissue binding gradually returns, as does the staining. Examples of this can be seen in **figures 5.3j** and **5.3k**. **Figure 5.3j** shows the quenching of immunostaining in the adrenal zona fasciculata/reticularis by the preincubation of the anti-11β-hydroxylase antibody 11BIA4 with the peptide against which it was raised. **Figure 5.3k** shows the quenching of immunostaining in the cerebellar Purkinje cells by preincubation of the anti-aldosterone synthase antibody AS IE6 with its immunogenic peptide.

Finally, given the interest in cardiovascular production of corticosteroids (see **section 1.6.1**), attempts were made to extend the CSA kit immunostaining protocol to paraffin-embedded heart tissue. The protocol was identical to that used for the successful adrenal and brain immunostainings but heart tissue stained non-specifically. Sections incubated with Mouse IgG1 Negative Control were indistinguishable from those exposed to the specific monoclonal antibodies (see **figure 5.3l**).

5.4 Discussion

Antigens are better preserved during cryosection preparation than with paraffin-embedded sections. However, the frozen sections do tend to have inferior morphological detail. For these reasons, the initial intention was to identify specific

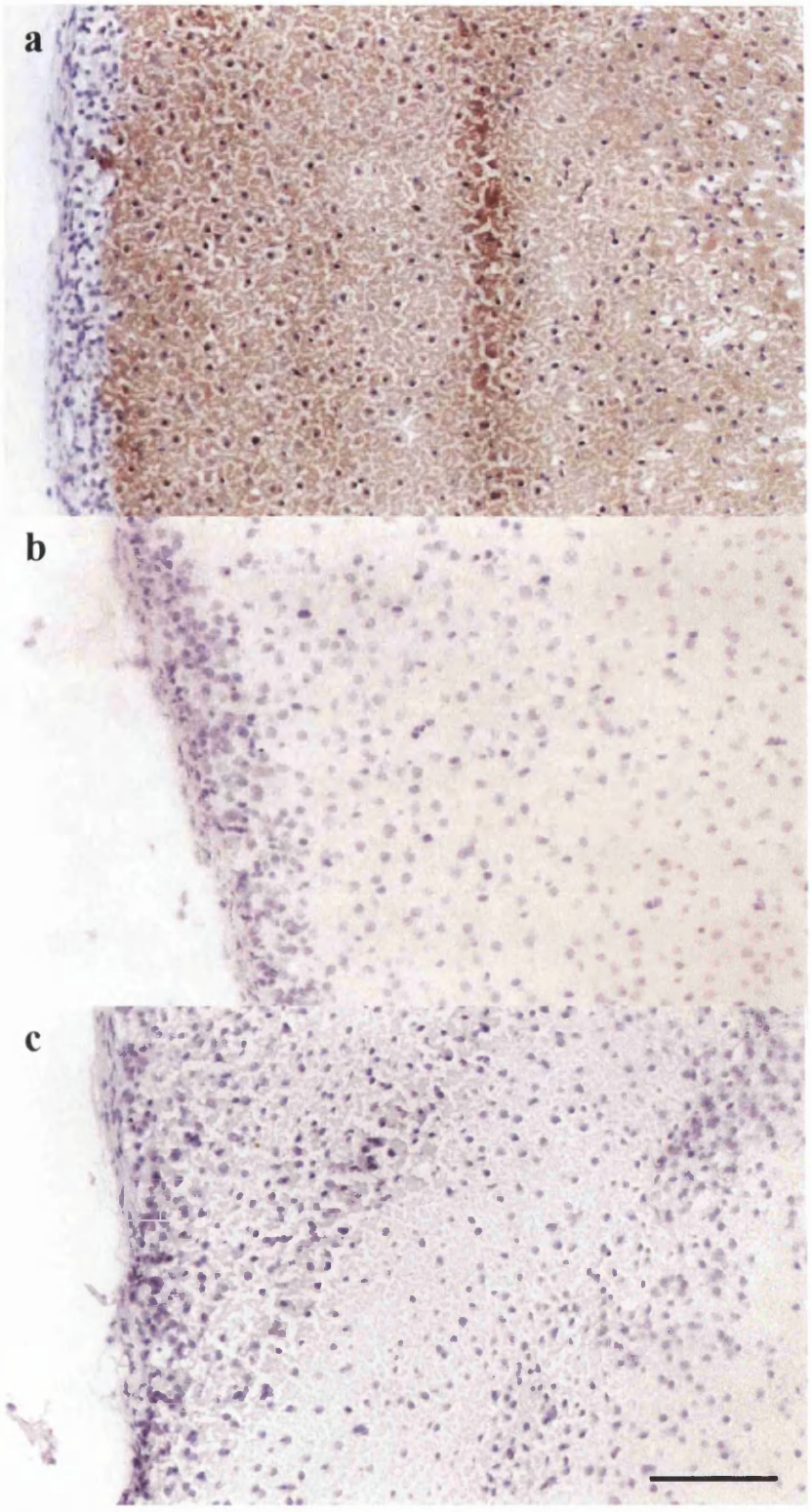


Figure 5.3a. Immunostaining for 11β-hydroxylase and aldosterone synthase in adrenal tissue cryosection using the ABC protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 100μm.

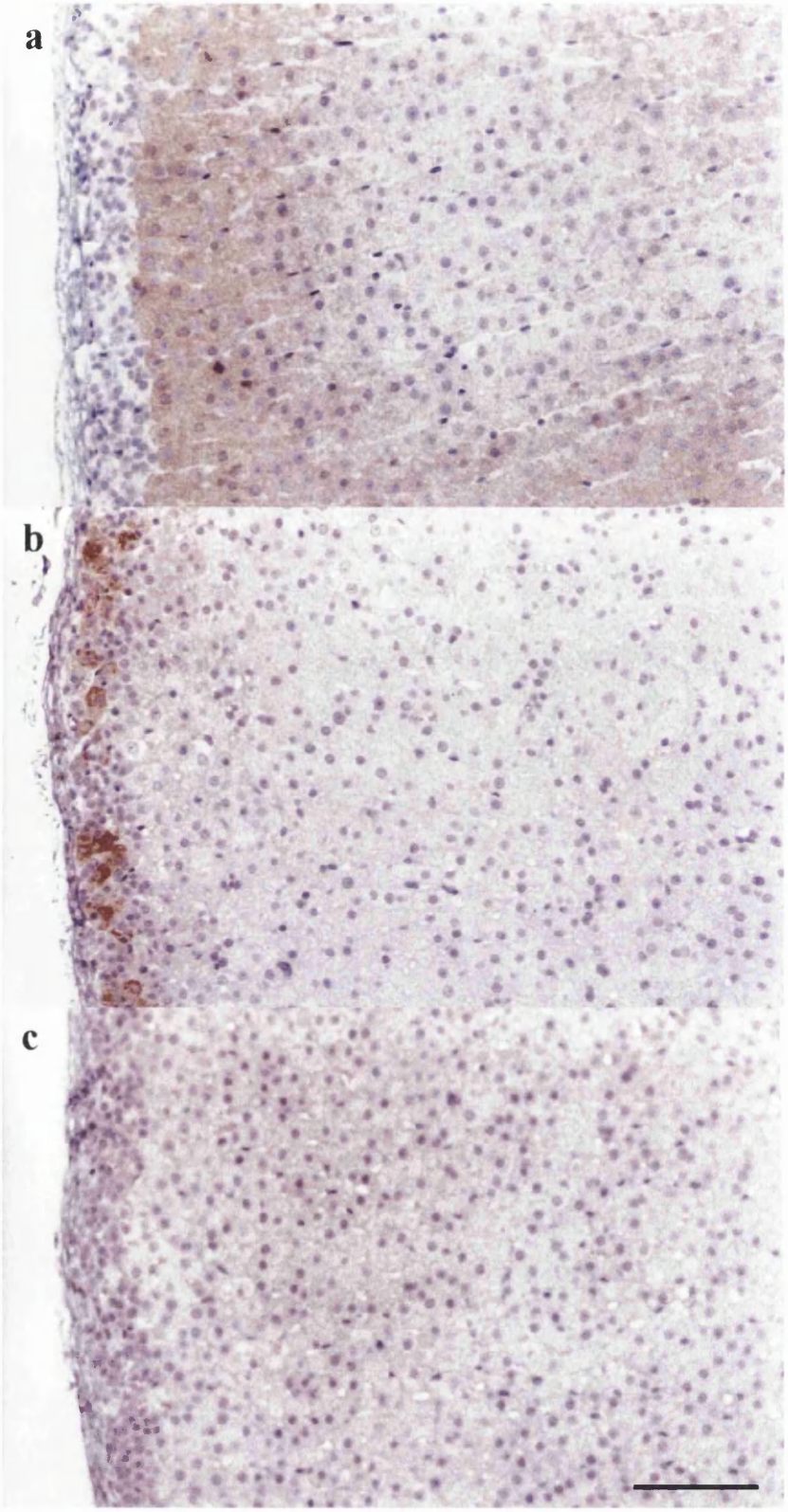


Figure 5.3b. Immunostaining for 11β-hydroxylase and aldosterone synthase in paraffin-embedded adrenal tissue sections using the ABC protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 100μm

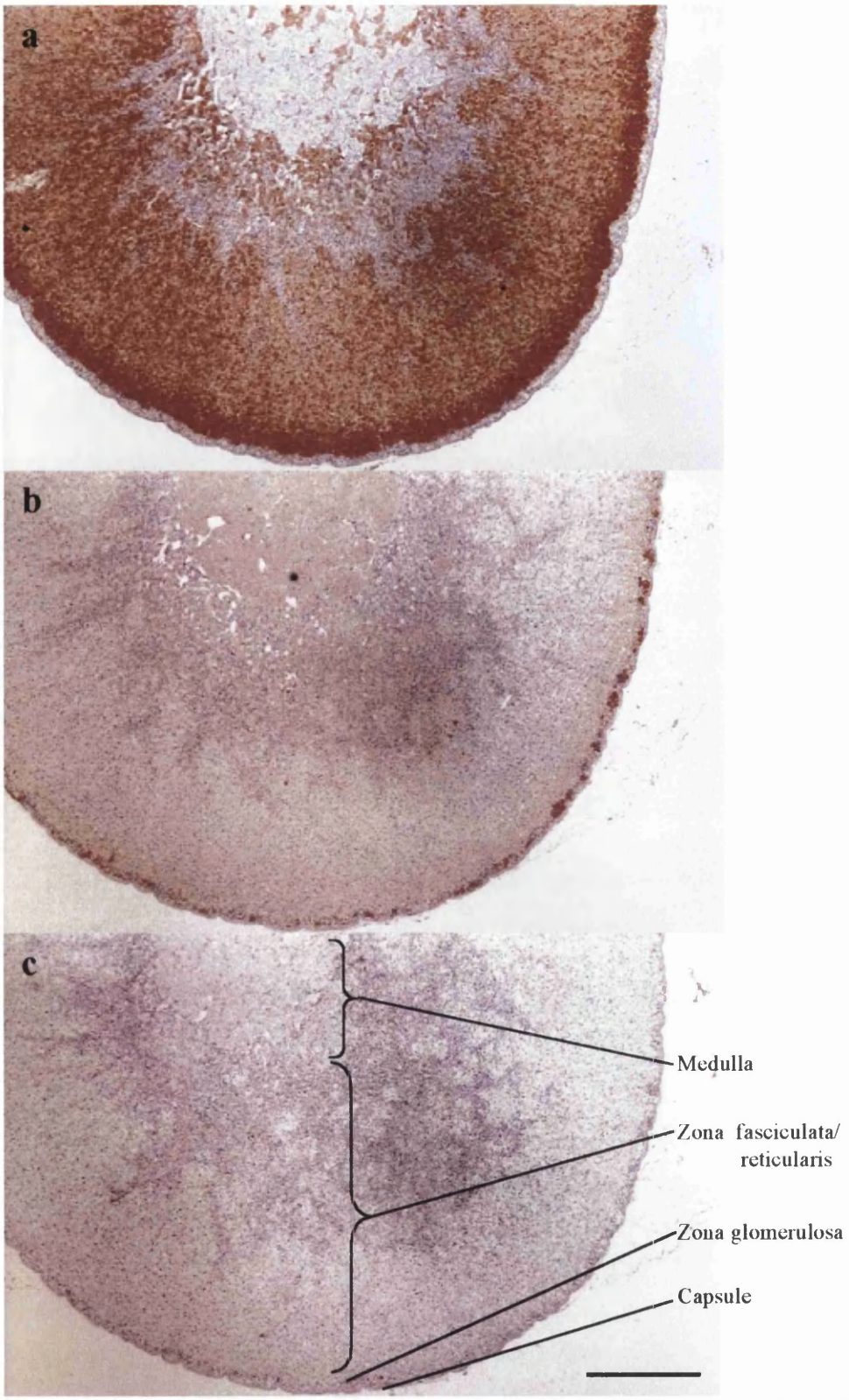


Figure 5.3c. Immunostaining for 11β-hydroxylase and aldosterone synthase in paraffin-embedded adrenal tissue sections using the CSA kit protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 500μm. The regions of the adrenal gland are indicated on section c.

