THE REGULATION OF STEROIDOGENIC ENZYMES IN RAT ADRENAL GLAND

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Declaration of Originality

I declare that the composition of this thesis and the work presented herein is my own. Work performed by others as a part of collaboration is indicated in the text.

Yi-Chen Lo

I dedicate this thesis to my grandfather (1904-1977) and my grandmother (1904-1998)
who always insisted that education was a requisite of life

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Abstract

Functional zonation of the rat adrenal cortex may be defined by the expression of the mitochondrial enzymes aldosterone synthase (CYP11B2) and 11β-hydroxylase (CYP11B1) that regulate the secretion of the steroids aldosterone in the zona glomerulosa (ZG) and corticosterone in the zona fasciculata (ZF), respectively. Synthesis of these steroids is also dependent on steroidogenic acute regulatory (StAR) protein which limits the transport of cholesterol into the mitochondria. The main aims of this thesis were to study the distribution and expression of CYP11B2 and CYP11B1 in the adrenal cortex of Wistar adult female rats, pregnant rats, their fetuses and offspring, after maternal manipulation of (i) the renin-angiotensin system (RAS) by changing dietary sodium, and (ii) the hypothalamo-pituitary-adrenal (HPA) axis by chronic administration of dexamethasone (DEX). Thirdly, an antibody against a bovine StAR peptide sequence was characterised for future studies on StAR protein expression in the rat adrenal paradigms. Immunohistochemistry, immunoblotting and competitive reverse transcriptase-polymerase chain reaction (cRT-PCR) methods were developed to examine protein and mRNA expression for CYP11B2 and CYP11B1. Radioimmunoassays were used for plasma steroid levels.

In the first experimental series, fetal adrenocortical functions were compared at embryonic day 20 (E20) after feeding high (HS; 3% Na), normal (NS; 0.3%) and low (LS; 0.03%) sodium diets throughout pregnancy. The effects of dietary sodium on fetal adrenal histology were compared with those of adult adrenals of non-pregnant female and pregnant rats. As expected, compared with tissues of rats fed a NS-diet, LS-diet caused significant increases of ZG width in non-pregnant and pregnant rats

whereas HS-diet markedly reduced ZG width in both non-pregnant and pregnant rats. Cell hypertrophy was seen in the ZG of the adrenals from both the non-pregnant and pregnant LS-treated rats. In pregnant rats, the adrenal CYP11B2 level was 2.8-fold higher in LS-treated rats and 50% lower in HS-treated rats compared with NS-treated rats. Similarly, levels of CYP11B2 mRNA were significantly increased and decreased by LS- and HS-treatment, respectively. A HS-diet also significantly reduced the ZG staining for CYP11B2. There were no effects of sodium diets on either adrenal CYP11B1 protein or mRNA expression in pregnant rats. In fetal rats, plasma aldosterone levels were significant increased in the LS- compared to those in the NS- and the HS-groups (LS; 1005.0 ± 139.6 pmol/L, NS; 436.0 ± 46.4 pmol/L, HS; 520.9 ± 69.5 pmol/L). The staining intensity of CYP11B2 was markedly increased ($P<0.005$) in the LS- compared to the NS- and HS-groups. However, there were no significant differences of protein or mRNA expression of CYP11B2 among the groups and no differences of any measured variable between NS- and HS-groups. Plasma corticosterone levels and the protein and mRNA expression levels of the fetal adrenal CYP11B1 were not affected by maternal sodium intake. These results suggest that dietary sodium restriction may activate the RAS to induce the expression of CYP11B2 in fetal adrenals but that responses of protein and mRNA are muted compared with changes in maternal adrenal expression. These modest fetal effects are compatible with published data suggesting that aldosterone biosythesis is initiated only in the final few days of gestation.

In the second experimental series, chronic daily injections of DEX (100 μg/kg body weight/day) throughout gestation significantly reduced the protein and mRNA expression of the CYP11B1 and caused modest increases in CYP11B2 mRNA and protein in adrenals of DEX-treated pregnant rats. Regulation of protein and mRNA

levels of CYP11B2 and CYP11B1 in these E20 adrenals appeared to be similar to those in maternal adrenals. These results suggest that DEX readily crosses the placenta and down-regulates fetal HPA axis activity, and are compatible with previous *in vitro* studies demonstrating that fetal adrenal CYP11B1 activity is regulated from E15. Intrauterine DEX-treated rats exhibited fetal growth retardation with differential effects on heart, kidney and adrenal weights. Some of these effects persisted after birth when DEX-treatment had been stopped. Postnatal body weights continued to be lower at age 7-, 28-day and 4-month-old whereas heart weight was proportionately greater after DEX-treatment *in utero*, particularly at days 7 and 28. In addition to CYP11B1 and CYP11B2, transcripts of CYP11B3 a third gene encoding an 11β-hydroxylase enzyme were found in 7- and 28-day-old rat adrenals, but not in E20, 4-month-old and pregnant rat adrenals. The relative amount of CYP11B3 mRNA was higher in 28-day-old than in 7-day-old rat adrenals. No obvious effect of the prenatal DEX-treatment on the amount of the CYP11B3 transcripts was observed. Long-term DEX exposure throughout gestation causes significant changes of the expression of the CYP11B1 in the adrenals of pregnant rats. In the adrenals of the 7-, 28-day- and 4-month-old rat offspring, gene and protein expression of CYP11B2 and CYP11B1 was regulated in a different manner from those in E20 and pregnant rats. Intrauterine DEX-treatment slightly increased the CYP11B1 and CYP11B2 mRNA and protein expression, especially in the 4-month-old offspring. The results suggest that maternal DEX-treatment may programme the physiological state of the adult offspring *via* changes in the activity of the HPA axis or possibly the RAS system.

Immunohistochemical studies in adult rat adrenal glands, using a peptide StAR antibody, showed the distribution of StAR protein in the ZG and ZF with less intense

staining in the zona reticularis (ZR). In addition, intense immunoreactivity was seen in the adrenal medulla. The specificity of the peptide StAR antibody was examined by immunoblotting. Consistent with other studies, a 30 kDa band, corresponding to StAR, was detected mainly in the total protein homogenate of the adrenal and ovary and the mitochondria of the ZG and ZF. These results suggest that StAR protein may have some other as yet unidentified functions in the adrenal gland, especially in the medulla. Alternatively, the adrenal medulla may contain a protein closely related to StAR, sharing common antigenicity. These preliminary studies emphasise the need for further studies on the role of StAR (and related) proteins in the adrenal gland and in the other endocrine organs in different physiological situations.

Chapter 1 Introduction

1.1 The adrenal gland

In mammalian species, the adrenal glands are located above the kidneys. The adrenal glands develop into two different endocrine tissues, the cortex and the medulla. These two distinct tissues have different morphologies, functions and embryological origins.

1.1.1 Morphology and function of the adult rat adrenal gland

The adult rat adrenal glands are encapsulated by fibrous collagen. The adrenal cortex is divided into three zones, the zona glomerulosa (ZG), the zona fasciculata (ZF) and the zona reticularis (ZR) (Fig. 1.1). Underneath the capsule, the ZG contains aggregated rounded cells which secrete mineralocorticoids, aldosterone being the most potent one. Mineralocorticoids are essential for maintaining the balance of water and electrolytes, and hence for the regulation of blood pressure. About 75% of the cortex consists of the ZF. The ZF cells form finger-like columns following the vasculature and synthesise glucocorticoids, principally cortisol in primates and corticosterone in rodents. These cells also contain high levels of cholesterol and cholesterol esters. Glucocorticoids are involved in carbohydrate, protein and fat metabolism and they also regulate blood pressure by positive modulation of the noradrenergic pressor response and probably through other, as yet, unidentified mechanisms. The ZR, which is responsible for adrenal C_{19} steroid biosynthesis has a granular, compact appearance and lies in direct contact with the medulla.

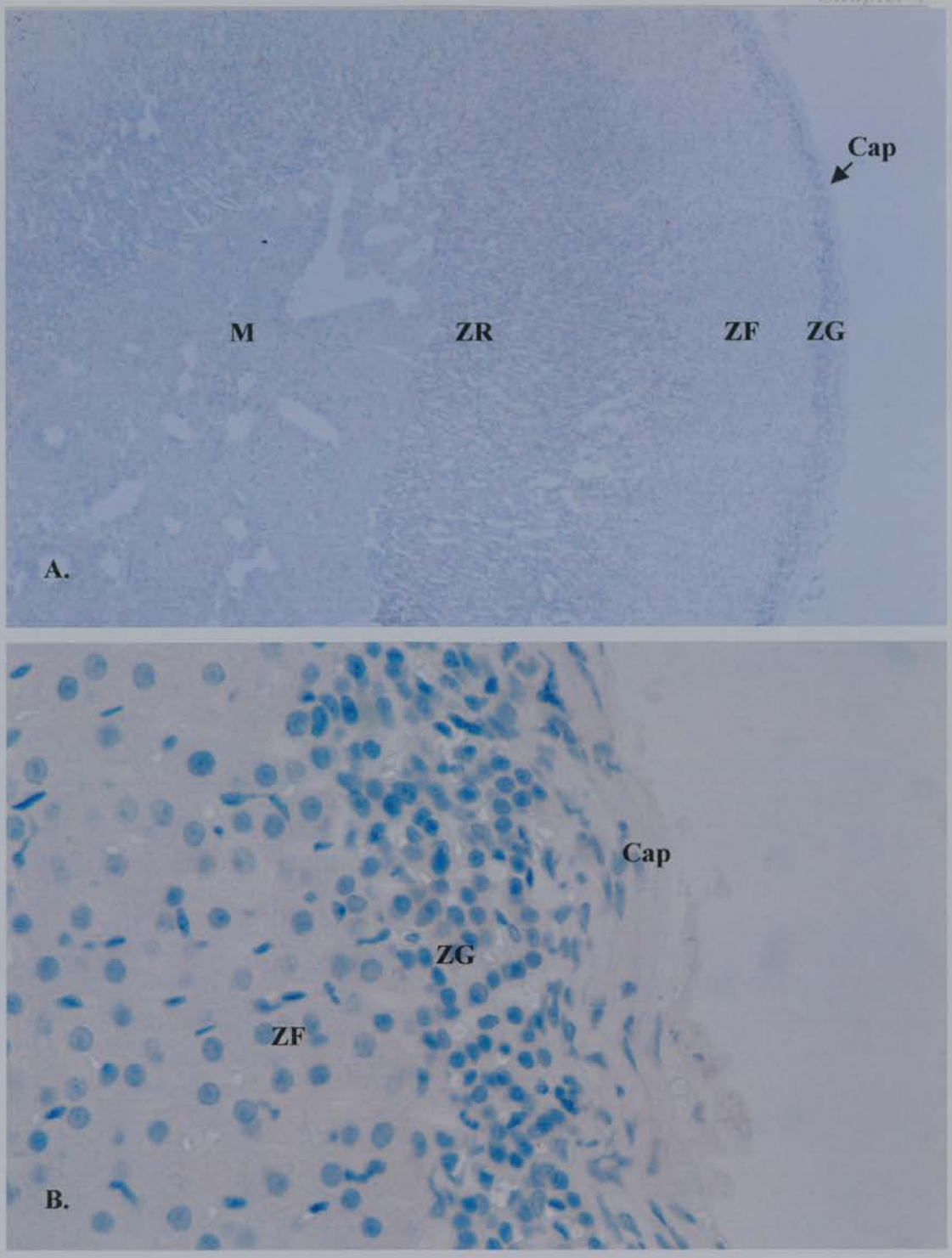


Fig. 1. 1 Adrenal morphology. Adrenal sections of adult Wistar rats were stained by haematoxylin. A. Magnification 48X. B. Magnification 480X. C, ZG, ZF, ZR and M denote capsule, zona glomerulosa, zona fasciculate, zona reticularis and medulla, respectively.

In addition to these zones, a cell layer, described previously as the intermediate or transitional zone (Deane and Greep, 1946; Nicander, 1952; Cain and Harrison, 1950; Josimovich et al., 1954), has been reported to exist between the ZG and ZF. Mitani et al. (1994) confirmed that this Sudan-unstained and tightly packed cell layer corresponded to the intermediate or transitional zone by using various immunohistochemical methods. Furthermore, the cells are regarded as inert in relation to the biosynthesis of aldosterone and corticosterone (Mitani et al. 1994).

The medulla consists of the non-steroidogenic chromaffin cells, which are derived from neuroectoderm and synthesise norepinephrine or epinephrine. However, small islands of cortical tissues surrounding the central adrenal vein in the medulla have been reported. Some chromaffin cells also transverse the adrenal cortex (Bornstein et al. 1994; Bornstein et al., 1997). The physiological function of these cells, interwoven in the cortex and medulla is still unclear.