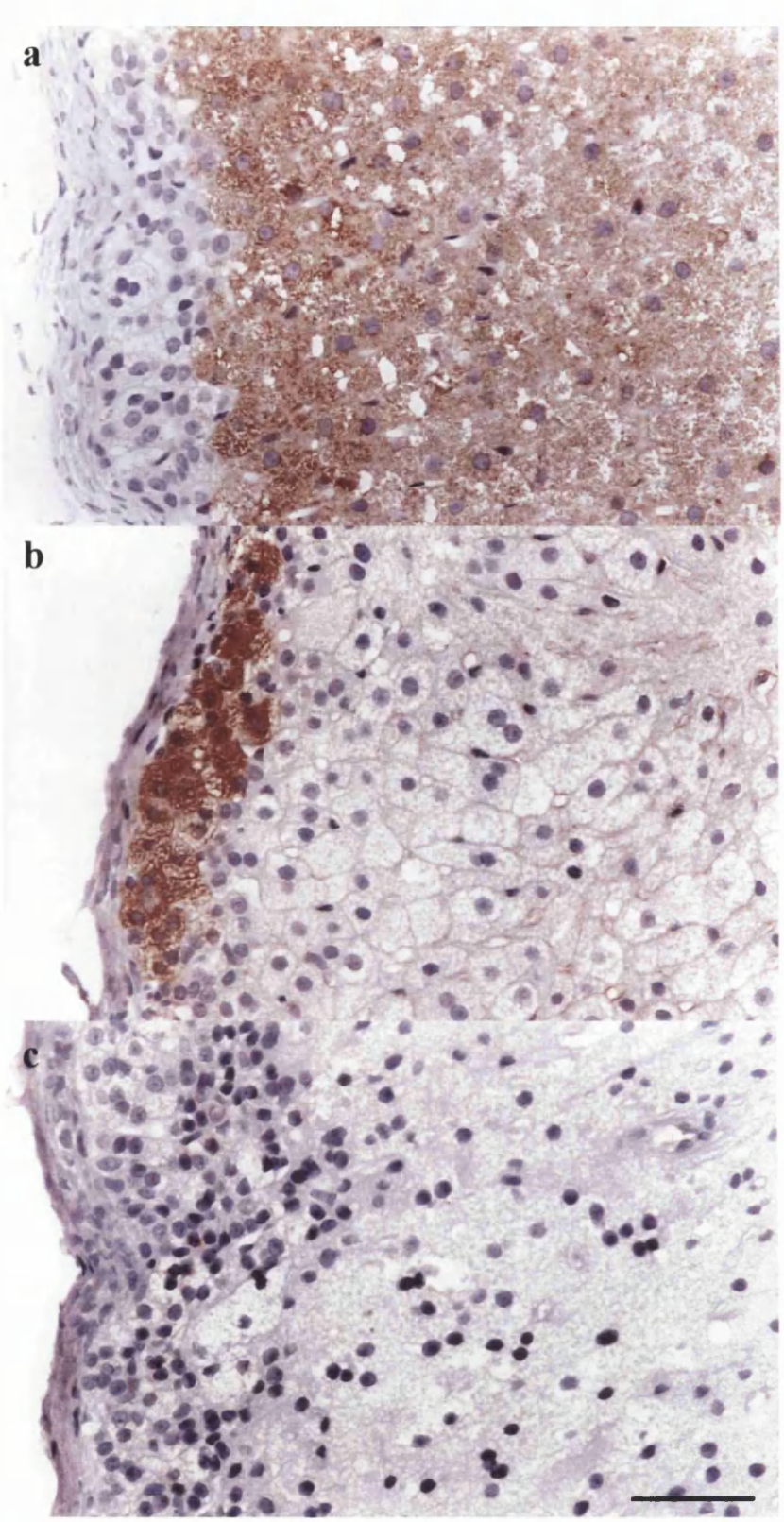


Figure 5.3d. Immunostaining for 11β-hydroxylase and aldosterone synthase in paraffin-embedded adrenal tissue sections using the CSA kit protocol. a: 11β-Hydroxylase; b: aldosterone synthase; c: control. Scale bar = 50μm.

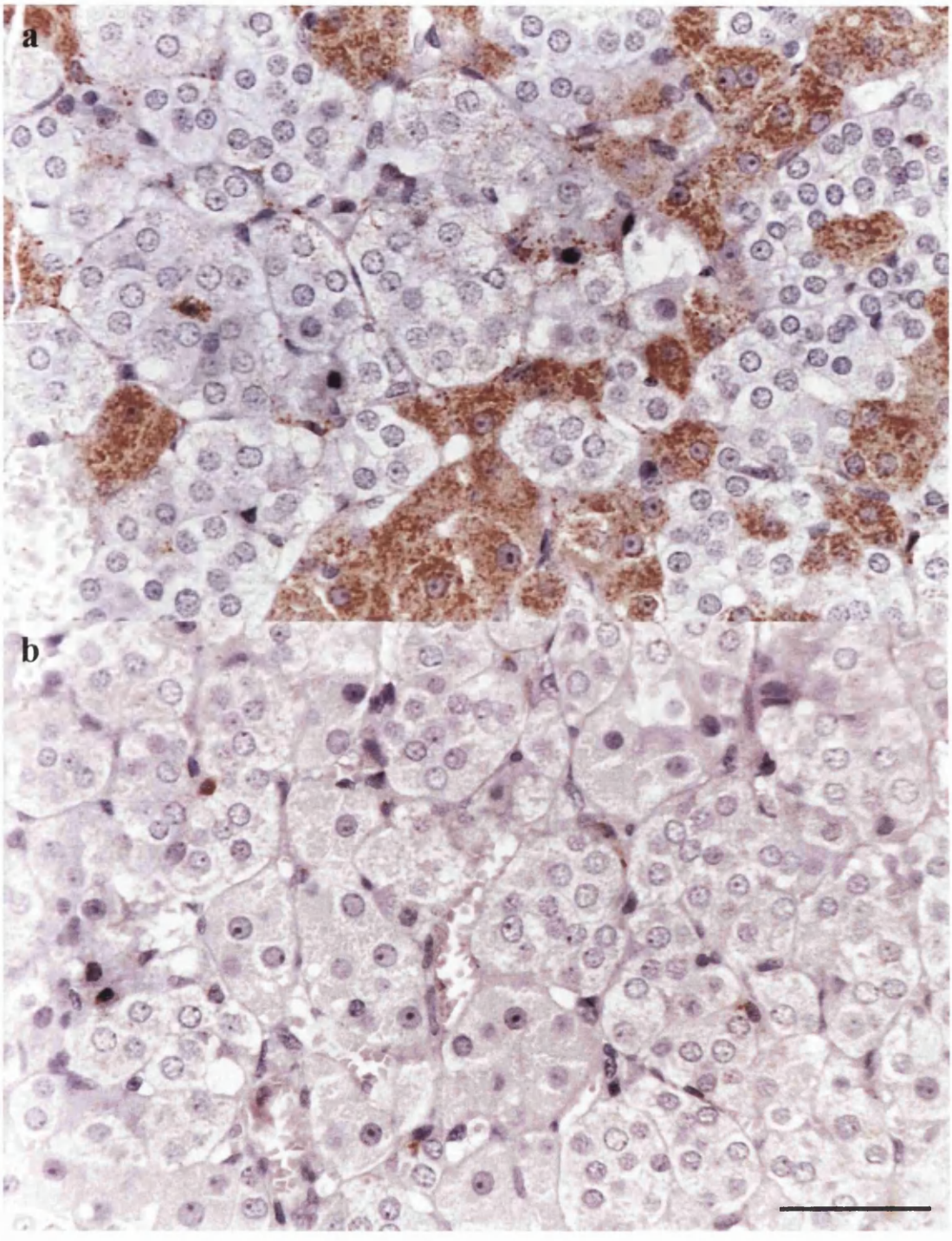


Figure 5.3e. Immunostaining for 11β-hydroxylase in adrenal medulla cells of paraffin-embedded adrenal tissue sections using the CSA kit protocol. a: 11β-hydroxylase; b: control. Scale bar = 50μm.

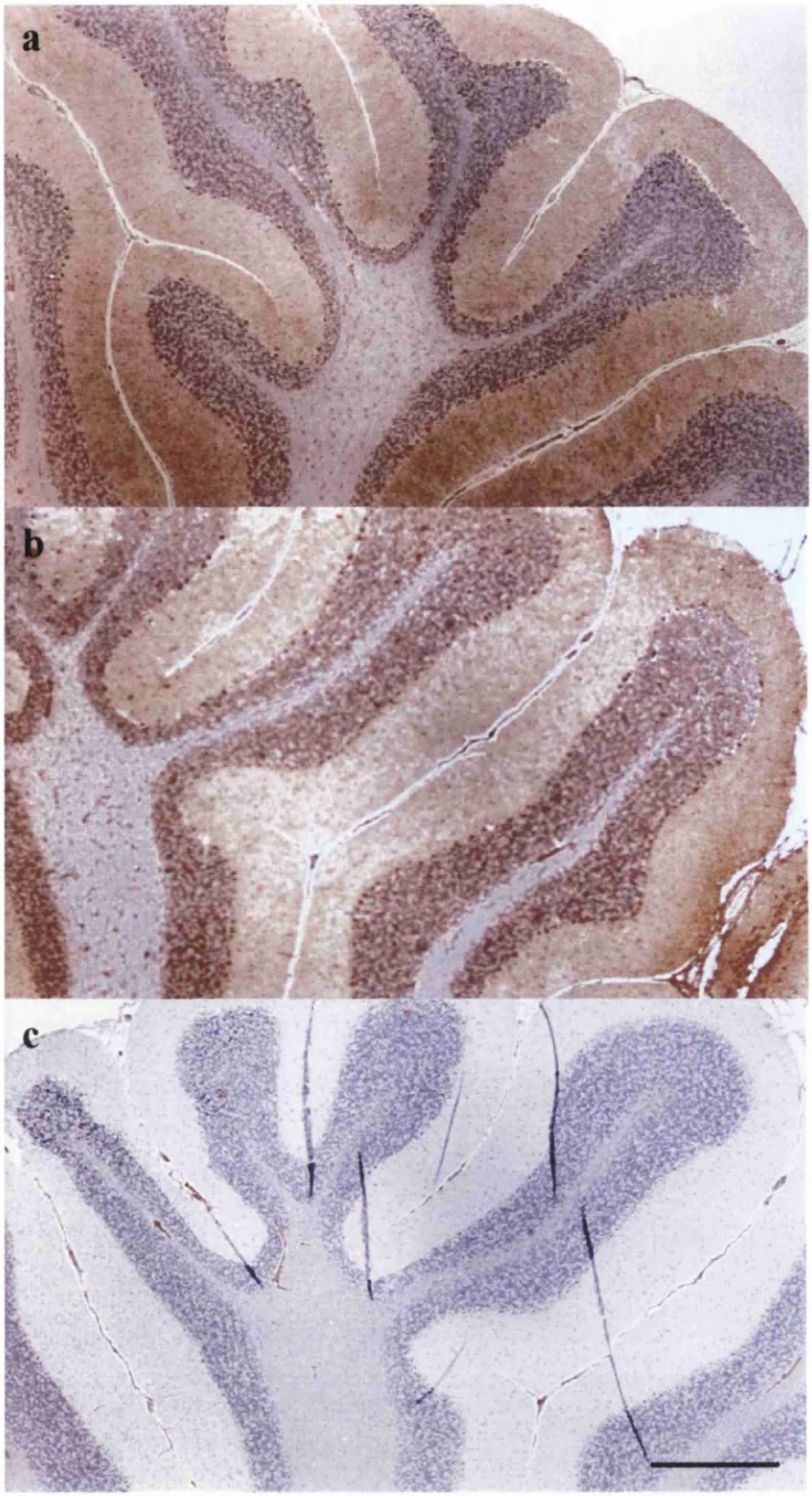


Figure 5.3f. Immunostaining for 11β-hydroxylase and aldosterone synthase in the cerebellum of paraffin-embedded brain sections using the CSA kit protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 500μm.

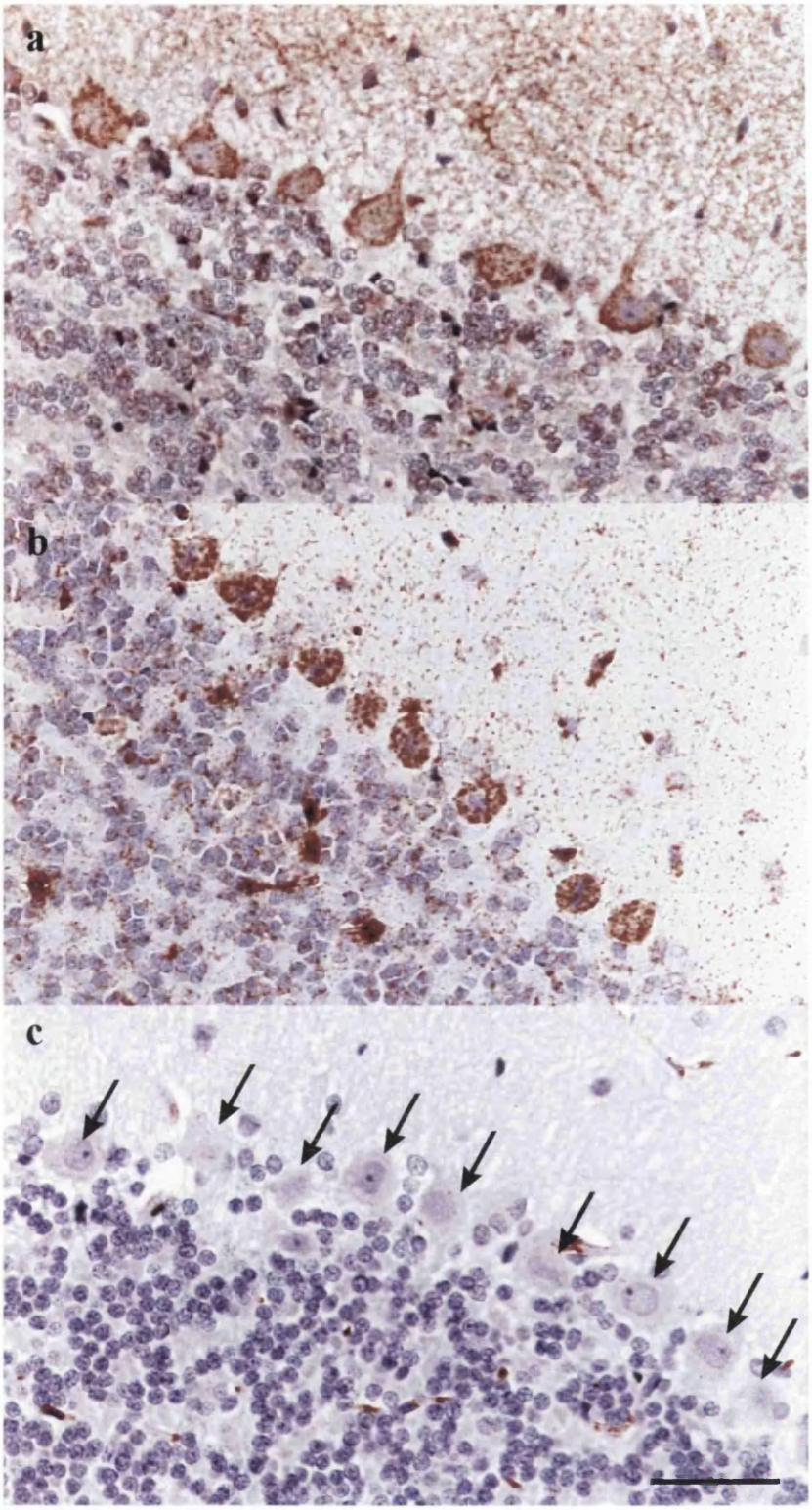


Figure 5.3g. Immunostaining for 11β-hydroxylase and aldosterone synthase in cerebellar Purkinje cells of paraffin-embedded brain sections using the CSA kit protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 50μm. Arrows indicate the positions of Purkinje cells on section c.

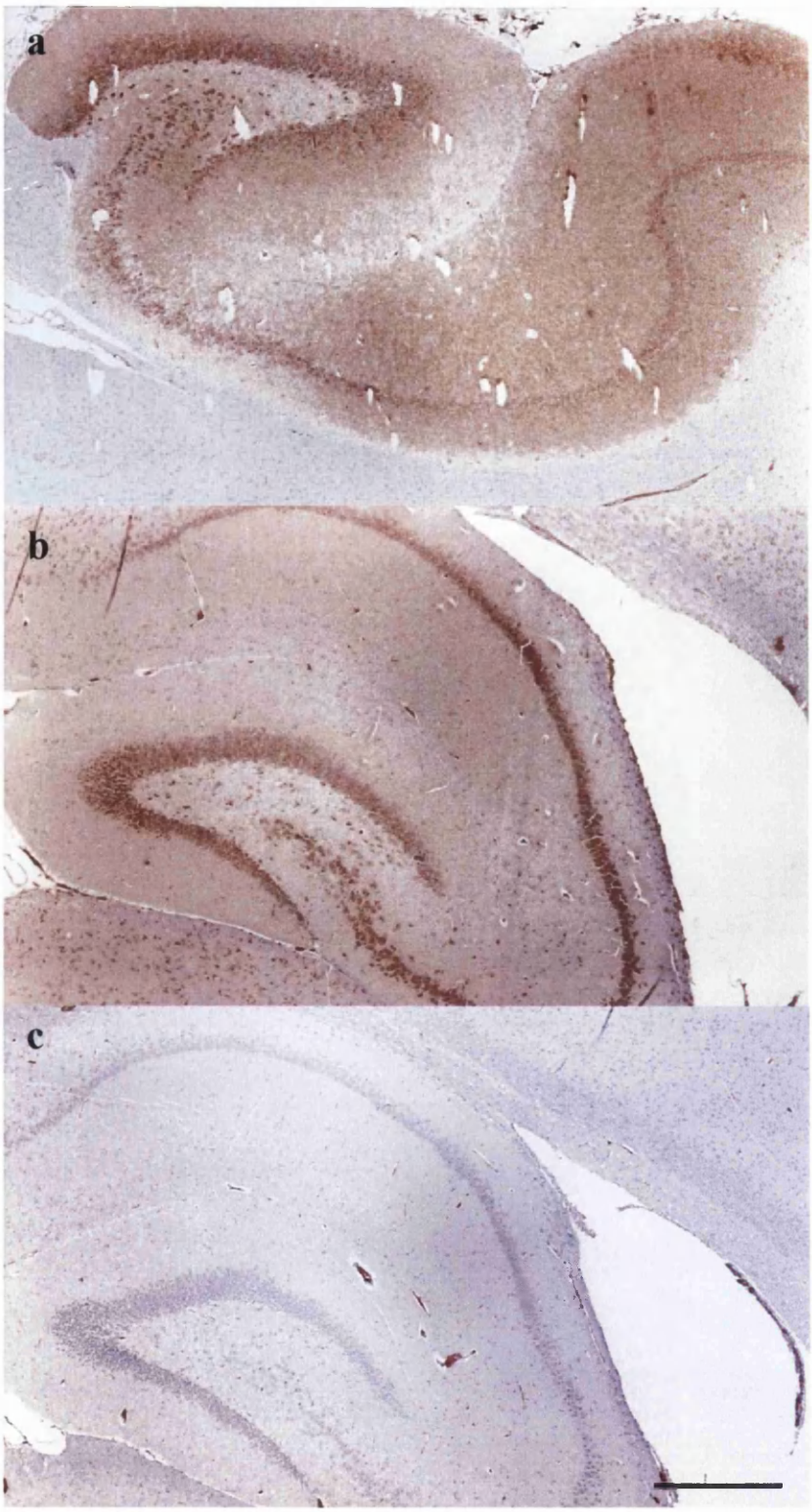


Figure 5.3h. Immunostaining for 11β-hydroxylase and aldosterone synthase in the hippocampus of paraffin-embedded brain sections using the CSA kit protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 500μm.

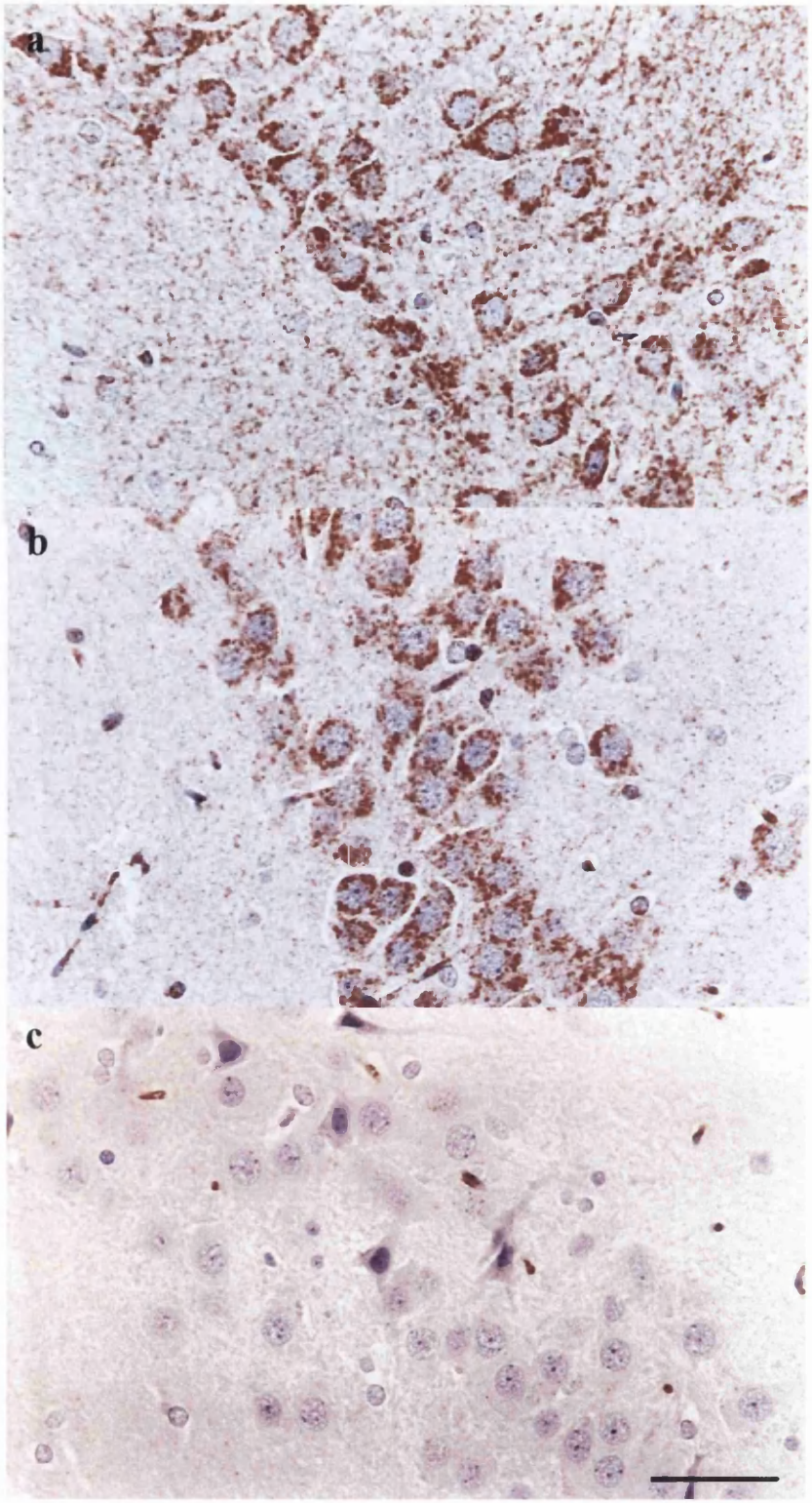


Figure 5.3i. Immunostaining for 11β-hydroxylase and aldosterone synthase in the CA3 cells of paraffin-embedded brain sections using the CSA kit protocol. a: 11β-Hydroxylase; b: aldosterone synthase; c: control. Scale bar = 50μm.

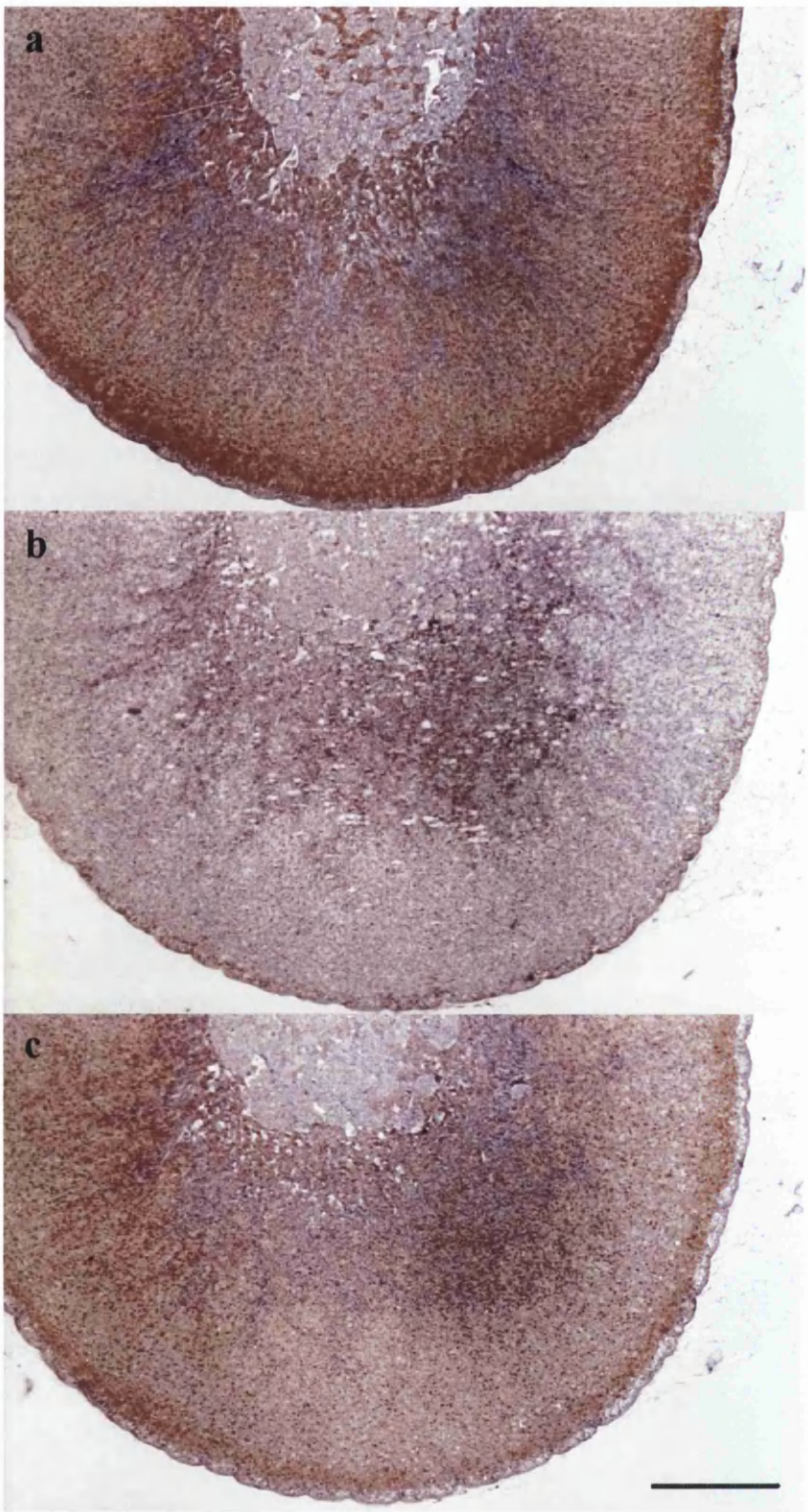


Figure 5.3j. Quenching of immunostaining in adrenal tissue by preincubation of anti-11β-hydroxylase antibody 11BIA4 (1:100 dilution) with its immunogenic peptide. The CSA kit protocol was used. a: Antibody without peptide; b: antibody with 10μM peptide; c: antibody with 0.1μM peptide. Scale bar=500μm.

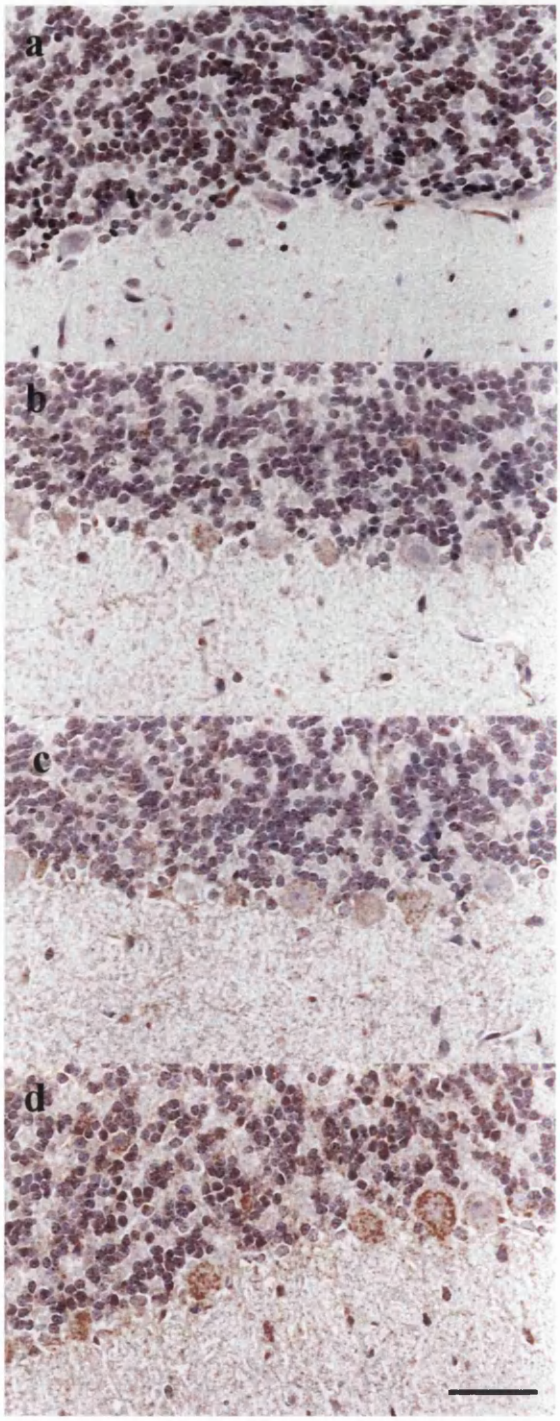


Figure 5.3k. Quenching of immunostaining in cerebellar Purkinje cells by preincubation of anti-aldosterone synthase antibody ASIE6 (1:50 dilution) with decreasing concentrations of its immunogenic peptide. The CSA kit protocol was used. a: antibody with 1mM peptide; b: antibody with 10μM peptide; c: antibody with 100nM peptide; d: antibody with 1nM peptide. Scale bar = 50μm.