1.1.2 Adrenal embryology and development

The adrenal gland is derived from mesenchymal tissue next to coelomic epithelium (Fig. 1.2). The adrenal cortex originates from mesodermal tissue and the medulla is of neuroectodermal origin and migrates from the neural crest into the adrenal primordium. These two tissues then become encapsulated.

Evidence for visible adrenal differentiation in rats was not seen prior to 11.5 days of gestation. However, at this stage, adrenal blastema cells, showing small, multiple nucleoli, were seen beneath the surface of the urogenital ridge. Fetuses in their 14th day of development had well-developed nests of presumptive adrenocortical cells distributed along the angle of the dorsal mesentery. Mesenchymal cells started to be flattening out as capsular tissue along the adrenal. At 15th embryonic day, the nests of adrenocortical cells were enlarged and more readily distinguishable (Roos, 1967). In the 16.5 day of fetuses, a narrow layer immediately beneath the capsule consisted of relatively small, tightly packed cells, which were correspondent to the ZG cells of the

adult. At this stage, the adrenal cells appeared virtually fat-free (Josimovich et al., 1954). The adrenals of mature fetuses (18.5 day) were completely formed with a distinct capsule and a basophilic ZG. The cells in the inner ZF and ZR were similar, with swollen cytoplasm and a central nucleus (Roos, 1967). Small lipid droplets occurred in all of the cortical cells. However, a lipid-free transitional zone became detectable between the ZG and the ZF (Josimovich et al., 1954).

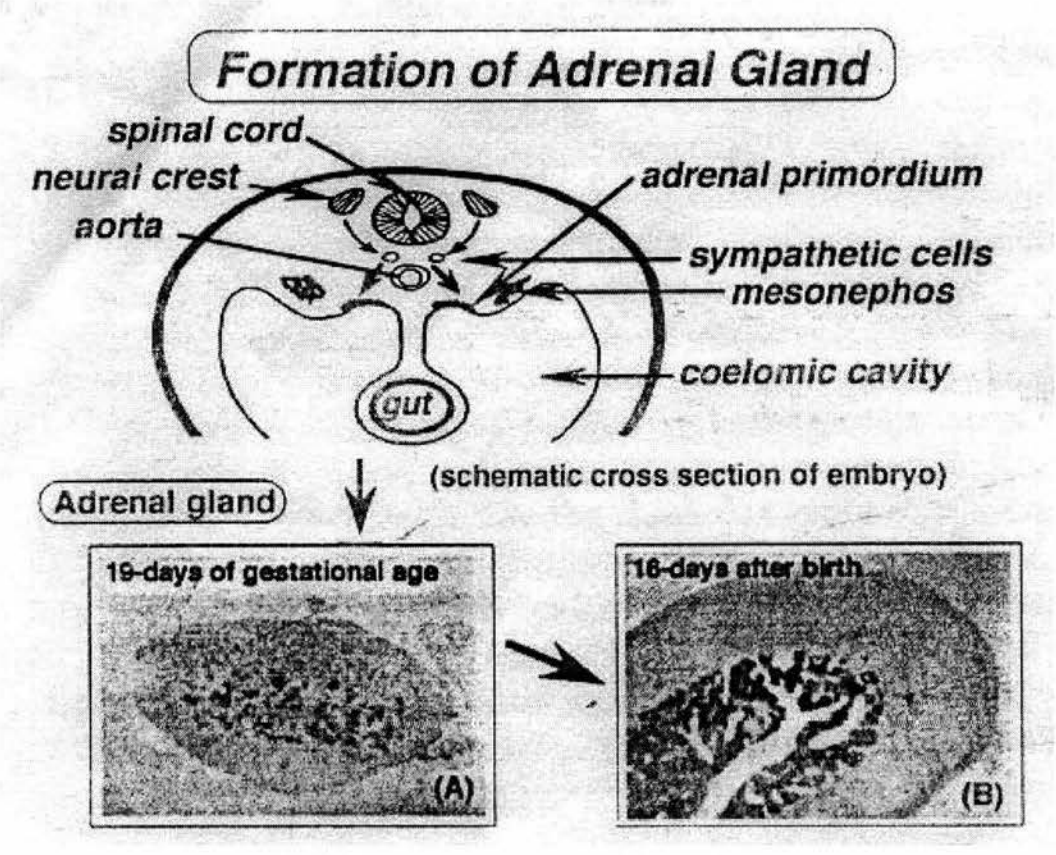


Fig. 1. 2 Formation of adrenal gland (Adapted from Mitani et al., 1997).

Blood vessels and nerve cells appeared within the developing adrenal during the 15th embryonic day (Roos, 1967). A cluster of prospective medullary cells was able to be seen outside of the adrenal gland at 16.5 day rat fetus. After that, the medullary nests migrated toward the centre of the glands and gradually coalesced (Josimovich et al., 1954, Mitani et al., 1997). The medullary cells enlarged only slightly before birth (Fig. 1.2). Both hypertrophy and hyperplasia accounted for the further growth after birth. However, even at 2.5 days after birth, the medullary tissue still occurred in separate clusters surrounded by strands of cortical cells (Josimovich et al., 1954).

1.1.3 Adrenal zonation

The development of functionally distinct cells in relation to the zonation of the adrenal cortex has been studied over several decades. A number of theories have been proposed to account for the observed structure and function of the adrenal cortex.

Cell migration theory

The cell migration theory is also called the escalator theory. Gottschau first proposed that adrenal cortical cells arose from a common stem cell in and beneath the capsule or in the subcapsular region, that then migrated centripetally and degenerated at the border between the ZR and the medulla (reviewed by Long, 1975). This theory was also supported by studies which showed that the original ZF and ZG cells died and were absorbed after adrenal cortex enucleation or autotransplantation. However, the adrenal capsule formed a sphere, within which the glomerulosa developed well and generated new functional fasciculata cells (Belloni et al., 1991; Vendeira et al., 1996). More recently, Morley et al. (1996) confirmed that the adrenal parenchyma is composed of clonally related cords of cells that extend centripetally from the ZG in to the ZF/R in the adult mouse using a 21-hydroxylase/β-galactosidase transgenic mouse model (Morley et al., 1996). According to a mitotic study, cell proliferation is greater in the ZG and ZF related to the higher rate of mitosis in these zones (Mitchell, 1948).

In contrast, cell death is indicated by more apoptotic bodies and pyknotic nuclei, which are more prominent in the ZR than in the other two zones (Wyllie et al, 1973a, 1973b). These observations are consistent with the model proposed by Mitchell who modified the cell migration theory to propose a non-migrating, self-sustaining ZG and a second proliferating population of cells in the outer part of the ZF that subsequently migrated centripetally (Mitchell, 1948).

Zonal theory

The zonal theory originated from the relatively independence of pituitary control on the ZG cells, while, in contrast, the inner zones (ZF and ZR) relied on the regulation of pituitary adrenocorticotropic hormone (ACTH) (reviewed by Long, 1975). In addition, each zone in the cortex had its own distinct function and replenished its own cells, which subsequently died locally.

Transformation field theory

This theory is based on certain histological changes of the ZG cells during various phases of activity. Continuous stimulation of rat ZG cells in culture with ACTH led to the loss of mineralocorticoid secretory capacity in favour of glucocorticoids, with a concomitant histological transformation from a typical glomerulosa to a more fasciculata-like appearance (reviewed by Estivariz et al. 1992).

However, none of the above theories could explain the adrenal zonation completely. More recently, Mitani et al. defined a progenitor cell layer in the rat adrenal cortex between the ZG and ZF, based on functional and morphological observations (Mitani et al., 1994). This finding was in accordance with the intermediate or transitional zone previously suggested by others (Deane and Greep, 1946; Nicander, 1952; Cain and Harrison, 1950; Josimovich et al., 1954). As a consequence of the occurrence of DNA-synthesizing cells present in and around the

intermediate zone, and the fact that the cells labelled with 5-bromo-2'-deoxyuidine (BrdU) migrated inward from around this zone in a time-dependent manner, it was proposed that the intermediate zone is an undifferentiated zone and could potentially be the stem cell zone of the adrenal cortex (Mitani et al., 1994) (Fig. 1.3).

1.2 Steroidogenesis in the adrenal cortex

Adrenal steroid biosynthesis is the result of sequential hydroxylation, dehydrogenation and isomerisation reactions that convert cholesterol to the physiologically active mineralocorticoids, glucocorticoids and adrenal androgens. The synthesis of the various adrenal hormones involves several proteins and key enzymes. The steroidogenic pathway and enzymatic steps are shown in Fig. 1.4. The key enzymes and proteins are discussed in this chapter.

1.2.1 Biosynthesis of steroid hormones

Cholesterol is the precursor for all steroid hormone biosynthesis. In the past, the conversion of cholesterol to pregnenolone by cytochrome P450 cholesterol side chain cleavage (CYP11A1) was thought to be the rate-limiting step for steroidogenesis (reviewed by Hall, 1987; Jefcoate et al., 1992). However, some researchers found that a protein synthesis inhibitor, cycloheximide retarded steroidogenesis and blunted the activity and amounts of CYP11A1 in response to acute hormone stimulation (Davis and Garren, 1968; Privalle et al., 1983). Thereafter, a rapidly synthesised, cycloheximide-sensitive protein, named steroidogenic acute regulatory (StAR) protein (Clark et al., 1994), was identified and proposed to act in the translocation of cholesterol through the aqueous intermembrane mitochondrial space to the inner mitochondria where CYP11A1 is localised (Cherradi et al., 1997) and where pregnenolone is synthesised. As pregnenolone is a more hydrophilic metabolite than cholesterol, it can then be transported to the endoplasmic reticulum, where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase

space to the inner mitochondria where CYP11A1 is localised (Cherradi et al., 1997) and where pregnenolone is synthesised. As pregnenolone is a more hydrophilic metabolite than cholesterol, it can then be transported to the endoplasmic reticulum, where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase (3β-HSD), which is expressed in both the endoplasmic reticulum and in the mitochondrial compartments (Cherradi et al., 1997) and is also present in all cortical cells (Ishimura and Fujita, 1997).

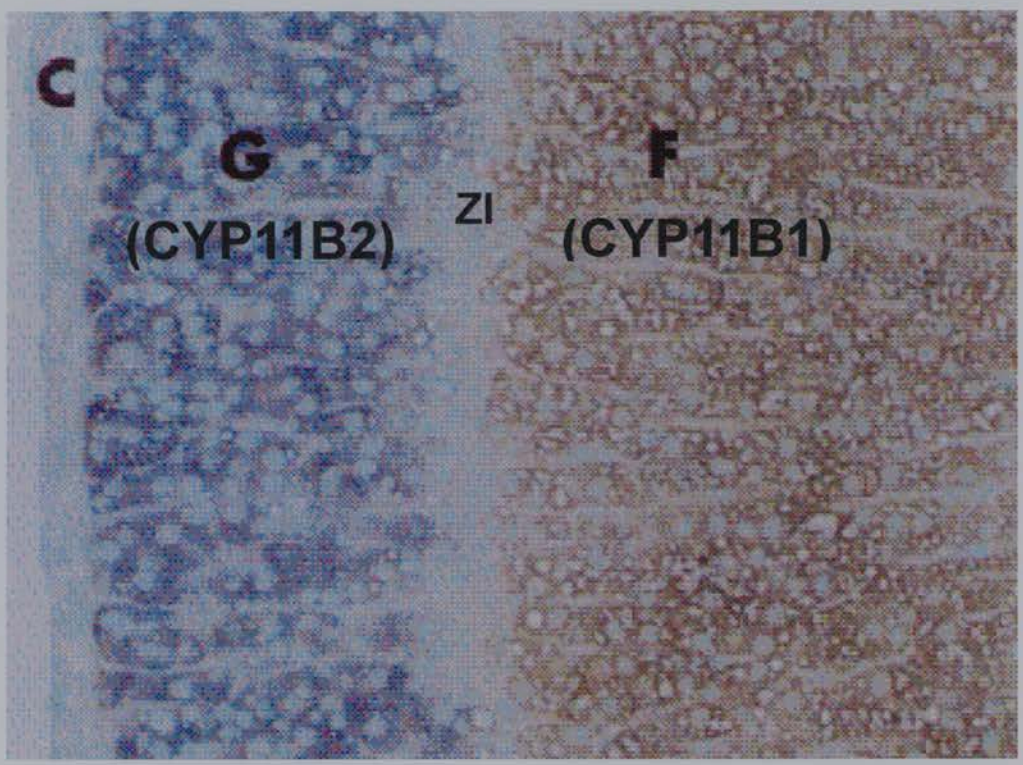


Fig. 1. 3 Adrenal zonation. An adrenal section from a sodium-deficient rat for 20 days was stained with the anti-CYP11B2 and the anti-CYP11B1 simultaneously. C, G, ZI and F denote capsule, zona glomerulosa, zona intermediate and zona fasciculate. Violet and brown colours show the presence of CYP11B2 and CYP11B1, respectively (Adapted from Mitani et al., 1994).