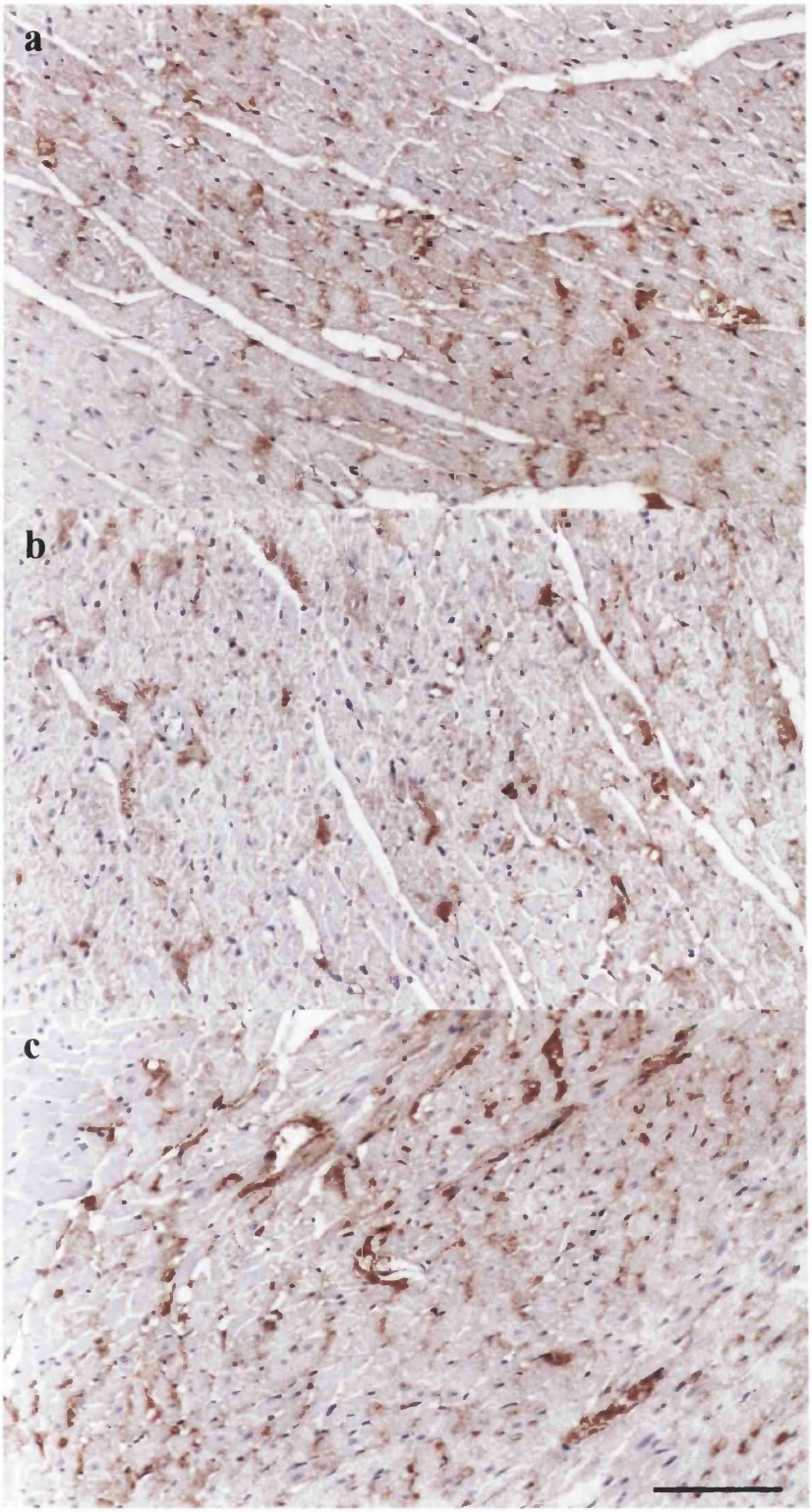


Figure 5.3l. Immunostaining for 11β-hydroxylase and aldosterone synthase in paraffin-embedded heart sections using the CSA kit protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 100μm.

staining in adrenal tissue cryosections before adapting this same protocol for use with paraffin sections of adrenal and extra-adrenal tissue. Using cryosections, anti-11β-hydroxylase staining was identified. This followed the known distribution of the enzyme throughout the zona fasciculata-reticularis, with the outer zona glomerulosa completely clear. However, this ABC method could not produce positive results for aldosterone synthase in the same tissue. Attempts were also made to stain for 11β-hydroxylase in brain tissue but this was unsuccessful. Despite these results, it was decided to make further attempts at immunostaining using paraffin-embedded tissue.

The adrenal tissue used had been fixed in formalin solution (40% w/v formaldehyde in water) for 24 hours. Such formaldehyde-based fixatives form cross-linking methylene bridges between basic amino acids. Although this action preserves the tissue, it can also mask the antigen, preventing access of the antibody to its specific binding site. For this reason, certain pretreatments – such as proteolytic digestion or heating of the tissue in citrate buffers – are necessary to expose the antigen once more. When using the ABC method on paraffin-embedded tissue, proteolytic treatment followed by microwave heating were found to be necessary and, under these conditions, specific staining for both 11β-hydroxylase and aldosterone synthase in adrenal tissue was achieved. However, brain tissue still gave negative results.

Nevertheless, these results did confirm the specificity of the antibodies for their respective enzymes. The antibodies bound specifically to the expected regions of the adrenal cortex (see **section 1.2.4**) and were apparently free of cross-specificities. This is an important consideration given the high degree of amino acid sequence homology between 11β-hydroxylase and aldosterone synthase.

The failure to detect the enzymes in extra-adrenal tissue was, as with the immunoblotting in the previous chapter, attributed to the lower levels of these enzymes that were expected to be present. Therefore, a biotinylated tyramide method was adopted in order to increase the intensity of staining resulting from any bound primary antibody. In such protocols, a streptavidin-biotin-peroxidase complex is formed at the site of the bound primary antibody, as with the ABC method. However, instead of performing the chromogenic reaction at this point, the peroxidase enzymes that have been assembled at the site are reacted with biotinylated tyramide. This adds biotin at the site which then binds streptavidin-peroxidase conjugates, thus greatly increasing the number of peroxidase enzymes in the region of the bound primary antibody. Therefore, the intensity of the final chromogenic reaction is greatly enhanced (see **figure 5.4a**). The DAKO catalysed signal amplification (CSA) system kit which works on this principle was used in all subsequent immunostaining experiments. It is appropriate only for paraffin-embedded tissue.

Immunostaining of tissue using the CSA method required some changes to the pretreatment of tissue. First, formalin-fixed tissue gave heavy background staining. More specific immunostaining was achieved using 4% w/v paraformaldehyde as a fixative; this was the recommended fixative for use with the kit. Protease pretreatment also resulted in intense background staining. This step was omitted but microwave pretreatment was retained.

Staining of adrenal tissue using the CSA kit followed the same zonal distribution as with the ABC protocol although, as would be expected, staining was more intense. The zonal distribution of these enzymes has been shown previously by immunohistochemistry using these antibodies (Holzwarth *et al.* 1996; Engeland *et al.* 1996) and also polyclonal antibodies raised in rabbits (Mitani *et al.* 1994). Although

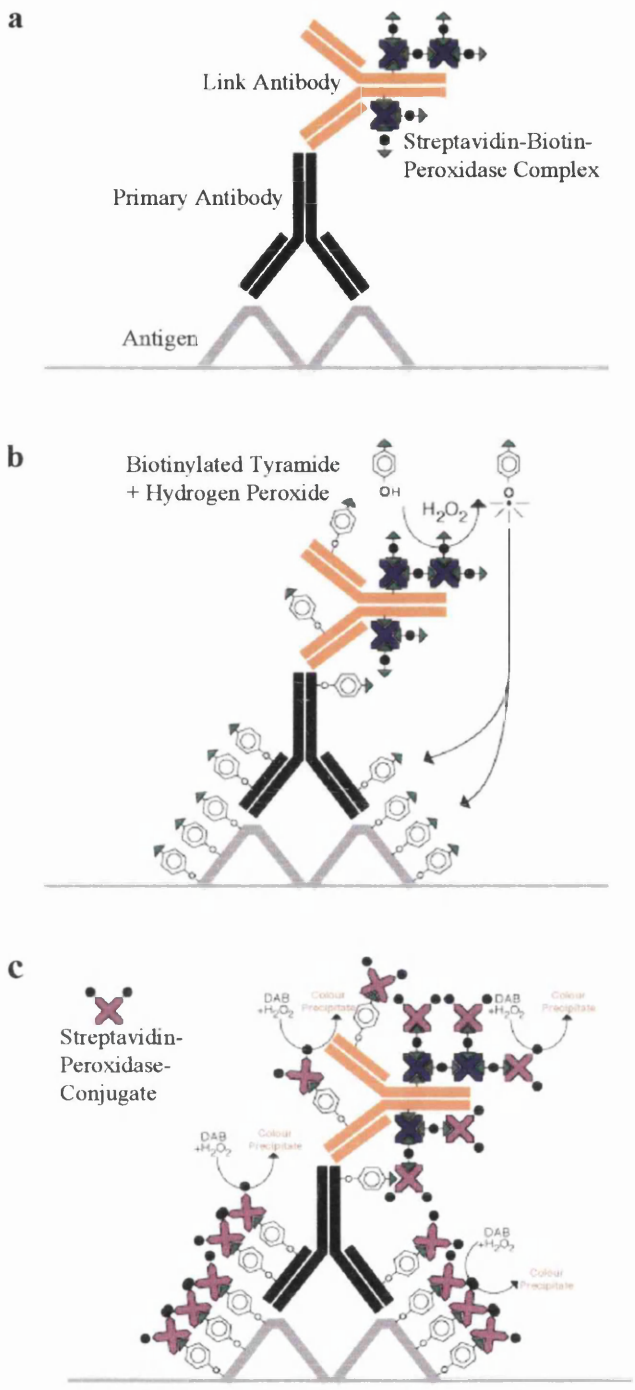


Figure 5.4a Schematic diagram of immunostaining methods. The ABC protocol forms a complex at the site bound by the primary antibody (a). The CSA method amplifies this signal further by precipitating biotinylated tyramide at the bound site (b) which then binds streptavidin-peroxidase conjugate (c). (From *www.dakoltd.co.uk*.)

not a main purpose of the study, the absence of 11β-hydroxylase from the zona reticularis suggests that it does not contribute significantly to glucocorticoid production. This deficiency may account for the relatively high DOC levels in the rat. A similar situation in man may explain its role as a source of adrenal androgens. By double-staining for both enzymes on the same tissue section, it has been shown that there is a layer of cells between the zona glomerulosa and the zona fasciculata-reticularis which expresses neither enzyme (Mitani *et al.* 1994). These groups also showed that a low Na^+ diet increased the width of the zona glomerulosa (Holzwarth *et al.* 1996). However, they failed to detect the surprising finding here that the adrenal medulla stained positively for 11β-hydroxylase. This staining had been faint and therefore ambiguous when using the ABC method but the CSA method gave an undisputable positive result with stained cells apparently extending from the zona reticularis into the medulla. Previously, 11β-HSD1 has been localised to the rat adrenal gland's corticomedullary junction. Its presence was thought to optimise the delivery of corticosterone from the cortex to the medulla, thereby stimulating adrenal catecholamine biosynthesis (Paxinos and Watson, 1998). Medullary 11β-hydroxylase may perform a similar function, either by converting precursor which passes into the medulla or by *de novo* synthesis, although the presence of other enzymes in this pathway within the medulla has not been demonstrated. Shimojo *et al.* were therefore surprised to find 11β-HSD2 expression within the medulla. This posed the question of why the adrenal medulla should produce corticosterone only to inactivate it? The discovery of 11β-hydroxylase expression within the medulla only adds to this puzzle. Further, more precise histology will be required to ascertain whether the 11β-hydroxylase-expressing cells are typical chromaffin cells, displaced adrenocortical cells or even part of the local vascular system.

Consistent staining in brain tissue proved more difficult to achieve. As with the immunoblotting experiments, various antibody dilutions, incubation times and temperatures were used before settling on the final protocol given in **section 2.4.5**. This method gave reproducible staining in brain tissue. The staining varied greatly in intensity throughout the section, suggesting that it was not simply blanket background staining. The staining was most intense in two particular regions of the brain, the cerebellum and the hippocampus. In marked contrast to the adrenal staining for the two enzymes, the brain staining for 11β-hydroxylase and aldosterone synthase colocalised. Staining for both was visible in the cerebellum and the hippocampus. Colocalisation may imply interaction. The enzymes may compete for their principal substrate, DOC. 11β-Hydroxylase synthesises corticosterone but this is a poor substrate for aldosterone synthase. The level of interaction will obviously depend on relative expression rates and on the relative affinities of the brain enzymes for DOC. These remain to be determined.

Although the distribution of 11β-hydroxylase and aldosterone synthase throughout the brain has not previously been investigated in such detail, those of several other enzymes in the corticosteroid pathway have. The distribution of *CYP11A1*, 3β-HSD and StAR transcripts has been analysed by *in situ* hybridisation and the results of these studies were recently published. Purkinje cells contained transcripts from all three of the genes investigated (Guennon *et al.* 1995; Furukawa *et al.* 1998; Ukena *et al.* 1999). These cells also produced intense immunostaining when incubated with a P450scc-specific antibody (Ukena *et al.* 1998). This matches the findings here, where cerebellar staining for 11β-hydroxylase and aldosterone synthase was most intense within the Purkinje cells. The other studies are also in

broad agreement with the presence of these enzymes in the molecular and granular layers of the cerebellum but not the white matter.

The hippocampal expression of *CYP11B1* and *CYP11B2* also agrees with investigations into the distribution of StAR, P450scc and 3β-HSD. Aldosterone synthase and 11β-hydroxylase were detected throughout the pyramidal cells of the hippocampus, in the CA1, CA2 and CA3 cells of Ammon's horn as well as in the dentate gyrus. StAR transcript levels are slightly lower in the hippocampus than in the cerebellum (which has the highest expression level within the brain) but, along with the olfactory bulb, these are the only brain regions to demonstrate strong StAR expression; levels in such regions as the thalamus and hypothalamus are far lower (Furukawa *et al.* 1998). *In situ* hybridisation to *CYP11A1* and 3β-HSD transcripts overlap with StAR's expression pattern in the cerebellum and hippocampus, except for the CA3 region of the hippocampus where 3β-HSD transcription cannot be detected. 3β-HSD has also been detected by immunohistochemistry in the rat hippocampus and cerebellum (Guennon *et al.* 1995).

These two brain regions also have the greatest density of MR (Agarwal *et al.* 1993). GR, although widespread throughout the brain, is highly expressed in the hippocampus. As 11β-hydroxylase and aldosterone synthase seem to colocalise to hippocampus and cerebellum, this raises the question of whether there is any MR/GR selectivity in these regions. 11β-HSD activity in homogenates of cerebellum and hippocampus is estimated to be at 10–30% of the level found in kidney and liver (Seckl, 1997). It is open to question whether this would confer MR selectivity on these regions of the brain.

Work on the presence of P450scc and 3β-HSD in the Purkinje cell has focussed on its possible developmental role. P450scc has a constant expression level

within the rat Purkinje cell from its differentiation at 3 days of age to the final formation of the cerebellum at around 21 days (Ukena *et al.* 1998). 3β-HSD transcript levels, on the other hand, are low at 3 days, high at the 10-day stage and then fall back by 60 days of age. Distribution of expression also changes. 3β-HSD transcripts are detectable in Purkinje and all granular cells at 10 days but become restricted to Purkinje cells and fewer granule cells by 60 days (Ukena *et al.* 1999). Ukena *et al.* (1999) are unsure whether steroids produced by these cells could perform a developmental role or whether the changing expression pattern of 3β-HSD simply reflects the increasing differentiation of the cells within the region. The presence of aldosterone synthase and 11β-hydroxylase in these cells suggests that the progesterone produced by 3β-HSD may only be an intermediate product and that it is required for the ultimate production of aldosterone and corticosterone by these cells.

The expression of these enzymes within Purkinje cells is an important finding for another reason. It has long been established that the glial cells of the nervous system are capable of neurosteroid production and, in culture, can express steroidogenic genes including *CYP11A1* (Mellon and Deschepper, 1993; Sanne and Krueger, 1995). The weight of evidence suggests that these neuronal cells are also capable of neurosteroidogenesis. (Further evidence in favour of steroid production by neurons is presented in the next chapter.)

The accumulation of evidence suggesting that all the components of the corticosteroidogenic machine are present in the hippocampus is also compelling although, as with the cerebellum, there is little understanding of what purpose these enzymes might serve. As reviewed in **chapter 1**, the corticosteroids have many effects on this region of the brain although it is still unclear whether locally-produced hormone is of physiological significance.

Although the expression of these genes appears strong in certain regions of the brain, it is confined to comparatively few cells. Such expression would probably be incapable of exerting any significant endocrine effects. Therefore, a more probable action would be through a paracrine or even autocrine mode. Work on the heart has shown that, even where expression is highly regional, large local concentrations of steroid can be achieved (Delcayre and Silvestre, 1999).

Finally, attempts were made to investigate the distribution of 11β-hydroxylase and aldosterone synthase within the rat heart, given previous evidence of their expression in that tissue (see **section 1.6.1**). However, non-specific staining was sufficiently strong to render the control sections indistinguishable from those incubated with primary antibody. It may be that specific staining did occur but that it was masked by the intensity of the non-specific staining. The staining was probably due to cross-reactivity with endogenous immunoglobulins which is acknowledged by the manufacturers as a limitation of the CSA kit. This results in 'staining in the negative control around blood and lymph vessels, in connective tissue, and in extravascular tissue spaces' as well as 'certain types of epithelium'. Antigen retrieval protocols such as the microwaving method used here are known to increase this effect. Therefore, this method is probably inappropriate for use on heart tissue. The rigorous controls performed on the adrenal gland and brain confirm that the immunostaining produced in those tissues is specific and does not result from the non-specific interactions encountered with the heart.

Having identified the presence of 11β-hydroxylase and aldosterone synthase within the rat brain, the next chapter assesses whether neurons derived from the fetal rat brain are able to synthesise corticosteroids.

Chapter 6

Results: Corticosteroid Production By Primary Cultures Of Fetal Rat Hippocampal Neurons

6.1 Introduction

The immunostaining results presented in the previous chapter highlighted the hippocampus as one of two regions of the adult rat brain that react positively with specific anti-11β-hydroxylase and anti-aldosterone synthase antibodies. It would be desirable to study such cells in isolation from other brain cell types in order to assess their corticosteroidogenic capabilities. Although adult rat neurons are not capable of cell division that would make their culture possible, fetal hippocampal neurons possess the ability to divide and grow in culture. Dr Margaret Lai of the Molecular Medicine Centre at the University of Edinburgh is skilled in the preparation of such cells and provided rat fetal hippocampal neurons for the work presented here.

The results of various experiments on these cells are presented in this chapter. First, their capacity to transcribe *CYP11B1*, *CYP11B2* and other steroidogenic genes was established by RT-PCR. Expression of 11β-hydroxylase and aldosterone synthase polypeptides was then confirmed by immunostaining. Finally, their enzymatic activity was assessed by incubating them with the corticosteroid precursor DOC and measuring aldosterone and corticosterone production.

6.2 Methods

The procedure used by Dr Lai to prepare the primary cultures of fetal hippocampal neurons for these experiments is described in **section 2.6.1**.

Dr Lai also provided total RNA isolated from these cells which was used for RT-PCR and Southern blotting experiments as described in **sections 2.1.3** to **2.1.9**. The RNAs for these experiments were subjected to DNase treatment before use, as described in **section 2.6.2**. Immunostaining of the cells was performed using the CSA kit protocol in **section 2.4.5**. No pretreatments were necessary.

The procedure for the incubation of cells with DOC and analysis of the steroid products is given in **sections 2.6.3** to **2.6.6**.

6.3 Results

6.3.1 RT-PCR

The cells were tested for the presence of the three types of *CYP11B* transcripts, and a positive result was observed for each of these (see **figure 6.3a**). The presence of adrenodoxin and *CYP11A1* transcripts was also established (see **figure 6.3b**). All controls were clear.

6.3.2 Immunostaining

The cells stained positively in the presence of the anti-11β-hydroxylase antibody 11BIA4 and the anti-aldosterone synthase antibody AS IE6. Controls were clear (see **figure 6.3c**). There appeared to be a difference in the subcellular localisation of staining; 11β-hydroxylase staining was more apparent in the dendrites while aldosterone synthase staining tended to be within the cell body.

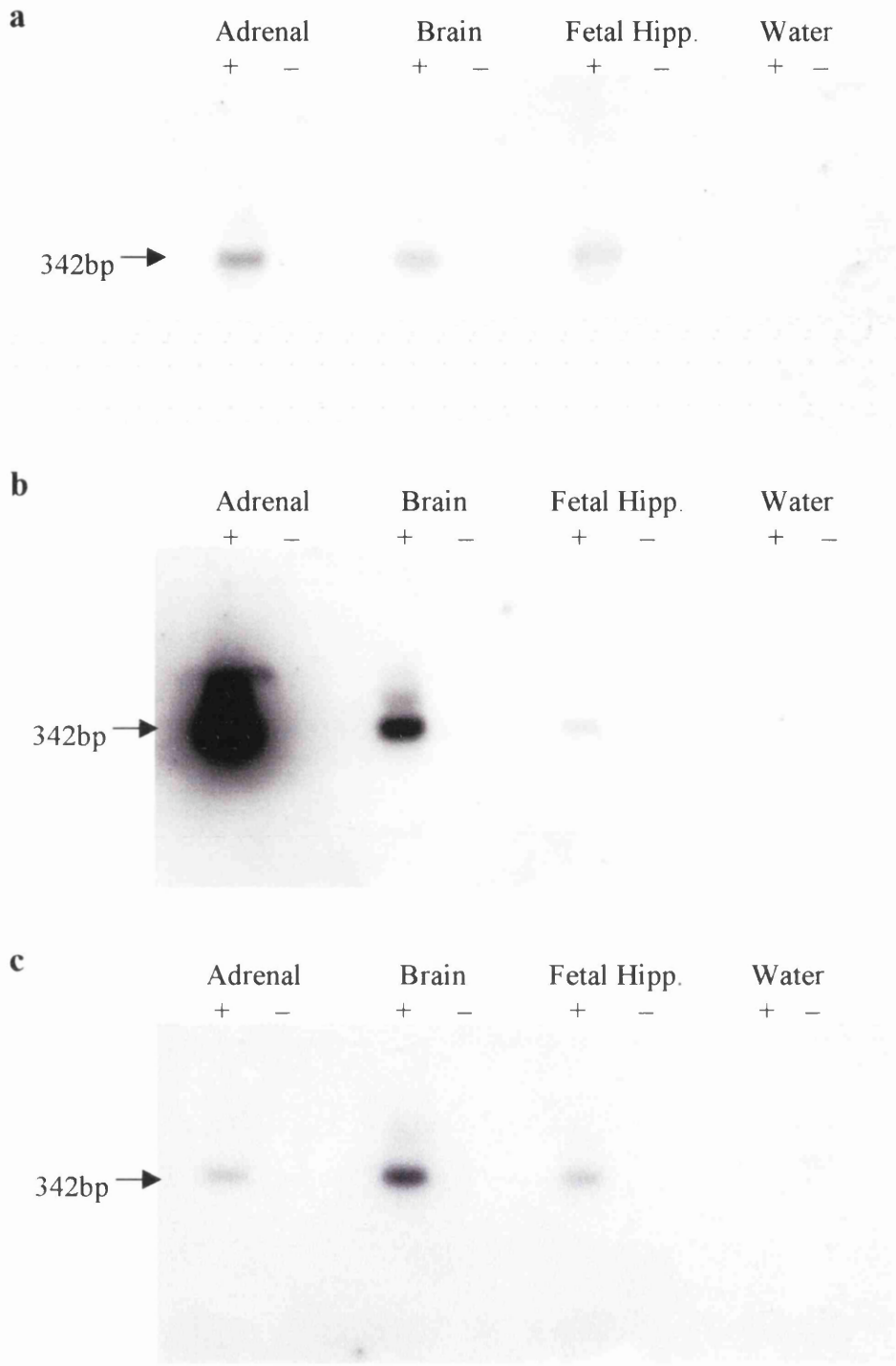
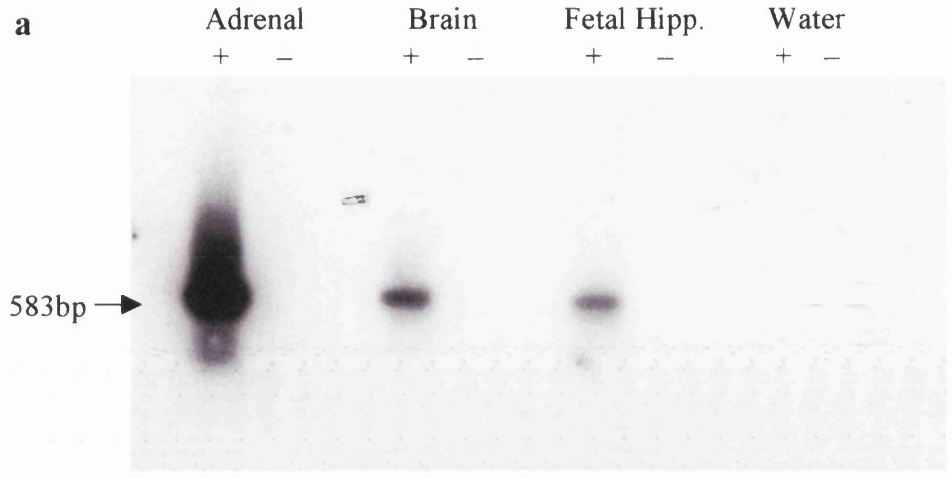


Figure 6.3a RT-PCR of *CYP11B* RNAs from adult rat adrenal gland, whole adult rat brain and primary cultures of fetal rat hippocampal neurons performed in the presence (+) and absence (–) of reverse transcriptase. (a) *CYP11B1*; (b) *CYP11B2*; (c) *CYP11B3*. Transcripts were detected by Southern blotting and subsequent hybridisation with a specific, radiolabelled probe.



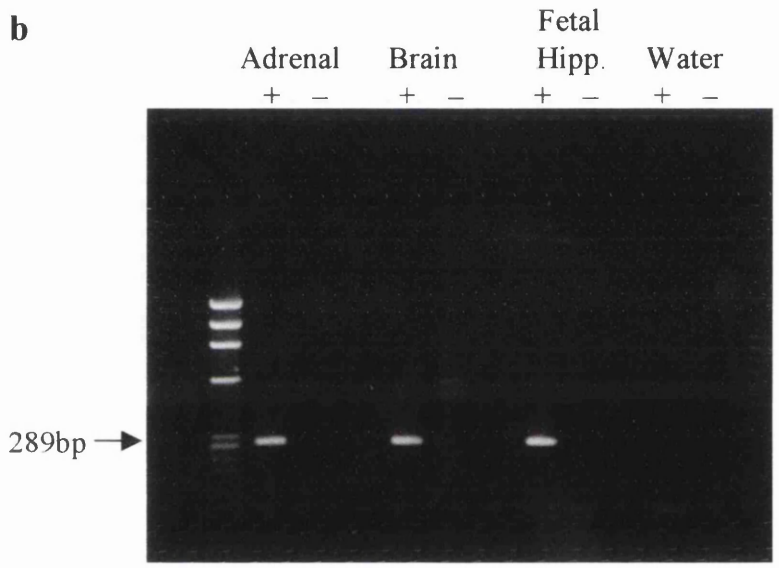


Figure 6.3b RT-PCR of RNAs from adult rat adrenal gland, whole adult rat brain and primary cultures of fetal rat hippocampal neurons performed in the presence (+) and absence (–) of reverse transcriptase. (a) *CYP11A1* transcripts detected by Southern blotting and subsequent hybridisation with a specific, radiolabelled probe.; (b) Adrenodoxin transcripts visualised on agarose gels under ultraviolet light.

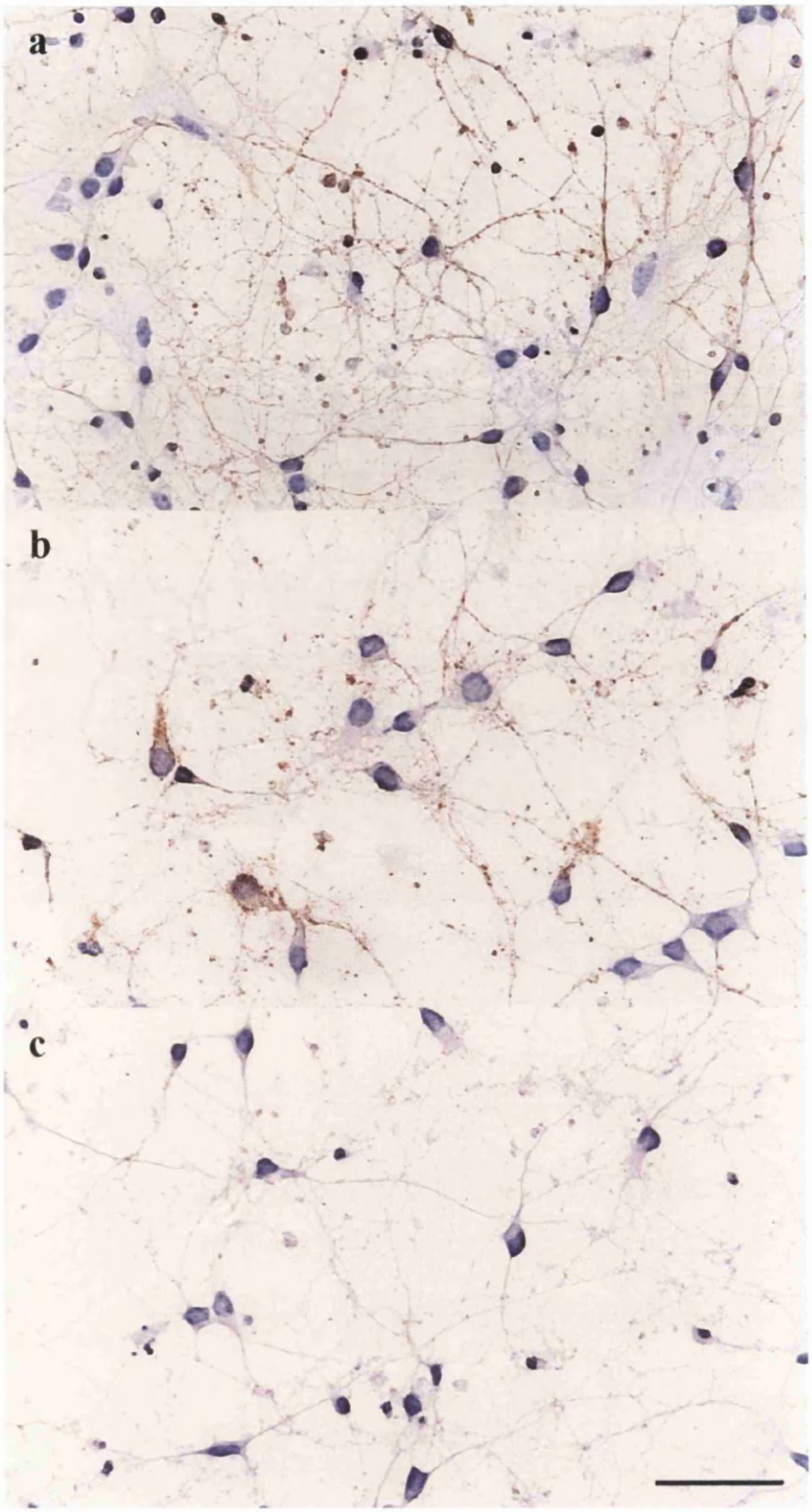


Figure 6.3c. Immunostaining for 11β-hydroxylase and aldosterone synthase in primary cultures of fetal rat hippocampal neurons using the CSA kit protocol. a: 11β-hydroxylase; b: aldosterone synthase; c: control. Scale bar = 50μm.