As shown in Fig.1.4, pregnenolone and progesterone can enter a number of biosynthetic pathways catalysed by various enzyme cascades. These different pathways produce a range of steroid hormones with various biological activities. However, rats have divergent metabolic pathways when compared to other species. It has been known that the rat adrenal cortex lacks steroid 17α-hydroxylase (CYP17) whereas the ZF and ZR contain CYP17 in many species, including the human and bovine species (Bell et al., 1979). Therefore, the progesterone is converted into deoxycorticosterone (DOC) by cytochrome P450 21-hydroxylase (CYP21) in the rat adrenal cortex. As a result, the cells in the ZF/R synthesise corticosterone rather than cortisol, which is synthesised in humans or other mammalian species, as the major glucocorticoid. Also, the ZR of the rat adrenal is presumably inert to synthesise C19 steroids (Bell et al., 1979). The nature of the enzyme involved in the final steps of aldosterone synthesis has only been established relatively recently. The rate-limiting steps of aldosterone biosynthesis are involved in the conversion of DOC to aldosterone in the adrenal ZG.

1.2.2 Mineralocorticoids (aldosterone) and glucocorticoids (corticosterone) biosynthesis

As illustrated in Fig. 1.4, the early steps in steroidogenesis are common to all cortical zones. In contrast, the late steps in the steroidogenic pathway are zone-specific. In the rat adrenal ZG, cytochrome P450 aldosterone synthase (CYP11B2) contains 11β-hydroxylase, 18-hydroxylase and 18-oxidase activities. The 11β-hydroxylation of DOC to corticosterone and its subsequent 18-hydroxylation of corticosterone to 18-hydroxycorticosterone (18-OH-B) and 18-oxidation of 18-OH-DOC to aldosterone are catalysed by CYP11B2. Expression of this enzyme is limited to the ZG so that it prevents the production of aldosterone in other adrenocortical zones (Ogishima et al., 1992; reviewed by Rainey, 1999).

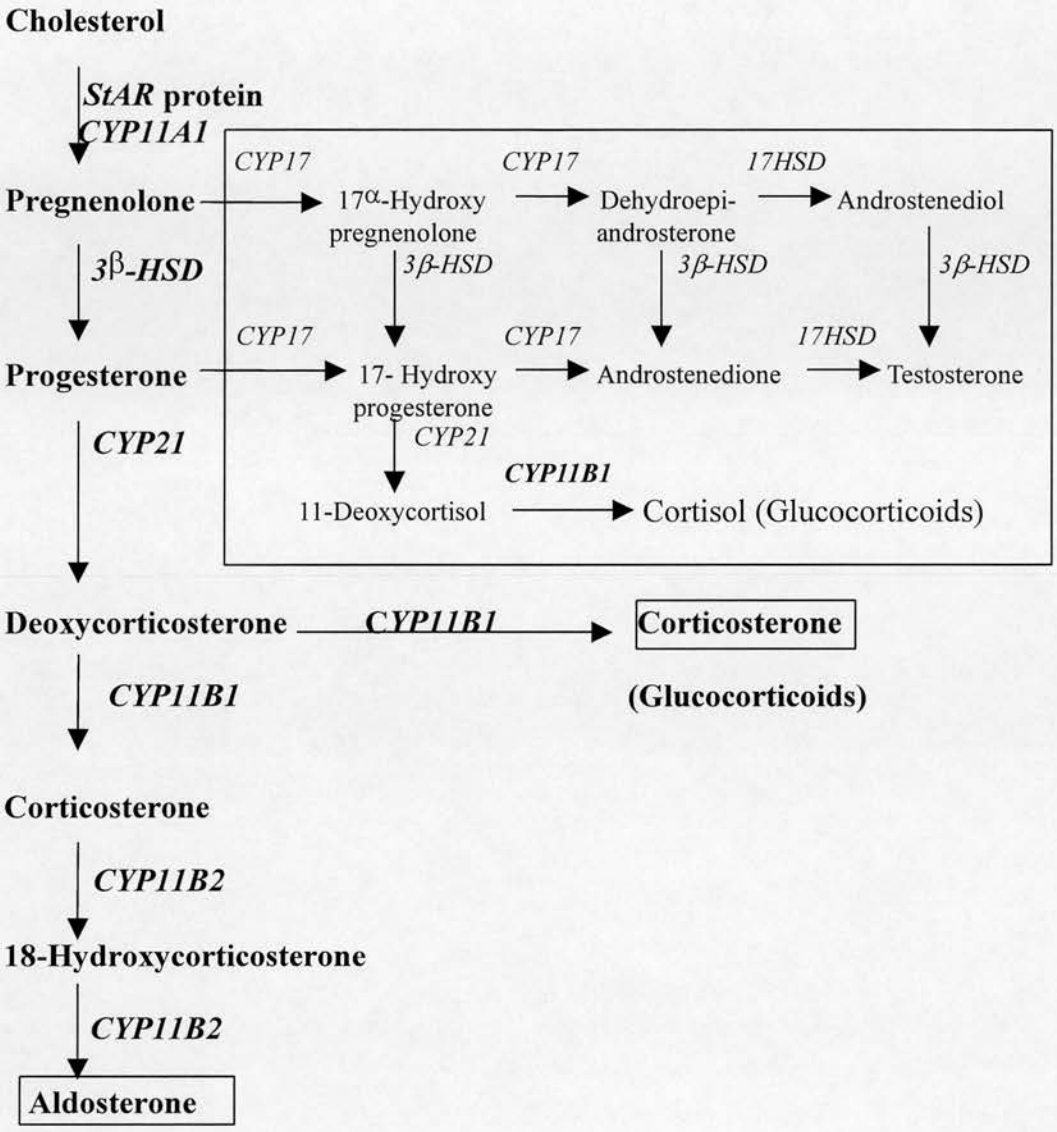


Fig. 1. 4 Principal pathways for adrenal steroid biosynthesis. Pathways which do not exist in the rat adrenal cortex are bordered by a solid line. StAR protein: Steroidogenic acute regulatory protein, CYP11A1: Cytochrome P450 cholesterol side chain cleavage enzyme, CYP17: Cytochrome P450 17α-hydroxylase, 3β-HSD: 3β-Hydroxysteroid dehydrogenase/ Δ5-4 isomerase, CYP21: Cytochrome P450 21-hydroxylase, CYP11B1: 11β-Hydroxylase, CYP11B2: Aldosterone synthase. (Adapt from Brook, C. & Marshall, N., 1996)

On the other hand, corticosterone biosynthesis is completed by cytochrome P450 11β-hydroxylase (CYP11B1). Although CYP11B1 has 18-hydroxylation activity, but unlike CYP11B2, CYP11B1 is unable to express 18-oxidase. Therefore, CYP11B1 has limited function to convert DOC to corticosterone in the ZF and ZR in rats (Ogishima et al., 1992; reviewed by Rainey, 1999).

1.2.2.1 Zone-specific enzymes: CYP11B2 and CYP11B1

Although the functional zonation of the rat adrenal cortex according to the morphological findings had been established for several decades, the enzymes responsible for the final steps of aldosterone and corticosterone biosynthesis in rats were not identified until recent years.

Treatment of rats with low sodium and high potassium diet markedly stimulated the production of aldosterone in rats (Meuli and Műller, 1983). Later, a protein of molecular size 49 kilodaltons (kDa) was isolated from ZG mitochondria of rats on a sodium-restricted and potassium-replete diet (Lauber et al., 1987). Another 51 kDa protein was identified from the ZF/R mitochondria of rats on normal diet (Ogishima et al., 1989) or on a sodium-replete and potassium-deficient diet (Lauber et al., 1987). These findings indicated the existence of two different forms of CYP11B in the rat adrenal cortex, with only one of them, i.e. the 49 kDa form, being capable of catalysing the two final steps for aldosterone biosynthesis *in situ* (Meuli and Műller, 1983; Lauber et al., 1987; Ogishima et al., 1989). Thereafter, several studies led to the isolation and purification of the 49 kDa protein, named CYP11B2, from the capsular portion of rat adrenals (Lauber and Műller, 1989; Ogishima et al., 1989). On the other hand, the 51 kDa protein, named CYP11B1, was found mainly in the decapsulated portion of the adrenals (Ogishima et al., 1989).

The zonal distribution of CYP11B2 and CYP11B1 was further confirmed by other techniques, i.e. immunohistochemistry and *in situ* hybridisation. CYP11B2 is

localised strictly in the ZG of the rat adrenal cortex, while CYP11B1 is present in the ZF and ZR and more abundant in the outer ZF. Also, the zonal distributions are principally unchanged following treatment with angiotensin II (AII) or by sodium depletion, but the number of CYP11B2-positive cells increase considerably in the ZG (Ogishima et al., 1992; Ho and Vinson, 1993; Mitani et al., 1994; Wotus et al., 1998; Mitani et al., 1999).

1.2.2.2 Molecular cloning of CYP11B2 and CYP11B1

Two discrete complementary DNAs (cDNAs), encoding CYP11B2 and CYP11B1, were cloned and identified from the adrenal capsular tissues of sodium-depleted, potassium-replete rats and from ACTH-stimulated rats, respectively. The nucleotide sequences of CYP11B2 cDNA contain a 74 base pairs (bp) 5'-noncoding region, a 1530 bp open reading frame and a 1217 bp 3'-noncoding region. The overall length of the CYP11B1 nucleotide sequence is 2747 bp with a 1247 bp 3'-noncoding region. The two proteins are highly homologous in their amino acid sequence with 88% similarity. Both the deduced amino acid sequences of CYP11B2 and CYP11B1 contain a conserved heme-binding site and steroid binding site (Nonaka et al., 1989; Matsukawa et al., 1990). Furthermore, expression of the proteins encoded by these cDNAs in nonsteroidogenic COS-7 cells confirmed the difference in their enzymatic activities (Matsukawa et al., 1990; Nonaka et al., 1991).

According to the analyses of the 5'-upstream regions, both the CYP11B2 and CYP11B1 genes contain a common sequence of TATA box, defining the transcription initiation site and 4 modified or conserved cAMP response elements (CREs; or *cis* elements) which are linked to cAMP / dibutyryl cAMP dependent stimulators (Mukai et al., 1993; Nomura et al., 1993). In addition, Mukai et al. (1993) also isolated CYP11B2 and CYP11B1 genomic clones with two additional genes, CYP11B3 and CYP11B4. Of these, CYP11B3 had the same number of exons and the same location of introns as those of CYP11B2 and CYP11B1. In the nucleotide sequences, the homologies between CYP11B2 and CYP11B3 and between CYP11B1 and CYP11B3

are 89% and 96%, respectively. However, no CYP11B3 gene product including transcript has been reported (Mukai et al., 1993; Nomura et al., 1993). As CYP11B4 lacks exon 3 and a part of exon 4, it is considered as a pseudogene (Mukai et al., 1993).

1.2.2.3 Expression of CYP11B2 and CYP11B1 in adrenal glands during development

According to functional and morphological studies, the rat adrenal gland at 16 days of gestation has a distinct capsule and distinguishable ZG and ZF structures (see section1.1.2). It was also reported that a considerable amount of glucocorticoid was produced at this stage of gestation (Roos, 1967; Kalavsky, 1971; Milković et al., 1973; Yamamoto et al., 1983; Yamamoto et al., 1986). Fetal adrenal glands were able to secret some mineralocorticoid at around 18 days of gestation (Milković et al., 1976; Churchill et al., 1981). The discrepancy between the morphological features and the steroidogenic capacity of adrenal glands was clarified at the molecular level.

The developmental expression of zone specific enzymes has been studied in detail by immunohistochemistry and *in situ* hybridisation. Although small clusters of CYP11B2-positive cells throughout the adrenal were detected at 16 days of gestation (E16) (Wotus et al. 1998), a clear expression of CYP11B2 and its mRNA was established between E18 and E20 (Mitani et al., 1997; Wotus et al. 1998, Mitani et al., 1999). This implies that aldosterone biosythesis is initiated only in the final few days of gestation. On the other hand, although the appearance of angiotensin receptor-type 1B (AT_{1B}) mRNA was apparent in the outer part of the adrenal (Mitani et al., 1999), the AT_{1B} receptor was thought to be not functionally mature or the concentration of AII in plasma was not sufficient at this stage, resulting in no expression of CYP11B2 until the time around birth (Mitani et al., 1999). A CYP11B2-positive layer was clearly recognized in the outer part of the adrenal gland and showed the number of functionally active ZG cells which increased one day after birth. The intensity of CYP11B2 staining then increased afterward with time until adulthood (Mitani et al.,

1999).

The onset of expression of CYP11B1 was detected throughout the adrenal gland and intermingled with medullary cells from E16. At the same time, CYP11A1 and CYP21 were also co-expressed in the inner part of the gland (Mitani et al., 1999). This suggests that the ZF cells start functioning as early as E16. At E20, CYP11B1 was more apparent in the ZF than in those of earlier days although the cortical cells remained mixed together with medullary cells, but most of them were found gathering at the centre of the gland. Furthermore, a zona intermediate, lacking CYP11B2 and CYP11B1, was first clearly identified at this stage and thereafter constantly observed in adult rats (Mitani et al., 1997; Wotus et al. 1998, Mitani et al., 1999).

1.3 Regulations of CYP11B2/aldosterone and CYP11B1/corticosterone biosynthesis

The regulation of steroid biosynthesis in the adrenal cortex is conducted mainly via the renin-angiotensin system (RAS) and the hypothalamo-pituitary-adrenal (HPA) axis. The RAS system is primarily involved in the regulation of mineralocorticoid (aldosterone) synthesis whereas the HPA axis is associated more with glucocorticoid synthesis. The nature of the adrenal cortex is to produce aldosterone and corticosterone distinctly. This mainly relies on the differential expression of CYP11B2 and CYP11B1.

1.3.1 Regulation of CYP11B2 and aldosterone biosynthesis

1.3.1.1 The renin-angiotensin system (RAS)

The RAS is the principal regulator of the secretion of aldosterone. The classical circulating RAS is important in the control of salt and water balance and blood pressure through actions in the kidney (Fig. 1.5).

Renin, a protease enzyme, is mainly synthesised by the juxtaglomerular cells of kidney and is responsible for the synthesis of angiotensin I (AI) from renin substrate (angiotensinogen). Angiotensin II (AII) is the effective peptide, which is cleaved from AI by angiotensin-converting enzyme (ACE). AII exerts two major actions, one as a direct arteriolar vasconstrictor and the other as a stimulus to aldosterone secretion. These two actions, in concert, maintain the volume and pressure of the arterial circulation (Fig 1.5). Sodium reabsorption in renal tubules and aldosterone secretion in the ZG of adrenal cortex are also regulated to some extent by AII.

Local RASs have now been identified in a number of tissues, including the brain, testes, ovaries and the adrenal gland. In the rat, the adrenal gland has the highest renin concentration next to the kidney. All of the components of RAS have also been identified in the adrenal cortex (reviewed by Mulrow, 1999; Lumbers, 1999).

Aguilera et al. (1981) demonstrated the presence of renin enzyme in the outer zone of adrenal cortex of the rat and noted that AII was present in the adrenal as well as in the plasma of nephrectomised rats, suggesting that the adrenal cortex was the source of the plasma AII (Aguilera et al., 1981). The presence and concentration of angiotensin peptides were not eliminated or reduced by nephrectomy. This also suggested that AII is produced locally rather than recycled from the circulation.

AII exerts its action on target organs by interacting with a receptor in the plasma membrane. There are several types of AII receptors. Angiotensin receptor-type 1 (AT_1) and angiotensin receptor-type 2 (AT_2) are the major ones (Murphy et al., 1991; Sasaki et al., 1991). The AT_1 receptor exists as two isoforms, angiotensin receptor-types 1A (AT_{1A}) and 1B (AT_{1B}). Most of the known biological actions of AII are mediated via the AT_1 and probably AT_{1A} except in the adrenal where control of aldosterone secretion appears to be mediated via the AT_{1B} (Gigante et al., 1997). By *in situ* hybridization and by using losartan and PD123319 as specific ligands to displace [^{125}I]AII from AT_1 and AT_2 receptors, respectively, the adrenal ZG was shown to contain both AT_1 and AT_2 receptors while the medulla contained only AT_2 receptors

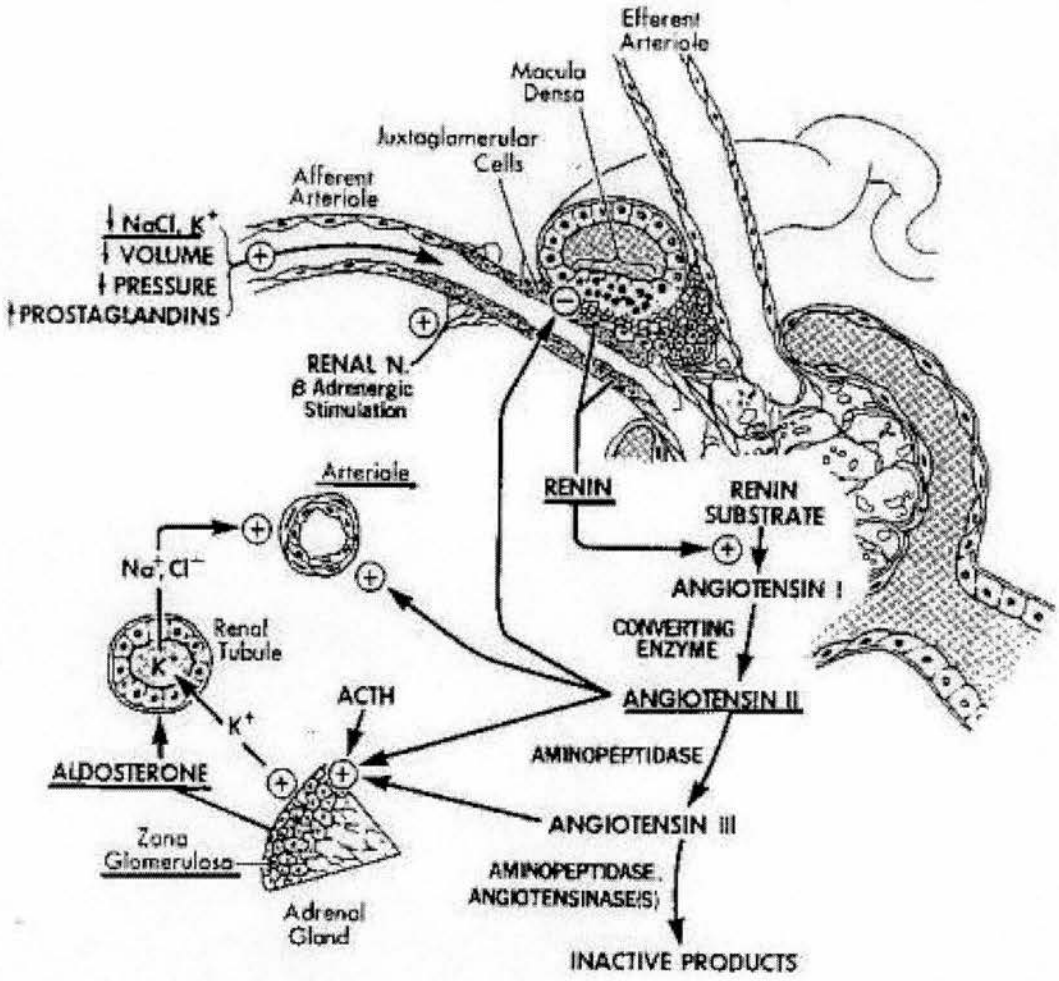


Fig. 1. 5 The renin-angiotensin system. Plus and minus signs indicate stimulation and inhibition, respectively. Only the major influences on the system are shown. (Adapted from Felig et al., 1995).

(Aguilera et al., 1992; Lehoux et al., 1997). However, the mechanisms by which the adrenal RAS acts on aldosterone remain unclear.

1.3.1.2 Factors regulate CYP11B2 and aldosterone biosynthesis in adult rat adrenal glands

1.3.1.2.1 Sodium

It is now well established that sodium restriction causes both hypertrophy and hyperplasia of the ZG and an increase in aldosterone (Pike et al., 1966; Pohanka and Pike, 1970; McEwan et al., 1999). The increase in basal and AII-stimulated aldosterone output from isolated ZG cells is due to the specific increase of CYP11B2 mRNA levels and its associated enzyme activities, but not the expression of CYP11A1 mRNA and its enzyme activities (Tremblay et al., 1992; Adler et al., 1993). Volpe et al. (1997) showed that salt depletion and losartan caused a rise in plasma and adrenal renin activities and its expression, whereas CYP11B2 mRNA and plasma aldosterone levels were increased by salt restriction and reduced by losartan in both intact and nephrectomised rats (Volpe et al., 1997). Lehoux et al. (1994) further extended these observations to the effective translation of AT_1 receptor mRNA into AT_1 receptor protein and established a correlation between the increased synthesis of aldosterone and the increased level of AT_1 receptor in addition to an increased capacity of adrenal cells to bind AII in the ZG of rats after sodium deprivation (Lehoux et al., 1997).

1.3.1.2.2 Potassium

Potassium supplementation specifically increased CYP11B2 mRNA levels in the ZG without affecting CYP11B1 expression in the ZF (Tremblay et al., 1992). The induction of adrenal CYP11B2 expression seen following potassium loading in rats has been thought to be associated with sodium loss rather than direct effects of potassium on the ZG. Potassium has been shown to markedly increase plasma

aldosterone and adrenal renin in rats, whereas plasma renin activity was suppressed (Nakamaru et al., 1985). In isolated ZG cells, AII secretion and renin activity were also stimulated by potassium loading (Doi et al., 1984; Shier et al., 1989; Yamaguchi et al., 1990). Numerous studies in isolated ZG cells have demonstrated that potassium stimulates aldosterone directly *in vitro* (Müller, 1987). Captopril inhibited both the inductions of CYP11B2 mRNA and plasma aldosterone levels in the potassium-treated rats (Nakamaru et al., 1985; Tremblay et al., 1992). However, recent studies using mice with targeted deletion of angiotensinogen (Okubo et al., 1997) or the AT_{1B} receptor (Chen et al., 1997) have demonstrated that potassium itself can be an alternative potent regulator of adrenal glomerulosa aldosterone production and increase CYP11B2 expression in the ZG during dietary sodium restriction.

1.3.1.2.3 AII

Short-term and chronic AII administrations induce a significant rise in plasma aldosterone and cause lipid depletion and hypertrophy of the ZG cells, especially the hypertrophy of smooth endoplasmic reticulum and mitochondrial compartment where the steroidogenic enzymes involved in aldosterone synthesis are located (Mazzocchi et al., 1980). AII also stimulates adrenal ZG cell proliferation as well as augmenting aldosterone synthesis (McEwan et al, 1996; McEwan et al, 1999). Iwai and Inagami (1992) demonstrated that continuous infusion of AII increased the level of AT_1 receptor mRNA in the adrenal and suggested that this receptor mRNA level is dependent on the RAS (Iwai and Inagami, 1992). The effects of AII can be inhibited by antagonists to the AT_1 receptor. Furthermore, blockade of AII receptors by the non-selective antagonist, saralasin, or the AT_1 selective antagonist, losartan, and ACE inhibition by captopril reduce aldosterone secretion from *in situ* perfused adrenals in rats kept on a sodium-restricted diet (Mazzocchi et al., 2000). Along with these observations, AII receptors exhibit a zonal distribution and suggest a role for AII in the ZG cell-specific expression of CYP11B2 (Donna et al., 1998).

1.3.1.2.4 ACTH

Short-term or acute administration of ACTH stimulates the early steps in aldosterone synthesis, i.e. prior to formation of pregnenolone (Lumbers, 1999) whereas ACTH has inhibitory effects on adrenal expression of CYP11B2 in the long-term experiments in rats (Mitani et al., 1996; Lumbers, 1999). Sander et al. (1994) compared the regulation of adrenal steroidogenesis after ACTH injection of [TGR(mREN2)27] transgene rats, and normotensive SD rats. Both groups of animals showed no difference in expression of CYP11A1 and CYP11B1 by ACTH. However, ACTH treatment rendered CYP11B2 mRNA undetectable and altered the morphology of adrenal cortex, resulting in a lack of ZG-like cells (Sander et al., 1994). Suppression of or stimulation of ACTH secretion resulted in an increase or a decrease in the number of CYP11B2-positive cells in ZG, respectively (Mitani et al., 1996). These results perhaps account for the transient nature of ACTH's stimulatory effect on aldosterone secretion. However, the physiological relevance and the mechanisms of the ACTH regulation in aldosterone production and CYP11B2 expression remain unclear.

1.3.1.2.5 Other factors

Additional regulators have also been proposed to affect aldosterone biosynthesis and CYP11B2 gene regulation. These factors include atrial natriuretic peptide (ANP), dopamine, noradrenaline, adrenaline and transcriptional regulation.