6.3.3 Conversion of tritiated DOC to aldosterone and corticosterone

The initial conversion experiments incubated primary cultures of fetal rat hippocampal neurons in medium containing 10μM DOC plus ~0.5×10^6c.p.m. of ^{3}H-DOC. After 24 hours, the medium was removed and aldosterone and corticosterone extracted from it. The ^{3}H content of these steroids was then measured after partial purification (see **section 2.6.4**). The results are given in **table 6.3a**.

Samples (n=10)	c.p.m. ±S.D.	% standard recovery	Corrected c.p.m. ±S.D.	% conversion
Aldosterone	2680.2±409.7	17.3	15519.0±2372.0	2.8
Corticosterone	1908.7±224.5	29.4	6606.8±777.1	1.2

Table 6.3a Conversion of ^{3}H-DOC to aldosterone and corticosterone by primary cultures of fetal rat hippocampal neurons in a 24-hour period. A total of 549,352 c.p.m. ^{3}H was added to each sample.

6.3.4 Conversion of unlabelled DOC to aldosterone and corticosterone

Subsequent conversion experiments incubated the cells in medium containing only unlabelled DOC at a concentration of 10μM. After a 24-hour incubation period, the medium was removed from the cells. Aldosterone and corticosterone were extracted from the medium and their concentrations measured by radioimmunoassay (see **section 2.6.5**). The total protein content of the cells in each sample was also measured. Control medium, which contained no DOC, was also analysed (see **table 6.3b** and **figure 6.3d**). There was an apparent strong correlation between aldosterone and corticosterone production. However, this may have been due to the variable state of the cells between aliquots.

Sample	Corticosterone		Aldosterone	
	pmol/L medium	pmol/mg protein	pmol/L medium	pmol/mg protein
Control 1	4175	79	550	10
Control 2	2300	78	550	19
Sample 1	149150	2451	103175	1696
Sample 2	74000	1138	61750	949
Sample 3	187375	2034	114925	1248
Sample 4	54150	941	825	14
Sample 5	46125	615	52350	698
Sample 6	75500	2267	62075	1864
Sample 7	1150	48	725	30
Sample 8	132625	15789	141300	16821
Sample 9	203975	5319	141575	3692
Sample 10	1150	35	275	8

Table 6.3b Conversion of 10μM DOC to aldosterone and corticosterone by primary cultures of fetal rat hippocampal neurons in a 24-hour period. Products were measured by radioimmunoassay and corrected for recovery and protein content. DOC was omitted from control sample medium.

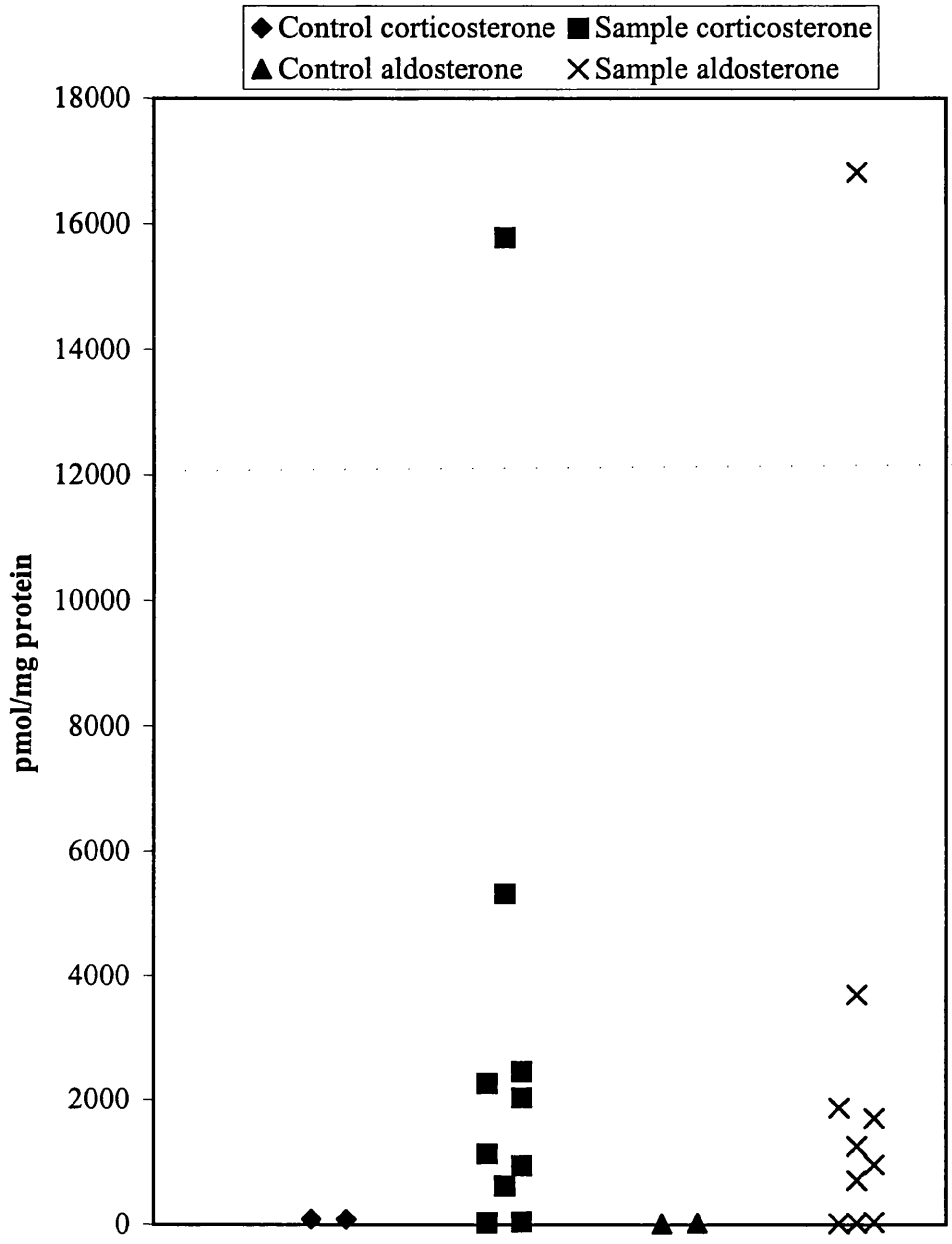
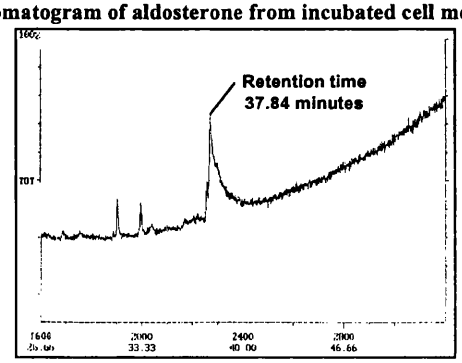


Figure 6.3d Scatter plot showing conversion of 10μM DOC to aldosterone and corticosterone by primary cultures of fetal rat hippocampal neurons in a 24-hour period. Products were measured by radioimmunoassay and corrected for recovery and protein content. DOC was omitted from control sample medium.

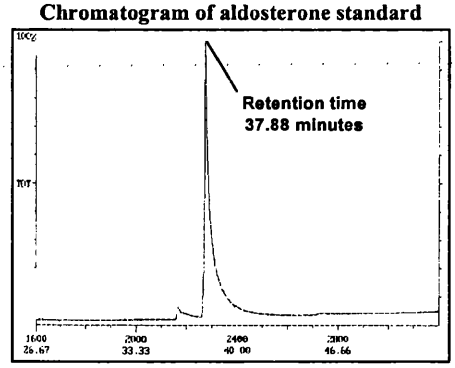
6.3.5 Identification of aldosterone by gas chromatography mass spectrometer (GCMS)

Incubated cell medium was subjected to paper chromatography in order to isolate any aldosterone within the sample. This partially purified sample was then injected on to a GCMS where it gave a distinctive peak with a retention time of 37.84 minutes. This matched the retention time of 37.88 minutes obtained for the aldosterone standard. (The trivial difference in retention time between sample and standard is due to the imprecision of manual injection on to the GCMS.) These peaks were found to have identical mass spectra. See **figure 6.3e**.

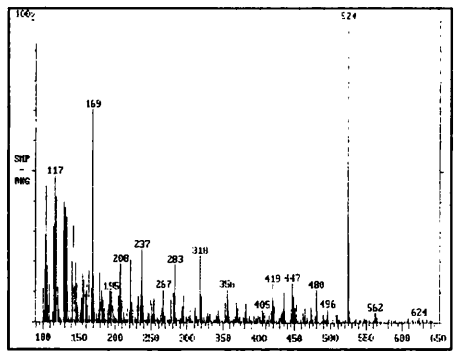
Chromatogram of aldosterone from incubated cell medium



Chromatogram of aldosterone standard



Spectrum of aldosterone from incubated cell medium



Spectrum of aldosterone standard

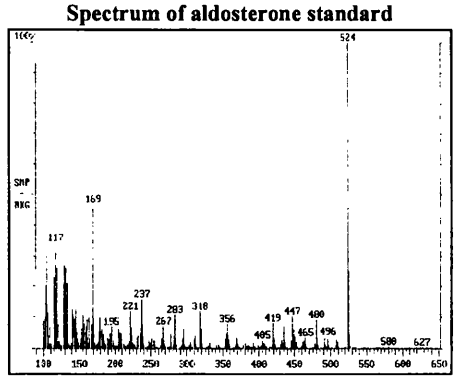


Figure 6.3e Chromatograms and mass spectra of aldosterone from incubated cell medium samples and standard solution.

6.4 Discussion

The RT-PCR results mostly confirmed the findings described in **chapter 3**. Transcription of the *CYP11B1*, *CYP11B2*, *CYP11A1* and adrenodoxin genes was demonstrated in fetal hippocampal neurons, and this is predictable in the light of those previous results.

More surprising was the discovery of *CYP11B3* transcription not just in the fetal rat hippocampal neurons but also in the adult rat adrenal and brain tissue homogenates which had been used as controls. These tissues had previously been found negative for *CYP11B3* transcription (see **chapter 3**). *CYP11B3* transcription has never before been detected in adult tissues (Mellon *et al.* 1995). The most likely explanation for this inconsistent result is the treatment of the RNA samples used in this chapter with DNase. This step was introduced because of genomic DNA contamination in the hippocampal cell RNA provided which led to PCR primers hybridising with genomic DNA sequences as well as any cDNA that may have been present. This effectively lowered the concentration of PCR primers within the reaction and any resulting bands were therefore fainter or undetectable. DNasing of the samples removed this problem and increased the sensitivity of the RT-PCR technique. The control RNA samples were also DNased. The detection of a *CYP11B3* signal in these adult RNAs therefore introduces the possibility that the results of **chapter 3** might have been different if all the RNA samples had been treated in the same way. However, although there may have been some falsely negative results produced in that work, this in no way throws into doubt the veracity of the positive results.

An alternative reason for the positive *CYP11B3* results is that the washes to remove any hybridised *CYP11B3* probes were somehow less stringent than those

used in **chapter 3**, despite the use of the same protocol. The nature of the *CYP11B* genes is such that some similarity between hybridising probes is inevitable. Although the probes for *CYP11B1* and *CYP11B2* are very different, the *CYP11B3*-specific probe has similarities to the *B1* and *B2* sequences at the hybridising region. Therefore, it is possible that, rather than detecting *CYP11B3*, this probe is detecting one or both of the other *CYP11B* genes.

The immunostaining results show that the *CYP11B1* and *CYP11B2* transcripts are translated into their respective polypeptide products within these cells. This, together with the RT-PCR findings, confirms the evidence in **chapter 5** that suggested neurons as well as glia can express steroidogenic enzymes such as aldosterone synthase and 11β-hydroxylase. From the subcellular staining of the fetal hippocampal neurons, there is a suggestion that the distribution of these two proteins might be different. 11β-hydroxylase staining tends to be within the dendrites projecting from the cell body while aldosterone synthase staining is seen more within the cell body itself. Mitochondria are found within both the cell bodies and the dendrites of neurons and these immunostaining results are therefore consistent with their cellular location.

The incubation experiments were performed initially with ^{3}H-DOC as it renders any conversion of DOC to aldosterone or corticosterone easier to detect. In the event, the detection of these products was not difficult and conversion occurred at a higher rate than had been expected. Having obtained these preliminary results, further incubations using 10μM concentrations of non-radioactive DOC were performed. Although the rates of conversion showed a great deal of sample-to-sample variation, in most cases the DOC-incubated samples contained much more

product than the controls. The identification of aldosterone within the incubated samples by GCMS confirmed this finding.

The ability of brain cells to perform reactions from the corticosteroid pathway has been tested before, although this has usually involved homogenates of brain tissue (Gómez-Sánchez *et al.* 1996; Ukena *et al.* 1999) or cultures of glial cells (Jung-Testas *et al.* 1989). Ukena *et al.* (1999) showed that cerebellar homogenates were able to convert pregnenolone to progesterone, suggesting the presence of active 3β-HSD. Gómez-Sánchez *et al.* (1996) demonstrated that minces of various brain regions including hippocampus, hypothalamus and cerebellum could convert ^{3}H-DOC to corticosterone, 11-dehydrocorticosterone and 18-OH-DOC. However, such methods cannot distinguish conversion by glial and neuronal cells and, as with RT-PCR, it is often difficult to dissect tissue precisely, especially in such a heterogenous tissue as the brain. The work presented here demonstrates conversion by neuronal cells of a single, homogenous type.

Having established that the cells perform the terminal stages of aldosterone and corticosterone production, the next logical step would be to explore the earlier stages of the pathway. Are the cells capable of *de novo* corticosteroid production from cholesterol? These cells may also be a good model for studying how corticosteroid production is regulated in neural tissue. Incubation of substrate together with potential modulators of corticosteroid production could give insights into such regulatory mechanisms and provide more detailed information about this potentially important site of extra-adrenal corticosteroidogenesis. It remains to be seen whether conventional factors such as ACTH, AngII or K+ also control brain expression or, more interestingly, whether control depends on local neurotransmitters such as serotonin or noradrenaline.

Chapter 7

General Discussion

Steroid hormones are ubiquitous switches controlling the rate of gene transcription. By binding to receptors, corticosteroids control genes involved in many metabolic processes. Over recent decades, our understanding of the biosynthesis, secretion and action of mineralocorticoids and glucocorticoids has become almost complete, although some details (e.g. transcription factors) are not fully resolved. The action of aldosterone on the distal nephron or of cortisol on liver or adipose tissue is now well understood. General attention in the next decades may concentrate on achieving a similar level of understanding of the brain.

Corticosteroids are of major importance in regulation of CNS function. Their varied effects on such complex aspects of behaviour as mood or memory have been established but are poorly understood. The effects of corticosteroids in the brain on blood pressure are clearly different from those exerted systemically. Further complexity must now result from the fact that the brain, or specific parts of it, receives its supply of corticosteroids from two distinct sources, the adrenal cortex and the brain cells themselves. The work in this thesis has contributed new information on the local neural synthesis of aldosterone and corticosterone in the rat. In particular, it confirms and strengthens the evidence that *CYP11B1* and *CYP11B2* are transcribed, that the enzyme proteins are formed and that, in rat fetal hippocampal cells at least, these enzymes can convert the proximal precursor to their respective products. Indeed, the identification of enzymes catalysing earlier steps in the biosynthetic pathway strongly supports the possibility of *de novo* synthesis from cholesterol within the brain. Strikingly, aldosterone synthase and 11β-hydroxylase

are coexpressed within the same brain cells, although not necessarily in the same intracellular compartment.

Thus, the rat brain makes aldosterone and corticosterone, although at a lower rate than the adrenal cortex. It is probable, therefore, that the human brain produces aldosterone and cortisol. The physiological significance of this local production, however, is not clear. It may merely be a device for achieving or maintaining local hormone levels, as with the high local cortisol levels that are necessary in the adrenal medulla. Alternatively, it may provide a mechanism for brain tissue to respond to a set of stimuli completely different from those encountered in the adrenal cortex. If this is the case, then the control of gene expression must be different. This exciting prospect could be investigated by, for example, analysing *in vitro* gene expression in response to particular agonists. Alternatively, it may be the production of conventional agonists such as AngII, which is also synthesised in the brain, that is controlled differently.

The clinical implications of neural corticosteroid synthesis are profound. Excess glucocorticoid has deleterious effects on brain function, so can local synthesis contribute to this? Very small increases in brain aldosterone levels markedly raise blood pressure. Where studies of systemic physiology have largely failed to account for common hypertensive syndromes, could aberrations of local brain synthesis provide an explanation? Investigation of these questions will require considerable ingenuity in both technical innovation and experimental design. Finally, if specific clinical significance is established, manipulation of these enzyme systems or of the brain receptors on which their products act may become a useful approach to therapy. For this, a detailed knowledge of brain corticosteroid molecular biology and

physiology is an absolute prerequisite. Thus, there remain numerous challenging avenues for research in the future.

Appendix 1: Nucleotide and deduced peptide sequences of *CYP11B1* and *CYP11B2*

Nucleotide Sequences The nucleotide sequences for rat *CYP11B1* and *CYP11B2*, obtained from the EMBL database, are shown below. The accession numbers for these sequences are:

	CYP11B1	*CYP11B2*
5' Region and exons 1–2	D14086	D14092
Exons 3–4	D14087	D14093
Exon 5	D14088	D14094
Exon 6	D14089	D14095
Exons 7–8	D14090	D14096
Exon 9	D14091	D14097

Exons, TATA boxes and cAMP-responsive elements (CREs) are shown in bold type. Introns are shown in lower case. The transcription initiation site is marked by an asterisk. The oligonucleotides listed in **appendix 2** are indicated by their name in bold letters above the relevant underlined sequence. Sense and antisense oligonucleotides are denoted by the symbols > and <, respectively.

Rat *CYP11B1*

```
agatctggac tcaggttctc atagttgacc aactaagtca tcctcttacc   50
ctatggcaaa tggagttcat tgttatttct caatttgaag agaaatgaga  100
aattctaaaa gccaacactt aaccgtaaat cacgacagtg agtaaagacc  150
cactccctaa atctggctag ggaattttaa actaggatga atcattcaag  200
gttccacaaa gggaaaatat gtgcatctga cggctctcac catgggggag  250
gagaaagaag gctctgacca ccattggact atttttgagt gttaaagtag  300
agtctgcacc ctcccaccca ccagcaggca ttgcagaggt aggaaaaggg  350
agaaagcctc tacctccaga agaaccatca gctcagtata catttctagg  400
```

CRE
gcaagtccag ggacacatcc tgcagtgaca ttatcagtca gcgatttata 450
TATA Box *
tcctcaagac aagataaaag gccacggact aaacacagga agagaggagg 500
Exon 1
atggcaATGG CTCTCAGGGT GACAGCAGAT GTGTGGCTGG CAAGACCCTG 550

GCAGTGCCTG CACAGGACGA GGGCACTGGG CACTACGGCA AAAGTGGCCC 600

CCAAGACACT GAAGCCCTTT GAAGCCATAC CACAATACTC CAGGAACAAG 650

TGGCTGAAGA TGATACAGAT CCTGAGAGAG CAGGGCCAAG AGAACCTACA 700
Intron 1
CCTGGAGATG CACCAGGCCT TCCAAGAGCT GGGGCCCATT TTCAGgtaag 750
(370bp)
ggctttcctt ccccatagcc aagcacagat atagcttgct gtcctctggt 800

tgcccaggct ctgcagggta ggaggtgctg cattctcctg taggcacccg 850

gcattccttc tttaggaaga cagggaggga gaacaggagg ttctgggact 900

gactcgtggg ctctccacag ggacagagaa aggcacttct cactgctgag 950

aggacagagt gctgaagtgg agtgcagaca ctgggagagt cctcctgtgg 1000

gggaagcaag ggctaggaga ggatgagatg agagagcaca ctgacagggc 1050

tggctgtgca gcgagctcag gtggaggaca ctccacttac agcagggctg 1100
Exon 2
tgtgcttcac cgcagGCACA GTGCAGGGGG AGCACAGATT GTGTCTGTGA 1150

TGCTGCCTGA GGACGCTGAG AAGCTGCACC AGGTGGAGAG TATCCTCCCG 1200
P5812>
CATCGGATGC CCCTGGAGCC GTGGGTGGCC CACAGAGAAC TCCGTGGCCT 1250
Intron 2 (1400bp)
GAGACGTGGT GTGTTCTTGC Tgtgagtgga c---------- ---------- 2661
Exon 3
tgttctgcag AAATGGGGCA GACTGGCGCT TCAACCGACT GCAGCTGAAT 2711

CCAAATATGC TGTCACCAAA AGCCATTCAA TCTTTTGTCC CCTTTGTGGA 2761

TGTGGTAGCA AGGGACTTTG TGGAAAACCT GAAGAAGAGA ATGCTGGAGA 2811
P5815>
ATGTTCATGG AAGCATGTCT ATAAACATTC AGTCCAATAT GTTCAACTAT 2861
Intron 3 (114bp)
ACCATGGAAG gtatgtatgg ggaaggctcc agcttgagag aggcagggac 2911

ggcagaggcc aggaaggagg aaaaatattg agagcaatga ctgccttggt 2961
Exon 4
tcattgataa tattctctct gcagCCAGCC ATTTTGTTAT TTCCGGAGAG 3011

CGTCTGGGCC TCACAGGCCA TGACCTGAAA CCTGAGAGCG TGACATTCAC 3061
P5813<
TCATGCTCTG CACTCAATGT TCAAGTCCAC CACACAGCTC ATGTTCTTAC 3111

CCAAGAGCTT GACTCGTTGG ACAAGCACCC GGGTGTGGAA AGAACACTTT 3161
Intron 4 (270bp)
GATTCCTGGG ATATCATCTC TGAGTATGgt aaggaccc-- ---------- 3449
Exon 5
tcttccgcag TCACAAAATG TATCAAGAAT GTGTATCGCG AACTGGCAGA 3499

GGGTCGCCAA CAGTCCTGGA GTGTCATATC CGAGATGGTA GCACAGAGTA 3549

CTCTGTCAAT GGATGCCATC CATGCCAACT CAATGGAACT TATTGCTGGA 3599

Intron 5 (1400bp)

AGTGTTGACA CGgtcaggat cc-------- ---------- ---------- 5001

Exon 6

gctcctgcag ACAGCAATCT CCTTGGTAAT GACCCTTTTT GAGCTGGCTC 5051

GGAACCCAGA TGTTCAGCAG GCCCTGCGGC AGGAGAGCCT AGCAGCTGAG 5101

GCCAGCATCG TTGCTAATCC CCAGAAGGCC ATGTCAGACC TGCCCTTGCT 5151

Intron 6 (240bp)

GCGGGCTGCC CTTAAAGAGA CCTTGAGgta ggtgctg--- ---------- 5408

Exon 7

tcatctccag GCTCTACCCT GTTGGTAGCT TTGTAGAGAG AATCGTACAC 5458

Intron 7 (87bp)

TCAGACCTGG TGCTTCAGAA CTATCATGTC CCTGCTGGGg tgagtgatct 5508

tcatgccctt accaactgcc cttccctcta tctcctcaaa aaagcagctg 5558

Exon 8

aacttaccct tggcctctgc ccacagACAT TCGTCATAAT TTATCTGTAC 5608

TCCATGGGCC GAAACCCTGC AGTGTTCCCA AGGCCTGAGC GCTACATGCC 5658

TCAGCGCTGG CTGGAGAGGA AAAGGAGTTT CCAGCATCTG GCCTTCGGCT 5708

TTGGGGTGCG CCAGTGCCTG GGGCGGCGCC TGGCAGAGGT GGAGATGCTG 5758

Intron 8 (250bp)

CTCCTGCTTC ACCATgtgag cacct----- ---------- ---------- 6013

Exon 9

tgtgttccag ATGCTGAAAA CCTTCCAAGT GGAGACACTG AGACAAGAGG 6063

ATATGCAGAT GGTTTTTCGC TTTCTTTTGA TGCCCAGCTC TAGTCCTTTC 6113

End of Exon 9

CTTACTTTCC GGCCTGTGAG CTAG 6137

Rat *CYP11B2*

aagctttctt catttttaa gaaagtaaat tcaaacttgt tgggggaaaa 50

ttacaatgct tttatgtact ttctagtctt tagatttatt ttcactaaaa 100

acacacacac acacacacac acacacacac acacacacac acacacacac 150

acccaagtct attaaaagaa accaaggtct tctaggatat cttcaaaaga 200

gaggatccat ccatcttccc ttttccatct atagtgagaa aagagtaatt 250

tcttcccctt ctacctcagg caacacagtt ccgcatgtgg acactacacc 300

tgctcttagt ttctaccccc cagcaggctt tgcaggtgtt ggggagctgg 350

tcatatgctt ctactgacag ttgagcccca accatgacca gagctcagat 400

```
                                        CRE
acctcagaca agtccaggga cagtttcttc catgacgtga ttagctgaac  450
                   TATA Box                          *
agtacagtac ttaggcagga taaaaggctg tgagctgaag ggaggaggat  500

    Exon 1
ggcaATGGCT CTCAGGGTGA CAGCAGATGT GTGGCTGGCA AGACCCTGGC  550

AGTGCCTGCA CAGGACGAGG GCACTGGGCA CTACGGCAAC ACTGGCCCCT  600

AAGACACTGA AGCCCTTTGA AGCCATACCA CAATACTCCA GGAACAAGTG  650

GCTGAAGATG ATACAGATCC TGAGGGAGCA GGGCCAAGAG AACCTACACC  700
                                                Intron 1
TGGAGATGCA CCAGGCCTTC CAGGAGCTGG GGCCCATTTT CAGgtaaggg  750
(370bp)
ctttccttcc ccatagccaa gcacagacat agcttgctgt cctctggttg  800

cccaggctct gcagggtacg aggtgctgca ttctccagta ggcaccaggc  850

attccatcat taggaagaca gggagggaga acaggaggtt ctgggactga  900

ctcgtgggct ctccacaggg acaaagaaaa gcacttctca ctgctgagag  950

gacagagtgc tgaagtggag tgcagacact gggagagtcc tcctgtgggg 1000

gaagcaaggg ctaggagagg atgagatgag agagcacact gacagggctg 1050

gctgtgcagc gagctcaggt ggaggacact ccacttacag cagggctgtg 1100
              Exon 2
tgcttcacca cagGCACAGT GCAGGGGGAG CACAGATTGT GTCTGTGATG 1150

CTGCCTGAGG ACGCTGAGAA GCTGCACCAG GTGGAGAGTA TCCTCCCGCG 1200
                                          P5812>
TCGGATGCAC CTGGAGCCGT GGGTGGCCCA CAGGGAACTC CGTGGCCTGA 1250
                    Intron 2 (1500bp)
GACGTGGTGT GTTCTTGCTg tgagtggac- ---------- ---------- 2759
           Exon 3
tgttctgcag AAATGGGGCT GAATGGCGCT TCAACCGACT GAAACTGAAC 2809

CCAAACGTGC TGTCACCAAA AGCTGTTCAA AATTTTGTCC CCATGGTGGA 2859

CGAGGTAGCA AGGGACTTCT TGGAGGCCCT GAAAAAGAAG GTGCGTCAGA 2909
                     P5814>
ATGCTCGAGG GAGCCTTACC ATGGATGTCC AGCAAAGTCT CTTCAACTAC 2959
          Intron 3 (115bp)
ACTATAGAAG gtatgtggcc agaggaaagt ctcaacttaa gggaggcagg 3009

gagggagaca gggagagaca aggcagtctt gaggacaggg attcccttac 3059
                          Exon 4
ctcagaggca ccattcattt tgcagCCAGC AACTTTGCAC TTTTTGGAGA 3109

GAGGCTGGGC CTCCTTGGTC ATGACCTGAA CCCTGGTAGC CTGAAGTTCA 3159
                    P5813<
TCCATGCCCT ACATTCAATG TTCAAGTCCA CCACACAGCT CCTGTTCTTA 3209

CCCAGAAGCT TGACTCGCTG GACAAGCACC CAGGTGTGGA AAGAACATTT 3259
                               Intron 4 (270bp)
TGATGCCTGG GATGTCATCT CTGAGTATGg tgagggccg- ---------- 3548
          Exon 5
acatctctag CCAACAGATG TATCTGGAAG GTGCACCAGG AACTCAGACT 3598
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CGGCAGCTCT CAGACCTACA GTGGCATTGT GGCAGCACTA ATAACTCAGG 3648

GAGCTTTACC TCTGGACGCC ATCAAAGCCA ACTCTATGGA GCTCACTGCT 3698

                Intron 5 (900bp)
GGGAGCGTTG ACACGgtcag gcggt----- ---------- ---------- 4613
           Exon 6
cctcctgcag ACAGCAATCC CCTTGGTAAT GACACTTTTT GAGCTGGCTC 4663

GGAACCCAGA TGTTCAGCAG GCCCTGCGGC AGGAGACCCT GGCAGCTGAG 4713

GCCAGCATCG CTGCTAATCC CCAGAAGGCC ATGTCAGACC TGCCCTTGCT 4763
                           Intron 6 (240bp)
GCGGGCTGCC CTTAAAGAGA CCTTGAGgta ggtgctg--- ---------- 5020
           Exon 7
tcatctccag ACTCTACCCT GTTGGTGGCT TTTTGGAGAG AATCCTAAAC 5070
                                              Intron 7 (87bp)
TCAGACCTGG TGCTTCAGAA CTATCATGTC CCTGCTGGGg tgagtgatct 5120

tcataccctc accaattgcc cttccctcta tctcctcaaa gaagcagctg 5170
                          Exon 8
actttatcct tggcctctgc ccacagACGT TGGTCCTACT TTATCTGTAC 5220

TCCATGGGCC GAAACCCTGC AGTGTTCCCA AGACCTGAGC GCTATATGCC 5270

TCAGCGCTGG CTGGAGAGGA AAAGGAGTTT CCAGCATCTG GCCTTCGGCT 5320

TTGGGGTGCG CCAGTGCCTG GGGCGGCGCC TGGCAGAGGT GGAGATGCTG 5370
                Intron 8 (250bp)
CTCCTGCTTC ACCATgtgag cacct----- ---------- ---------- 5605
           Exon 9
tgtcttccag ATGCTGAAAA CCTTCCAGGT GGAGACACTG AGACAAGAGG 5655

ATGTGCAGAT GGCCTATCGC TTTGTTTTGA TGCCCAGCTC TAGTCCTGTC 5705
                       End of Exon 9
CTCACTTTCC GGCCCATCAG CTAG                              5729
```