ANP is an important vasoactive peptide which has diverse biological actions, such as diuresis, natriuresis and vasorelaxation. ANP also inhibits the activity of the RAS through inhibition of renin secretion and blocks the actions of AII on aldosterone secretion in the ZG of the rat adrenal gland (Lumbers, 1999).

Dopamine has been suggested to exert an inhibitory influence on aldosterone

secretion. Infusion of dopamine into the isolated perfused rat adrenal gland resulted in a dose-related reduction of aldosterone secretion (Porter et al., 1992). Both epinephrine and norepinephrine stimulated aldosterone production by cultured adrenal capsules whereas a β-adrenergic antagonist, propranolol, inhibited the epinephrine effect (Pratt et al., 1985).

The molecular mechanisms that specify expression of the two CYP11B2 and CYP11B1 homologues in different adrenocortical zones remain unknown. However, the homology of the 0.5 kb 5'-flanking regions of CYP11B2 and CYP11B1 is only 50%. In further upstream regions up to 1.7 kb, the homology is approximately 45%. These low homologies are suggested to contribute to the differences in gene regulation of the two genes (Mukai et al. 1993; Nomura et al., 1993).

1.3.1.3 Regulation of CYP11B2 and aldosterone biosynthesis in fetal rat adrenal glands

During late gestation, fetal adrenal ZG cells can be morphologically identified (Josimovich et al., 1954; Roos, 1967) and produce amounts of aldosterone that can be found in the circulation of adrenolectomised mothers (Milković et al., 1976; Klepac, 1979; Churchill et al., 1981). Although the ZG cells are presumably able to express CYP11B2 and its gene transcripts at this stage (Mitani et al., 1997; Wotus et al., 1998), Mellon et al. (1995) suggested that the CYP11B2 in the fetal adrenal might be transcriptionally quiescent *in vivo* to intrauterine treatment (Mellon et al. 1995). However, hypertension has been reported in the late-gestation of the spontaneously hypertensive rat fetus and neonate relative to its normotensive genetic control (Mellon et al., 1995). The discrepancy between the gene regulation of CYP11B2 associated with aldosterone synthesis and the physiological changes in the rat fetus remains unclear.

On the other hand, renin is expressed in the kidney of fetal lambs (Iwamoto and

Rudolph, 1981; Siegel, 1981) and also in the adrenal tissue of the fetal mouse (Zemel et al., 1989; Jones et al., 1989). The concentration of AII binding sites continued to increase from the second half of intrauterine development, reaching more than twice the levels in the adrenal at 18 and 20 days of gestational age in rat fetuses (Jones et al., 1989). Feuillan et al. (1993) used subtype-specific AII antagonists to show that the adrenal capsule of rat fetus has both AT_1 and AT_2 receptors whereas the medulla contains mainly AT_2 receptor. Moreover, there was a greater proportion of AT_2 receptors in the adrenal capsule of the rat fetus than that has been reported in the adult (Feuillan et al., 1993). ACE and angiotensinogen have also been found to increase around the time of birth (Lee et al., 1987). In adult rats, both the systemic and local RAS have trophic effects on the regulation of aldosterone synthesis in the ZG of the adrenal gland. This has provoked interest as to whether the fetal and adult rats have a similar regulation of CYP11B2 and aldosterone biosynthesis.

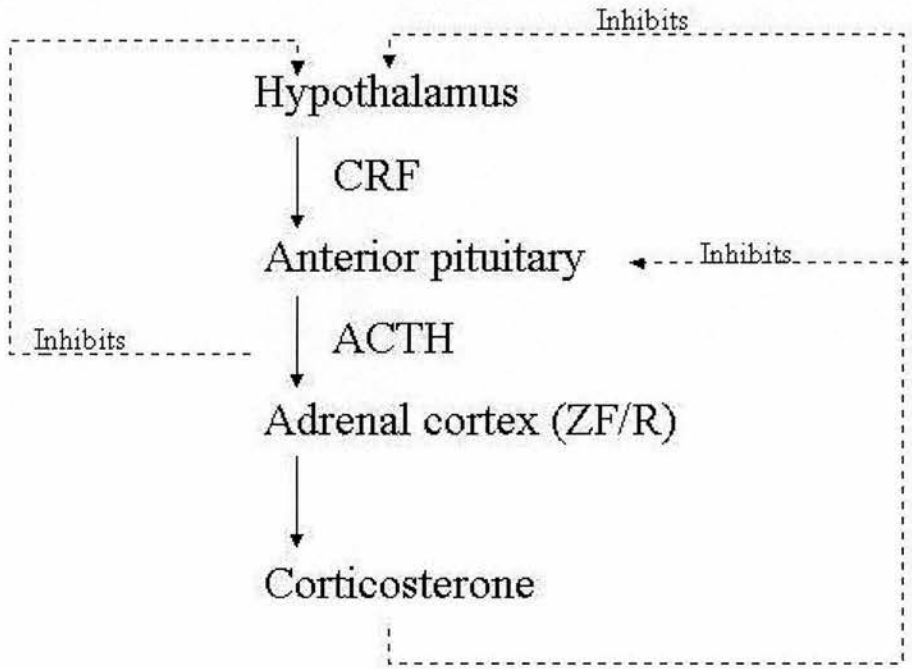


Fig. 1. 6 Mechanism for the regulation of glucocorticoid / corticosterone secretion in rats. Solid arrows indicate stimulation; dashed arrows indicate inhibition. Glucocorticoids are known to inhibit both pituitary ACTH and hypothalamic CRH secretion. ACTH also exerts a short negative feedback effect on CRH release.

1.3.2 Regulation of CYP11B1 and corticosterone biosynthesis

1.3.2.1 Hypothalamus-pituitary-adrenal gland (HPA) axis

The key components of the HPA axis include corticotrophin-releasing hormone (CRH) released from the hypothalamus and ACTH secreted from the anterior pituitary. The control of glucocorticoid synthesis is through a negative feedback regulatory process, whereby high plasma corticosterone levels inhibit both CRH and ACTH secretion, via specific glucocorticoid receptors. On the other hand, ACTH also exerts a short negative feedback effect on CRH release (Fig 1.6).

1.3.2.2 Factors regulate CYP11B1 and corticosterone biosynthesis in adult rat adrenal glands

1.3.2.2.1 ACTH

CYP11B1 expression in the ZF depends on normal plasma levels of ACTH. It has been demonstrated that ACTH stimulates corticosterodogenesis. In early studies an experimental anterior pituitary tumor that stimulated the secretion of ACTH, was shown to influence the adrenal morphology and also elevate the corticosterone secretion in rats (Ganten et al., 1974). Injection of high doses of ACTH increased CYP11B1 mRNA within the initial 24 hours and repeated injections maintained the elevated expression and increased the number of inner cortical cells expressing CYP11B1 mRNA (Ho and Vinson, 1993; Sander et al. 1994; Engeland et al., 1997). ACTH adminstration for 7 days increases corticosterone secretion *in vivo* and corticosterone production by adrenal tissue *in vitro*. The increases of corticosterone were accompanied by the increased activities of CYP11B1 as well as CYP11A1 (Kramer et al., 1977). Lehoux et al. (1998) reported that both acute and chronic ACTH treatments provoked changes in plasma corticosterone levels. However, there was little effect on the expression of CYP11B1 whereas the CYP11A1 mRNA levels were increased (Lehoux et al. 1998). Sanders et al. (1994) also found that neither adrenal CYP11B1 nor CYP11A1 mRNA levels were affected by ACTH

administration by immunocytochemistry (Sanders et al. 1994).

1.3.2.2.2 Glucocorticoids

Treatment with dexamethasone, a synthetic glucocorticoid, markedly decreased CYP11B1 mRNA levels in the rat adrenal given a normal diet. Normal levels were then restored by treatment with ACTH (Malee and Mellon, 1991; Oertle and Müller, 1993). However, the dexamethasone-induced virtual disappearance of CYP11B1 mRNA was associated with only minor decreases in CYP11B1 protein levels and also in the conversion of DOC to corticosterone and 18-OH-DOC by decapsulated adrenals (Oertle and Müller, 1993). The decreases in the CYP11B1 mRNA apparently reflected specific feedback inhibition in the glucocorticoid biosynthesis.

1.3.2.2.3 Other factors

Physiological stimuli produced by stress, for instance surgical stress, not only increased the number of cells expressing CYP11B1 mRNA, but also increased the amount of mRNA per cell (Engeland et al., 1997). Chronic stress in rats induced by immobilisation or repleated injection of hypertonic saline has been shown to activate the HPA axis and thereby to induce an increase in ACTH release accompanied by the increased plasma corticosterone levels. It also caused an increased thickness of the ZF and an expansion outward of the area of CYP11B1 mRNA expression (Aguilera et al., 1995; Pellegrini et al., 1998).

Adrenergic agonists can induce adrenal P450 gene expression *in vitro* indicating the possibility that neurotransmitters released from nerve terminals could act directly on cortical cells to increase CYP11B1 mRNA levels (Gűse-Behling et al., 1992).

1.3.2.3 Regulation of CYP11B1 and corticosterone biosynthesis in fetal rat adrenal glands

Fetal steroidogenesis has also been assessed using *in vitro* and *in vivo* conversions of radiolabelled precursors to a variety of products. Steroidogenesis, especially corticosterone biosynthesis, in fetal adrenal glands is highly active at the end of gestation (Dupouy et al., 1975; Dalle et al., 1978). Furthermore, the fetal HPA axis regulation seems to appear between day 16 and day 18 of gestation along with the increased CYP11B1 expression and corticosterone biosynthesis (Milković et al., 1976; Churchill et al., 1981; Mitani et al., 1997).

During the period around birth, maternal steroids obviously cross the placental barrier, thereby modulating the fetal HPA axis (Milković et al., 1973; Dupouy et al., 1975; Milković et al., 1976). However, whether the expression of CYP11B1 in the developing adrenals is affected by the maternal treatment and whether the changes of the gene expression in the development of the adrenal cortex would initiate programming of adult disease have not been fully elucidated.

1.4 Aims of the thesis

The main aims of this thesis were to study the distribution and expression of CYP11B2 and CYP11B1 in the adrenal cortex of Wistar adult female rats, pregnant rats, their fetuses and offspring, after various maternal manipulations.

(i) To investigate the effects of dietary salt treatments throughout pregnancy on the expression of CYP11B1 and CYP11B2 and their transcripts in the adrenals of adult female rats, pregnant rats and their fetuses.

(ii) To investigate the effects of maternal dexamethasone treatment, a modest dose of glucocorticoid hormone on the expression of CYP11B1 and CYP11B2 and their transcripts in the adrenals of pregnant rats and

their fetuses.

(iii) To investigate the effects of maternal dexamethasone treatment, a modest dose of glucocorticoid hormone on the expression of CYP11B1 and CYP11B2 and their transcripts in the developing adrenals.

(iv) To characterise a StAR protein antibody raised against a bovine StAR peptide sequence for further studies on StAR protein expression in the rat adrenal paradigms.

Chapter 2 Materials and methods

2.1 General protocols for immunohistochemistry

2.1.1 Tissue preparation

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin (Tissue-Tek II embedding wax) within 24-48 hours. Details of paraffin processing using an automatic tissue processor (Pathology Department, University of Edinburgh, Western General Hospital, Edinburgh) are summarised in Table 2.1. Microtome sections (4 μm) were mounted on superfrost plus slides (Merck, Lutterworth, UK) and were incubated at 40°C overnight to prevent tissue detaching from the slides.

Table 2. 1 Paraffin processing using an automatic tissue processor (overnight processing)

Steps	Solution	Concentrations	Time	Temperature
1	Buffered formalin	10%	45 mins	40°C
2	Buffered formalin	10%	45 mins	40°C
3	Alcohol	70%	45 mins	40°C
4	Alcohol	80%	45 mins	40°C
5	Alcohol	90%	60 mins	40°C
6	Alcohol	95%	60 mins	40°C
7	Alcohol	100%	60 mins	40°C
8	Alcohol	100%	60 mins	40°C
9	Xylene	100%	60 mins	40°C
10	Xylene	100%	60 mins	40°C
11	Wax		60 mins	60°C
12	Wax		60 mins	60°C
13	Wax		90 mins	60°C
14	Wax		90 mins	60°C

2.1.2 General immunostaining procedures

After deparaffinisation in xylene and graded rehydration in ethanol, sections were incubated with 3% hydrogen peroxide for 10 minutes to inhibit endogenous peroxidase activity. Antigen retrieval (Fig. 2.1) methods and avidin and biotin blocking kits (Vector, Peterborough, UK) may be needed for different antibodies (Shi et al., 1991; Shi et al., 1997). Each of the blocking solutions was applied for 15 minutes, and slides were rinsed with phosphate buffered saline (PBS, pH 7.4) between the two blocking solutions. All the slides were treated with 20% normal serum for 10 minutes to reduce non-specific binding. A two step indirect technique (Fig. 2.2A) or avidin-biotin complex immunolabelling technique (Fig. 2.2B) was used to detect antigens. The optimal incubation time and concentration varied according to different antibodies. 3'-Diaminobenzidine tetrahydrochloride (DAB) (Dako Ltd., Cambridgeshire, UK) was used as a chromogen substrate to detect the antigens. The sections were immersed in copper enhancement solution for 5 minutes and were counterstained with hematoxylin. The brown-coloured deposits were left at the antigen sites and the nuclei stained blue. Negative control slides were processed using the same protocol, except that no incubation was carried with the primary antibody. The slides were examined with a Nikon light microscope.