Peptide Sequences The peptide sequences were derived from the nucleotide sequences for the *CYP11B1* and *CYP11B2* coding regions (Nomura *et al.* 1993). The parts of the sequences corresponding to the immunogenic peptides used to generate the monoclonal antibodies are given in bold. These regions were chosen for their lack of homology between CYP11B1 and CYP11B2 and for their hydrophilic properties. Searches on the SWISSPROT protein sequence database failed to find any known mammalian proteins containing these peptide sequences.

Rat 11β-hydroxylase

MALRVTADVW LARPWQCLHR TRALGTTAKV APKTLKPFEA IPQYSRNKWL 50

KMIQILREQG QENLHLEMHQ AFQELGPIFR HSAGGAQIVS VMLPEDAEKL 100

HQVESILPHR MPLEPWVAHR ELRGLRRGVF LLNGADWRFN RLQLNPNMLS 150

PKAIQSFVPF VDVVARDFVE NLKKRMLENV HGSMSINIQS NMFNYTMEAS 200

HFVISGERLG LTGHDLKPES VTFTHALHSM FKSTTQLMFL PKSLTRWTST 250

RVWKEHFDSW DIISEYVTKC I**KNVYRELAE GRQQS**WSVIS EMVAQSTLSM 300

DAIHANSMEL IAGSVDTTAI SLVMTLFELA RNPDVQQALR QESLAAEASI 350

VANPQKAMSD LPLLRAALKE TLRLYPVGSF VERIVHSDLV LQNYHVPAGT 400

FVIIYLYSMG RNPAVFPRPE RYMPQRWLER KRSFQHLAFG FGVRQCLGRR 450

LAEVEMLLLL HHMLKTFQVE TLRQEDMQMV FRFLLMPSSS PFLTFRPVSS 500

PYIWGPS 507

Rat aldosterone synthase

MALRVTADVW LARPWQCLHR TRALGTTATL APKTLKPFEA IPQYSRNKWL 50

KMIQILREQG QENLHLEMHQ AFQELGPIFR HSAGGAQIVS VMLPEDAEKL 100

HQVESILPRR MHLEPWVAHR ELRGLRRGVF LLNGAEWRFN RLKLNPNVLS 150

PKAVQNFVPM VDEVARDFLE ALKK**KVRQNA RGSLTMDVQQ** SLFNYTIEAS 200

NFALFGERLG LLGHDLNPGS LKFIHALHSM FKSTTQLLFL PRSLTRWTST 250

QVWKEHFDAW DVISEYANRC IWKVHQELRL GSSQTYSGIV AALITQGALP 300

LDAIKANSME LTAGSVDTTA IPLVMTLFEL ARNPDVQQAL RQETLAAEAS 350

IAANPQKAMS DLPLLRAALK ETLRLYPVGG FLERILNSDL VLQNYHVPAG 400

TLVLLYLYSM GRNPAVFPRP ERYMPQRWLE RKRSFQHLAF GFGVRQCLGR 450

RLAEVEMLLL LHHMLKTFQV ETLRQEDVQM AYRFVLMPSS SPVLTFRPIS 500

SPYIWGPN 508

Appendix 2: Sequences of oligonucleotide primers

Primers for RT-PCR experiments

The melting temperature (T_m) for PCR primers was calculated by the formula:

$$T_m = 2(A + T) + 4(G + C)\ °C$$

where A, T, G and C respectively represent the number of adenine, thymine, guanine and cytosine bases in a given primer's sequence. Generally, the annealing temperature used for a particular primer in a PCR was 5°C lower than its melting temperature.

The primer pairs used in the RT-PCR detailed in **chapter 3** are given below.

Name	Primer	Sequence (5'–3')	Product
P5812	*CYP11B1/2/3* (S)	AAC TCC GTG GCC TGA GAC G	342bp
P5813	*CYP11B1/2/3* (AS)	GCT GTG TGG TGG ACT TGA AC	
V7953	Adrenodoxin (S)	GAC TCT CTG CTA GAT GTT GTG ATT	289bp
V7954	Adrenodoxin (AS)	ATT CTT GCT CAT GTC AAC AGA CTG TCG	
V7955	*CYP11A1* (S)	CAA CAT CAC AGA GAT GCT GGC AGG	583bp
V7956	*CYP11A1* (AS)	CTC AGG CAT CAG GAT GAG GTT GAA	
P5817	GAPDH (S)	ACC ACA GTC CAT GCC ATC AC	450bp
P5818	GAPDH (AS)	TCC ACC ACC CTG TTG CTG TA	

Primers for Southern blot hybridisation

The sequences of the probes hybridised to Southern blots are given below.

Name	Primer	Sequence (5'–3')
P5815	*CYP11B1* Probe	TAA ACA TTC AGT CCA ATA
P5814	*CYP11B2* Probe	TGG ATG TCC AGC AAA GTC
P5816	*CYP11B3* Probe	TGG ACA TTC AGT CCA ATG
V8822	*CYP11A1* Probe	GGT GGA GTC TCA GTG TCT CCT TGA TGC TGG CTT TGA G

Appendix 3: Reagents

Unless stated otherwise, all standard chemicals and reagents were purchased from Sigma (Sigma-Aldrich Chemical Co. Ltd., Poole, Dorset, U.K.).

RT-PCR and Southern blotting

DEPC-H_2O: 0.1% (v/v) diethyl pyrocarbonate in sterile H_2O

6× Loading dye: 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 15% (w/v) Ficoll in sterile H_2O

GeneAmp RNA-PCR core kit: (Perkin Elmer, Warrington, U.K.)

Reagents supplied in kit:

-Recombinant Moloney Murine Leukemia Virus (MuLV) RT (50U/μl)

-AmpliTaq DNA polymerase (5U/μl)

-RNase inhibitor (20U/μl)

-dATP, dCTP, dGTP, dTTP (10mM each)

-GeneAmp 10×PCR buffer II

-$MgCl_2$ solution (25mM)

-Oligo $d(T)_{16}$ primers (50mM)

-Random hexamer primers (50mM)

Tris-Acetate (TAE) buffer: 40mM tris-acetate, 1mM EDTA

Denaturing buffer: 0.5M sodium hydroxide, 1.5M sodium chloride

Neutralising buffer: 1.3M tris, 4M sodium chloride, pH 7.2

20×SSC buffer: 3M sodium chloride, 0.3M sodium citrate, pH 7.2

Hybridizing solution: 6×SSC, 0.1% (w/v) SDS, 0.25% (w/v) dried skimmed milk

SDS-PAGE and immunoblotting

Homogenization buffer: 10mM tris, 1mM EDTA, pH 7.5

Isolation buffer: 225mM mannitol, 75mM sucrose, 0.5mM EGTA, 2mM MOPS, pH 7.4

CTC: 0.1% copper sulphate, 0.2% sodium potassium tartrate, 10% sodium carbonate

Reagent A: 0.025% copper sulphate, 0.05% sodium potassium tartrate, 2.5% sodium carbonate, 2.5% SDS, 0.2M sodium hydroxide

Reagent B: 1 volume Folin and Ciocalteu's phenol reagent to 5 volumes H_2O

Bio-Rad protein assay kit: (Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K.)

Reagents supplied in kit:

-Biorad protein assay dye reagent concentrate

-Bovine serum albumin (1.42mg/ml)

Buffer 1: 1.5M tris, 0.4% SDS, pH 8.8

Buffer 2: 0.5M tris, 0.4% SDS, pH 6.8

Tank buffer: 0.025M tris, 0.2M glycine, 0.1% SDS

Laemmli buffer: 5M urea, 0.17M SDS, 0.4M dithiothreitol, 0.05M tris, pH 8.0. A few crystals of bromphenol blue were added for colour.

Blotting buffer: 0.2M glycine, 0.025M tris, 20% (v/v) methanol

Coomassie brilliant blue G stain: 45% (v/v) H_2O, 45% (v/v) methanol, 10% (v/v) glacial acetic acid. A few crystals of Coomassie brilliant blue G were added for colour.

Destain: 45% (v/v) H_2O, 45% (v/v) methanol, 10% (v/v) glacial acetic acid

Tris-buffered saline (TBS): 0.5M NaCl, 0.02M tris, pH 7.5. TBS with added Tween 20 (usually 0.05%) was termed TBST.

Blocking solution: 5% (w/v) dried skimmed milk in TBS.

Primary antibody solution: 1% (w/v) dried skimmed milk, 0.05% (v/v) Tween 20 in TBS, pH 7.5. An amount of antibody was added to give the desired dilution. A pinch of thimerosal was added, allowing the solution to be stored at -20°C and reused twice.

Secondary antibody solution: As for primary antibody solution, but omitting thimerosal as this interferes with the ECL detection systems. For this reason the secondary antibody solution cannot be reused.

Native loading buffer: 10% glycerol, 0.06M tris, pH 6.8. A few crystals of bromphenol blue were added for colour.

ECL Plus Western blotting detection system: (Amersham Pharmacia Biotech U.K. Ltd., Little Chalfont, U.K.)

Reagents supplied in kit:

-Solution A: ECL Plus substrate solution with tris buffer

-Solution B: Stock acridan solution in dioxane and ethanol

Supersignal chemiluminescent substrate for Western blotting: (Pierce & Warriner, Cambridge, U.K.)

Reagents supplied in kit:

-Luminol/enhancer solution

-Stable peroxide buffer

Supersignal ULTRA chemiluminescent substrate: (Pierce & Warriner)

Reagents supplied in kit:

-Luminol/enhancer solution

-Stable peroxide buffer

Immunostaining

Paraformaldehyde: Heat 200ml distilled water to 65°C in a fume hood. Add 40g of paraformaldehyde and a few drops of 1M sodium hydroxide solution until the solution becomes clear. Filter the solution and add 200ml of 0.2M phosphate buffer. Make up to 1 litre with distilled water and store at 4°C for one week.

0.2M phosphate buffer: For 1 litre, mix 190ml of 0.2M NaH_2PO_4 solution and 810ml of 0.2M Na_2HPO_4 solution.

Phosphate-buffered saline (PBS): 0.01M sodium phosphate (monobasic, anhydrous), 0.15M sodium chloride, pH 7.5

3,3'-Diaminobenzidine tetrahydrochloride (DAB) staining solution: Dissolve 1 DAB tablet (Sigma) in 15mL 50mM Tris (pH 7.6). Add 12µl hydrogen peroxide, mix and leave for 10 minutes before use. The solution is prepared in a Universal tube covered with tin foil to omit light.

ChemMate Buffer for Antigen Retrieval: (DAKO) Supplied as ×10 concentrate.

0.01M anhydrous citric acid, pH 6.0

Avidin/Biotin Blocking Kit: (Vector Laboratories)

Reagents supplied in kit:

-Avidin D blocking solution

-Biotin blocking solution

DAKO Catalysed Signal Amplification (CSA) System, Peroxidase Kit: (Dako Ltd.)

Reagents supplied in kit:

-Hydrogen peroxide: 3% hydrogen peroxide in water

-Protein block: serum-free protein in PBS with 0.015M sodium azide

-Link antibody: biotinylated rabbit anti-mouse IgG in Tris-HCl buffer with carrier protein and 0.015M sodium azide

-Streptavidin-biotin complex, reagent A: streptavidin in PBS with anti-microbial agent

-Streptavidin-biotin complex, reagent B: biotin conjugated to horseradish peroxidase in PBS with anti-microbial agent

-Streptavidin-biotin complex, diluent: PBS containing carrier protein and anti-microbial agent

-Amplification reagent: Biotinyl tyramide and hydrogen peroxide in PBS containing carrier protein and an anti-microbial agent

-Streptavidin-peroxidase: Streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and an anti-microbial agent

-Substrate tablets, DAB chromogen: Tablets containing 10mg 3,3'-diaminobenzidine tetrahydrochloride (DAB)

-Substrate, Tris buffer concentrate: Tris-HCl buffer concentrate

-Substrate, Hydrogen Peroxide: 0.8% hydrogen peroxide in water

Fetal Hippocampal Neuron Studies

Coat-A-Count Aldosterone Kit: (Diagnostic Products Ltd.)

Reagents supplied in kit:

-Aldosterone antibdy-coated tubes

-^{125}I-Aldosterone

-Aldosterone Calibrators

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