2.2 General protocols for protein work

2.2.1 Protein extraction

Frozen tissues were homogenised in extraction buffer (Table 2.2). The homogenate was centrifuged at 4500rpm for 10min to remove tissue debris. The supernatant was spin at 12500rpm at 4°C to obtain a mitochondrial pellet. The pellet was then resuspended in 20-40μl of extraction buffer. Protein concentration was determined by the Bradford method (Bradford, 1976).

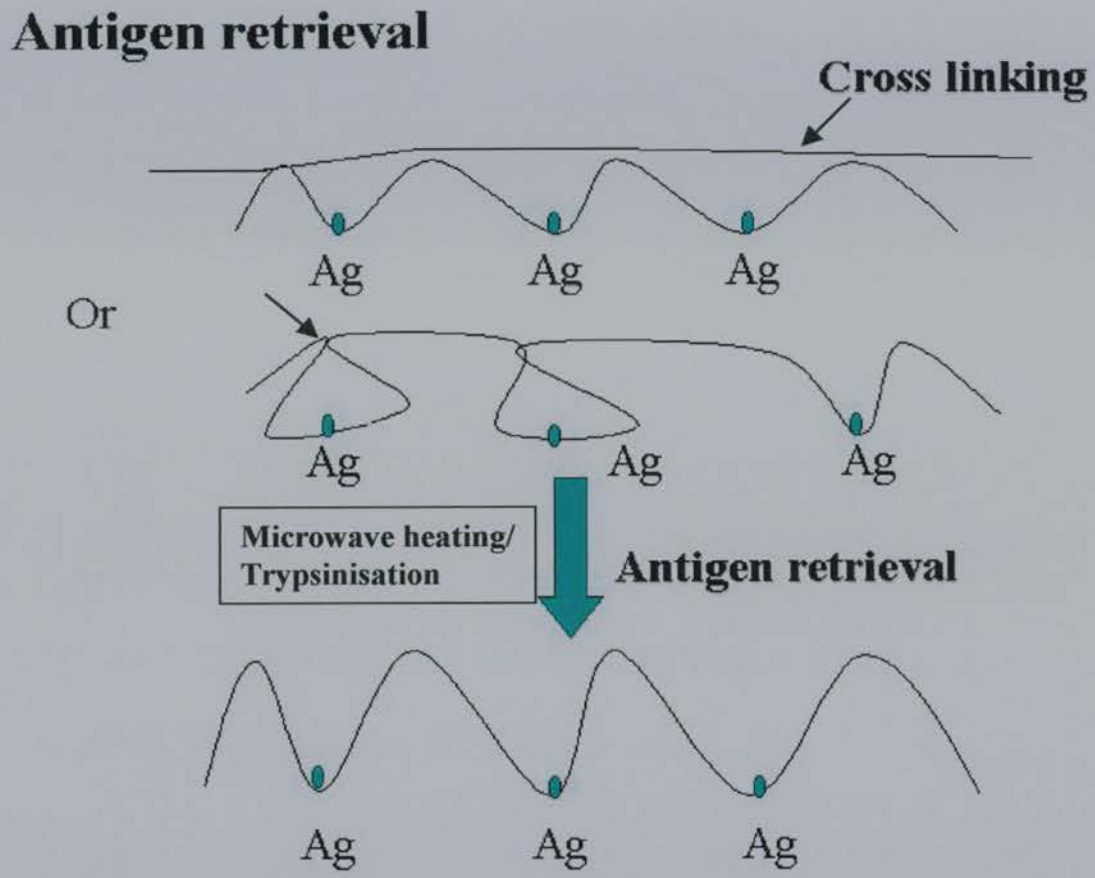
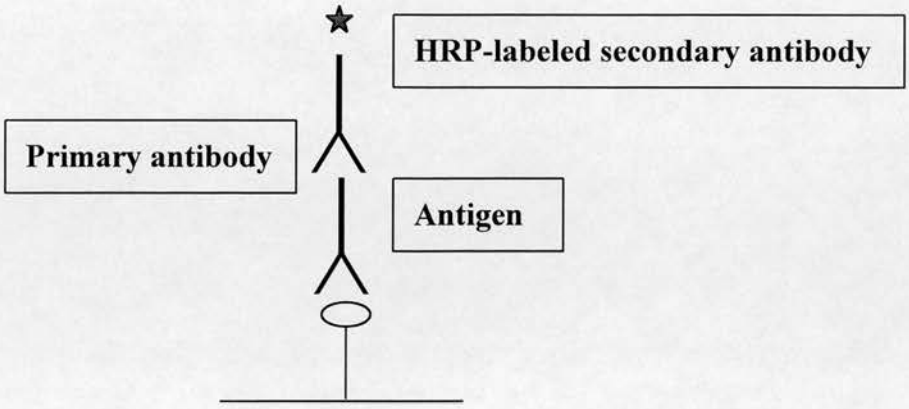


Fig. 2. 1 Antigen retrieval methods. Formalin fixation may form cross-linking between epitopes (antigens) and unrelated proteins. Breaking the cross-linking by various techniques, for example microwave heating or enzyme digestion, can uncover antigens. Ag denotes antigen; arrows indicate cross-linking.

A.



B.

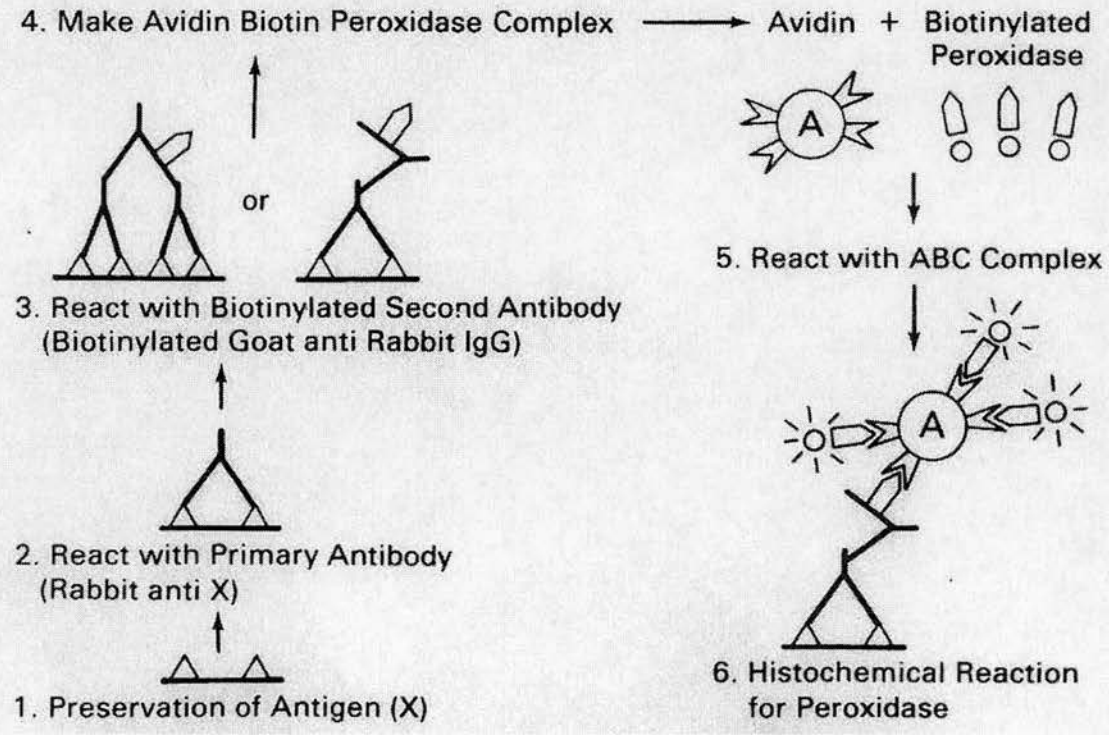


Fig. 2. 2 Procedures for two-step indirect (A) and avidin-biotin complex immunolabelling (B) techniques. **(Figures adapted from Bullock and Petrusz, 1992)**

Table 2. 2 Protein extraction buffer (10 ml)

Components	**Volume**	**Final concentration**
1M Tris (pH 7.5)	0.5 ml	50 mM
1M Sucrose	2.5 ml	250 mM
250 mM EDTA	200 µl	5 mM
1 mg/ml Leupeptin	20 µl	2 µg/ml
1 mg/ml Aprotinin	20 µl	2 µg/ml
1 mg/ml Trypsin and antipain	20 µl	2 µg/ml
100 mM PMSF (Phenylmethylsulfonyl Fluoride)	100 µl	1 mM
dH_2O	6.64 ml	

2.2.2 SDS-polyacrylamide gel electrophoresis

A Bio-Rad mini-gel apparatus (Bio-Rad, North Yorkshire, UK) was used for gel electrophoresis. CYP11B2 and CYP11B1 proteins were separated in a 10% SDS-polyacrylamide gel. The gradients of the 10% separating gel (Table 2.3) and 4% stacking gel (Table 2.4) were prepared first. Samples (20-30 µg) were diluted at least 1:4 with sample buffer (Table 2.5), and then heated at 95°C for 5 minutes prior to loading onto the gel. The condition used for separating CYP11B2 and CYP11B1 proteins was running the gel at constant voltage, 150V in 1× running buffer (Table 2.6) for 3-4 hours.

Table 2. 3 Separating gel, 10% gel in 0.375 M Tris, pH8.8 (10 ml)

Components	**Volume**
1.5 M Tris-HCl (pH 8.8)	2.5 ml
10% SDS	0.1 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	3.3 ml
Ammonium persulfate	0.05 ml
TEMED	0.005 ml
Distilled water	4.05 ml

Table 2. 4 Stacking gel, 4% gel in 0.125M Tris, pH6.8 (5 ml)

Components	**Volume**
1.5 M Tris-HCl (pH 6.8)	1.25 ml
10% SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
Ammonium persulfate	0.015 ml
TEMED	0.025 ml
Distilled water	3.3 ml

Table 2. 5 Sample loading buffer

Components	**Volume**
0.5M Tris-HCl	1.0 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
β-Mercaptoethanol	0.4 ml
Bromophenol blue	0.2 g
dH_2O	4.0 ml

Table 2. 6 Running buffer, pH8.3 (5x)

Components	Weight / Volume
Tris Base	15 g
Glycine	72 g
SDS	5 g
dH_2O	Add to 1 litre

2.2.3 Western blotting

A normal SDS protein gel was run on a small BioRad protein gel system. Then the gel was soaked in 1× transfer buffer (0.025 M Tris-HCl, 0.192 M glycine) for ten minutes to leach out the SDS in the gel. At the same time a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK.) as well as two sheets of 3MM papers were presoaked in the transfer buffer.

Proteins from the gel were transfered to the nitrocellulose membrane using Bio-Rad transfer apparatus in the same transfer buffer at 250 mA for two hours. After transfer, the membrane was washed with water and then blocked with blocking buffer (5 % non-fat dry milk, Bio-Rad, North Yorkshire, UK) in TBS (50 mM Tris-HCl pH7.4, 150 mM NaCl) overnight in order to block any non-specific sites on the membrane. Monoclonal CYP11B2 (1:100) or CYP11B1 (1:3500) antibody was incubated for 2 hours and then the membrane was washed three times in TBS/0.5% Tween 20, 15 minutes for each wash. The CYP11B2 and CYP11B1 antibodies were raised in mice, as described by Tam (1988), with the peptide KVRQNARGSLTMDVQQ or KNVYRELAEGRQQS, respectively. (Generously provided by Dr. Celso E. Gomez-Sanchez, Department of Internal Medicine, University of Missouri-Columbia and the Harry S. Truman Memorial Veterans Hospital.)

Sheep anti-mouse-HRP conjugated secondary antibody (1:2000) (Amersham Pharmacia Biotech, Buckinghamshire, UK.) was incubated for 1 hour, then the membrane was washed three times in TBS, 15 minutes for each wash. ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK.) was applied to detect the signal. The X-OMAT film was exposed to the membrane from 30 min to 1 hour for the CYP11B2 antibody and for 2-5 min for the CYP11B1 antibody.

2.3 General protocols for RNA work

2.3.1 RNA extraction

Total RNA from frozen tissues was extracted by Rneasy Mini Kits (QIAGEN, UK). Samples were homogenised in 350 µl of GITC-containing buffer. After removing tissue debris by 3 min maximunm speed of centrifugation, a further 350µl of 70% ethanol was added into the lysate mixture. The lysate was centrifuged in a collection tube at 8000x g for 15 sec. Then, 700µl of washing buffer (buffer RW1) was applied on to the Rneasy column and centrifuged for 15 sec at 8000x g. The column was washed twice by adding 500µl of RPE buffer and was centrifuged at full speed. The total RNA was eluted by 25 µl of Rnase-free water.

2.3.2 Competitive reverse transcription-polymerase chain reaction (cRT-PCR) amplification

2.3.2.1 Reverse transcription

Three hundred nanograms and 5 nanograms of RNA templates were used in the analyses of CYP11B2 mRNA and CYP11B1 mRNA, respectively. RNA template was reverse transcribed into complementary DNA (cDNA) using Gene Amp kit (PE Biosystems, Cheshire, UK.). Templates were hybridised at 25°C for 10 min. Reverse

transcription was performed at 42°C for 12 min. The composition of a 20 μl RT reaction mixture are listed in the Table 2.7.

2.3.2.2 PCR amplification

The composition of a 30 μl PCR reaction mixture are listed in the Table 2.8. The cDNAs of CYP11B2 and CYP11B1 were amplified through 28 cycles. The amplification programmes are listed below.

PCR amplification programme:

Step 1. Denature: 95°C for 10 minutes

Step 2. Denature: 95°C for 45 seconds

Step 3. Annealing: 51°C for 45 seconds

Step 4. Extension: 72°C for 45 seconds

Step 5. Repeat step 2-step 4 for 27 cycles.

2.3.2.3 Primers

CYP11B2 primers

The CYP11B2 primers amplified a 297 bp fragment corresponding to the sequence 657-954 of CYP11B2 (Oaks and Raff, 1995). This fragment includes sequence from exon 3 to exon 5 and cross two introns. The sequences of sense and antisense of CYP11B2 were as described (Fig 2.3).

Forward **5' ACCATGGATGTCCAGCAA 3'**

Reverse **5' GAGAGCTGCCGAGTCTGA 3'**

Table 2. 7 RT mixture (20 µl)

Components	Volume (µl)	Final concentration
10x RT PCR buffer	2	1x
$MgCl_2$ (25 mM)	2	2.5 mM
dNTP blend (Deoxyribonucleotide triphosphates) (10 mM)	2	1 mM (250 µM of each dNTP)
DTT (100 mM) (Dithiothreitol)	2	10 mM
Random Hexamers (50 µM)	0.5	1.25 µM
MultiScribe™ Reverse Transcriptase (50 Units/µl)	0.3	15 Units/20 µl
Rnase inhibitor (20 Units/µl)	0.5	10 Units/20 µl
RNA template	1	Various*
Rnase-free water	9.7	-

*300ng and 5 ng of RNA templates were used in the analyses of CYP11B2 mRNA and CYP11B1 mRNA, respectively.

Table 2. 8 PCR mixture (30 µl)

Components	Volume (µl)	Final concentration
10x RT PCR buffer	3	1x
$MgCl_2$ (25 mM)	2	1.67 mM
dNTP blend (Deoxyribonucleotide triphosphates) (10 mM)	2	0.67 mM (167.5 µM of each dNTP)
AmpliTaq Gold DNA Polymerase	0.5	2.5 Units/30 µl
Primers (upstream)	1	10 pmole/30 µl
Primers (downstream)	1	10 pmole/30 µl
Rnase-free water	19.5	-
Non-homologous DNA competitor	1	Various*

*See serial dilution table in Fig. 2.1.

CYP11B1 primers

The CYP11B1 primers amplified a 312 bp fragment corresponding to the sequence 528-840 of CYP11B1 (Oaks and Raff, 1995). This fragment includes sequence from exon 3 to exon 5 and cross two introns. The sequences of sense and antisense of CYP11B1 were as described (Fig. 2.4).

Forward 5' GCTGGAGAATGTTCATGG 3'

Reverse 5' CTCTGCCAGTTCGCGATA 3'

2.3.2.4 Competitors

Non-homologous competitors (generously provided by Sue Lloyd-MacGlip, Molecular Medicine Centre, Western General Hospital, Edinburgh) were synthesised by amplifying onion fragment from onion alliinase gene with the combination of CYP11B2 or CYP11B1 primers and primers, which were specific to onion fragment. The cRT-PCR protocol is schemed in Fig. 2.5. The sense and antisense sequences of both CYP11B2 and CYP11B1 competitors were as followed.

CYP11B2 competitor

Forward 5' ACCATGGATGTCCAGCAAGTTGCTCATGCCCC 3'

Reverse 5' GAGAGCTGCCGAGTCTGACGTAATCCGCTGCA 3'

CYP11B1 competitor

Forward 5' GCTGGAGAATGTTCATGGGTTGCTCATGCCCC 3'

Reverse 5' CTCTGCCAGTTCGCGATACGTAATCCGCTGCA 3'

2.3.2.5 Quantitative PCR

Serial dilutions of PCR competitors (see Fig. 2.5) were added to PCR amplification reactions containing constant amounts of the experimental RNA/ cDNA samples. The PCR competitor and target templates thus competed for the same primers in the same reaction. By knowing the amount of PCR competitor added to the reactions, the amount of target template, i.e. the initial mRNA levels can be determined.

2.4 Statistical analyses

Data were analysed by one-way or two-way ANOVA analyses, followed by Tukey's test or the Student's t test to establish if the differences of variables were significant ($P<0.05$).

CYP11B2 sequence

ATGGCTCTCA GGGTGACAGC AGATGTGTGG CTGGCAAGAC CCTGGCAGTG
CCTGCACAGG ACGAGGGCAC TGGGCACTAC GGCAACACTG GCCCCTAAGA
CACTGAAGCC CTTTGAAGCC ATACCACAAT ACTCCAGGAA CAAGTGGCTG
AAGATGATAC AGATCCTGAG GGAGCAGGGC CAAGAGAACC TACACCTGGA
GATGCACCAG GCCTTCCAGG AGCTGGGGCC CATTTTCAGG CACAGTGCAG
GGGGAGCACA GATTGTGTCT GTGATGCTGC CTGAGGACGC TGAGAAGCTG
CACCAGGTGG AGAGTATCCT CCCGCGTCGG ATGCACCTGG AGCCGTGGGT
GGCCCACAGG GAACTCCGTG GCCTGAGACG TGGTGTGTTC TTGCTAAATG
GGGCTGAATG GCGCTTCAAC CGACTGAAAC TGAACCCAAA CGTGCTGTCA
CCAAAAGCTG TTCAAAATTT TGTCCCCATG GTGGACGAGG TAGCAAGGGA
CTTCTTGGAG GCCCTGAAAA AGAAGGTGCG TCAGAATGCT CGAGGGAGCC

TT*ACCATGGA TGTCCAGCAA* AGTCTCTTCA ACTACACTAT AGAAGCCAGC
AACTTTGCAC TTTTTGGAGA GAGGCTGGGC CTCCTTGGTC ATGACCTGAA
CCCTGGTAGC CTGAAGTTCA TCCATGCCCT ACATTCAATG TTCAAGTCCA
CCACACAGCT CCTGTTCTTA CCCAGAAGCT TGACTCGCTG GACAAGCACC
CAGGTGTGGA AAGAACATTT TGATGCCTGG GATGTCATCT CTGAGTATGC
CAACAGATGT ATCTGGAAGG TGCACCAGGA AC*TCAGACTC GGCAGCTCTC*
AGTCTGAG CCGTCGAGAG
AGACCTACAG TGGCATTGTG GCAGCACTAA TAACTCAGGG AGCTTTACCT
CTGGACGCCA TCAAAGCCAA CTCTATGGAG CTCACTGCTG GGAGCGTTGA
CACGACAGCA ATCCCCTTGG TAATGACACT TTTTGAGCTG GCTCGGAACC
CAGATGTTCA GCAGGCCCTG CGGCAGGAGA CCCTGGCAGC TGAGGCCAGC
ATCGCTGCTA ATCCCCAGAA GGCCATGTCA GACCTGCCCT TGCTGCGGGC
TGCCCTTAAA GAGACCTTGA GACTCTACCC TGTTGGTGGC TTTTTGGAGA
GAATCCTAAA CTCAGACCTG GTGCTTCAGA ACTATCATGT CCCTGCTGGG
ACGTTGGTCC TACTTTATCT GTACTCCATG GGCCGAAACC CTGCAGTGTT
CCCAAGACCT GAGCGCTATA TGCCTCAGCG CTGGCTGGAG AGGAAAAGGA
GTTTCCAGCA TCTGGCCTTC GGCTTTGGGG TGCGCCAGTG CCTGGGGCGG
CGCCTGGCAG AGGTGGAGAT GCTGCTCCTG CTTCACCATA TGCTGAAAAC
CTTCCAGGTG GAGACACTGA GACAAGAGGA TGTGCAGATG GCCTATCGCT
TTGTTTTGAT GCCCAGCTCT AGTCCTGTCC TCACTTTCCG GCCCATCAGC
TAG

Fig. 2.3 Nucleotide sequence of CYP11B2 gene. The underlined sequence indicates the primers sequence.

CYP11B1 sequence

ATGGCTCTCA GGGTGACAGC AGATGTGTGG CTGGCAAGAC CCTGGCAGTG
CCTGCACAGG ACGAGGGCAC TGGGCACTAC GGCAAAAGTG GCCCCCAAGA
CACTGAAGCC CTTTGAAGCC ATACCACAAT ACTCCAGGAA CAAGTGGCTG
AAGATGATAC AGATCCTGAG AGAGCAGGGC CAAGAGAACC TACACCTGGA
GATGCACCAG GCCTTCCAAG AGCTGGGGCC CATTTTCAGG CACAGTGCAG
GGGGAGCACA GATTGTGTCT GTGATGCTGC CTGAGGACGC TGAGAAGCTG
CACCAGGTGG AGAGTATCCT CCCGCATCGG ATGCCCCTGG AGCCGTGGGT
GGCCCACAGA GAACTCCGTG GCCTGAGACG TGGTGTGTTC TTGCTAAATG
GGGCAGACTG GCGCTTCAAC CGACTGCAGC TGAATCCAAA TATGCTGTCA
CCAAAAGCCA TTCAATCTTT TGTCCCCTTT GTGGATGTGG TAGCAAGGGA

CTTTGTGGAA AACCTGAAGA AGAGAATGCT GGAGAATGTT CATGGAAGCA
TGTCTATAAA CATTCAGTCC AATATGTTCA ACTATACCAT GGAAGCCAGC
CATTTTGTTA TTTCCGGAGA GCGTCTGGGC CTCACAGGCC ATGACCTGAA
ACCTGAGAGC GTGACATTCA CTCATGCTCT GCACTCAATG TTCAAGTCCA
CCACACAGCT CATGTTCTTA CCCAAGAGCT TGACTCGTTG GACAAGCACC
CGGGTGTGGA AAGAACACTT TGATTCCTGG GATATCATCT CTGAGTATGT
CACAAAATGT ATCAAGAATG TGTATCGCGA ACTGGCAGAG GGTCGCCAAC
ATAGCGCT TGACCGTCTC
AGTCCTGGAG TGTCATATCC GAGATGGTAG CACAGAGTAC TCTGTCAATG
GATGCCATCC ATGCCAACTC AATGGAACTT ATTGCTGGAA GTGTTGACAC
GACAGCAATC TCCTTGGTAA TGACCCTTTT TGAGCTGGCT CGGAACCCAG
ATGTTCAGCA GGCCCTGCGG CAGGAGAGCC TAGCAGCTGA GGCCAGCATC
GTTGCTAATC CCCAGAAGGC CATGTCAGAC CTGCCCTTGC TGCGGGCTGC
CCTTAAAGAG ACCTTGAGGC TCTACCCTGT TGGTAGCTTT GTAGAGAGAA
TCGTACACTC AGACCTGGTG CTTCAGAACT ATCATGTCCC TGCTGGGACA
TTCGTCATAA TTTATCTGTA CTCCATGGGC CGAAACCCTG CAGTGTTCCC
AAGGCCTGAG CGCTACATGC CTCAGCGCTG GCTGGAGAGG AAAAGGAGTT
TCCAGCATCT GGCCTTCGGC TTTGGGGTGC GCCAGTGCCT GGGGCGGCGC
CTGGCAGAGG TGGAGATGCT GCTCCTGCTT CACCATATGC TGAAAACCTT
CCAAGTGGAG ACACTGAGAC AAGAGGATAT GCAGATGGTT TTTCGCTTTC
TTTTGATGCC CAGCTCTAGT CCTTTCCTTA CTTTCCGGCC TGTGAGCTAG

Fig. 2.4 Nucleotide sequence of CYP11B2 gene. The underlined sequence indicates the primers sequence.

Prepare Target Sequences & Reverse Transcription

Isolate RNA from tissue samples

↓

RT reaction: Synthesise 1st-strand cDNA (20 μl)

↓

Conduct Competitive PCR

↓

Make serial dilutions of PCR Competitor

Serial dilutions of PCR Competitor

	CYP11B2 Competitor concentration (M)	**CYP11B1 Competitor concentration (M)**
Competitor 1	3.16×10^{-12}	1.0×10^{-11}
Competitor 2	3.16×10^{-13}	3.16×10^{-12}
Competitor 3	1.0×10^{-13}	1.0×10^{-12}
Competitor 4	3.16×10^{-14}	1.0×10^{-13}

↓

Add 1 μl of each PCR Competitor dilution to 1st-strand cDNA samples (20 μl)

↓

Add 29 μl of PCR reaction buffer to each sample

↓

Perform PCR (total volume: (50 μl))

↓

Electrophorese products (12.5 μl) on an agarose gel (2%)

↓

Analyse above data

Fig. 2. 5 Outline for competitive RT-PCR protocols.

2.5 The methods for salt diet experiment

2.5.1 Animals handling

Animal handling and sample collections followed standards conforming to "The Principles of Animal Care"(NIH publication No. 85-23, revised 1985) and Home Office regulations in the U.K. Female Wistar/Han rats of the same age and weight were maintained in a 12-hour light/dark cycle and controlled temperature with sexually mature males and had free access to normal rat chow and deionized water. After the detection of a vaginal plug, indicating that mating had taken place, females were housed individually in breeding cages. Female rats were divided into three groups and were fed high (3%, HS), normal (0.3%, NS) or low (0.03%, LS) sodium diets (Appendix I) throughout pregnancy. Another three groups of non-pregnant female rats were also fed the same diets for 20 days.

2.5.2 Sample collection

In the first experiment, non-pregnant and pregnant rats were euthanised after 20 days of diet. The weights of the heart, kidneys and adrenal glands were recorded. The adrenal glands were immersed in 10% neutral-buffered formalin and embedded in paraffin within 24 hours.

In the second experiment, pregnant rats were sacrificed at day 20 of gestation. The adrenal glands were removed from pregnant rats and their offspring. Adrenal glands were frozen at -80°C. Frozen samples were used for Western blotting and competitive RT-PCR analysis. The weights of the heart, kidneys and adrenal glands were also recorded. The excised adrenals were bisected and decapsulated by the method of Giroud et al. (1956). Capsular (zona glomerulosa) and decapsulated (zona fasciculate, zona reticularis and medulla) portions of the adrenal glands were immediately frozen and stored at -80°C.

2.5.3 Immunohistochemistry

The conditions for the antibodies used in the experiments are summarised in Table 2.9. Negative control slides were processed using the same protocols, except that no incubation was carried with the primary antibody. The 3β-HSD antibody was raised in rabbit against human placenta 3β-HSD (Doody et al., 1990). The details of the immunostaining methods employed are described in Section 2.1.2.

Table 2. 9 Conditions for the antibodies used for immunostaining.

Antibody\Conditions	AR method/Time[1]	1° /Time[2]	2° /Time[3]	StreptABC/Time[4]
CYP11B2	Microwave (20min)	1:50/1 hr	1:800/1 hr	30min
CYP11B1	Microwave (20min)	1:100/1 hr	1:400/1 hr	30min
3β-HSD	Trypsin (30min)	1:400/30min	1:400/30min	30min

(AR method/Time[1]: antigen retrieval method and time.)

(1° /Time[2]: primary antibody dilution and incubation time.)

(2° /Time[3]: secondary antibody dilution and incubation time.)

(StreptABC/Time[4]: streptavidine-biotin complex method and incubation time.)

2.5.3.1 Scoring for immunostaining results

Slide labels were masked and coded to obtain objectivity in the readings. The score for the immunostaining was defined from 0 (no staining) to 9 (strong staining for throughout ZG or ZF). Score from 0 to 3 represents weak staining. Score from 4 to 6 stands for medium staining. Strong positive staining was scored from 7 to 9. All slides were examined carefully. The negative control slides showed no staining at all.

2.5.3.2 Measurement of ZG zona width and cell size in the ZG and the ZF

To identify ZG and ZF cells, sections were stained with hematoxylin and eosin. The width of the ZG was measured at a magnification of x400. The average width of ZG was determined by 8 counts from each adrenal section (2 counts per subfield for each of 4 subfields). A group of ZG and ZF cells was counted at a magnification of x100 (10 counts per subfield for each of 4 subfields) by computer-assisted grain counting using an image analysis system (MCID 4.0 software package).

2.5.4 Radioimmunoassays

2.5.4.1 Aldosterone

Plasma aldosterone levels were measured (in cooperation with Dr. Christopher Kenyon) using Coat-A-Count® Aldosterone kit (Diagnostic Products Corporation, Los Angeles, USA). According to the manufacturers' instruction, aldosterone-specific antibody immobilised to the wall of a polypropylene tube. Plasma samples were added into the tubes prepared. ^{125}I-labeled aldosterone (1.0 ml) was added to each tube and mixed briefly and gently. Labeled aldosterone was incubated for 3 hours at 37°C with plasma samples. All visible moisture was removed after incubation. Samples and standards were counted for 1 minute in an LKB Wallac Microbeta counter after the tubes were drained for 2 or 3 minutes. The percent cross reactivity of corticosterone in the aldosterone assay was 0.002%. The intraassay variation was 3.2%.

2.5.4.2 Corticosterone

Plasma samples were diluted tenfold with borate buffer (0.133 M boric acid, 67.5mM NaOH - HCl, pH 7.4 containing 0.5% bovine serum albumin, 1% methanol and 0.1% ethylene glycol) (in cooperation with Dr. Christopher Kenyon). After heating samples at 80°C for thirty minutes to denature corticosterone binding

globulin, aliquots (25µl) of diluted plasma were incubated with rabbit antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK.)to corticosterone (final dilution 1:40,000), ^{3}H-corticosterone (specific activity 80Ci/mmol; final concentration 1.5nM, Amersham Pharmacia Biotech, Buckinghamshire, UK.) and anti- rabbit scintillation proximity assay reagent (25µl diluted suspension) were mixed in a final volume of 100µl borate buffer. After incubation overnight at room temperature, samples and standards were counted in an LKB Wallac Microbeta counter. Intra and inter assay variations were 9.4 and 9.2% respectively. Compared with corticosterone (100%), cross reactivities for progesterone, deoxycorticosterone and cortisol are 7.7, 6.5 and 5.3 % respectively.

2.5.5 Western blotting and cRT-PCR

The protocols for western blotting and cRT-PCR were described in section 2.2 and 2.3. Twenty micrograms and thirty micrograms of protein samples extracted from adult and fetal adrenals, respectively, were used for western blotting analyses. Three hundred nanograms and 5 nanograms of RNA templates were used for CYP11B2 and CYP11B1, respectively.

2.6 The methods for dexamethasone experiments in fetal and pregnant rat adrenal glands

2.6.1 Animal handling

Animal handling and sample collections followed standards conforming to "The Principles of Animal Care"(NIH publication No. 85-23, revised 1985) and Home Office regulations in the U.K. Female Wistar/Han rats of the same age and weight were maintained in a 12-hour light/dark cycle and controlled temperature with sexually mature males and had free access to normal rat chow and deionized water.

After the detection of a vaginal plug, indicating that mating had taken place, females were housed individually in breeding cages. Female rats were divided into two groups. The experimental group was injected subcutaneously with 100 μg/kg body weight/day dexamethasone in 0.1 ml 4% ethanol-saline throughout pregnancy (20 days of gestation). The control group was treated with saline (called saline-treated or DEX-treated in the following sections).

2.6.2 Sample collection

Pregnant rats were euthanised at 20 days of gestation. The adrenal glands were removed from pregnant rats and their offspring and trimmed of fat. A fetal adrenal gland, from two pups of each pregnant rat was immersed in 10% neutral buffered formalin and embedded in paraffin within 24 hours. Other fetal adrenal glands were frozen at -80°C. Frozen samples were used for western blotting and competitive RT-PCR analysis of gene expression. The weights of the heart, kidneys and adrenal glands were also recorded.

2.6.3 Immunohistochemistry

2.6.3.1 Serial staining for CYP11B2, CYP11B1, 3β-HSD and chromogranin A

Serial sections of the adrenal glands were taken. These sections then were stained consecutively with antibodies specific for CYP11B2, CYP11B1, 3β-HSD and chromogranin A (generously provided by Dr. David Apps, Department of Biomedical Sciences and Clinical Laboratory, University of Edinburgh, UK). Negative control slides were processed using the same protocols, except that no incubation was carried with the primary antibody. The conditions for the antibodies used are summarised in Table 2.10. The details of the immunostaining methods employed are described in Section 2.1.

Table 2. 10 Conditions for the antibodies used for immunostaining.

Antibody\Conditions	AR method/Time[1]	1° /Time[2]	2° /Time[3]	StreptABC/Time[4]
CYP11B2	Microwave (20min)	1:200/ON[5]	1:400/1 hr	30min
CYP11B1	Microwave (20min)	1:200/ON	1:400/1 hr	30min
3β-HSD	Trypsin (30min)	1:400/30min	1:400/30min	30min
Chromogranin A	Microwave (20min)	1:25/1 hr	1:25/1 hr	-

(AR method/Time[1]: antigen retrieval method and time.)

(1° /Time[2]: primary antibody dilution and incubation time.)

(2° /Time[3]: secondary antibody dilution and incubation time.)

(StreptABC/Time[4]: streptavidin-biotin complex method and incubation time.)

(ON[5]: overnight incubation.)

2.6.3.2 Scoring for CYP11B2 and CYP11B1 immunostaining

Slide labels were masked and coded to obtain objectivity in the readings. CYP11B2 and CYP11B1 staining methods are summarised in Table 2.10. The scoring for the immunostaining was defined over the range zero (no staining) to 9 (strong staining throughout the ZG or ZF). All slides were examined carefully. The negative control slide had to exhibit no staining at all.

2.6.3.3 Measurement of cell size in the ZF and medulla

To identify ZF cells and medullary cells, two consecutive sections were stained with antibodies for CYP11B1 and chromogranin A, respectively. A group of ZF or medullary cells was measured at a magnification of x400. The average cell size was

determined by 20 counts from each adrenal section by computer using an image analysis system (MCID 4.0 software package), which identified individual cell nuclei.

2.6.4 Western blotting and cRT-PCR

The protocols for western blotting and cRT-PCR were described in section 2.2 and 2.3. Twenty micrograms and thirty micrograms of protein samples extracted from adult and fetal adrenals, respectively, were used for western blotting analyses. Three hundred nanograms and 5 nanograms of RNA templates were used for CYP11B2 and CYP11B1, respectively. In order to obtain a visible signal for CYP11B1 transcripts from the dexamethasone-treated pregnant rat adrenals, it was necessary to increase the amount of the RNA template to 150 nanograms.

2.7 The methods for dexamethasone experiments in postnatal rat adrenal glands

Animal handling and sample collections followed standards conforming to "The Principles of Animal Care"(NIH publication No. 85-23, revised 1985) and Home Office regulations in the U.K. Sample collections were also made as for the previous experiments (see Chapter 4). Female rats were divided into two groups. The experimental group was injected subcutaneously with 100 μg/kg body weight/day dexamethasone in 0.1 ml 4% ethanol-saline or vehicle (saline) throughout pregnancy. No further treatment was given to mothers or pups after delivery. Male offspring (called saline-treated or DEX-treated in the following sections) were kept until they were 7-day, 28-day or 4-month-old. The protocols for serial staining, scoring and western blotting and cRT-PCR were described in Section 2.6.

2.7.1 Detection of CYP11B3 mRNA transcripts

CYP11B1 primers also amplified CYP11B3 mRNA transcripts. The RT-PCR method was described in section 2.6. Ten nanograms of RNA templates were amplified by CYP11B1 primers. Then, the RT-PCR products (15 μl) of CYP11B1 were digested using *Sma*I enzyme (0.1%) at room temperature for an hour. The digestion buffer is summarised in Table 2.11. CYP11B3 transcripts are resistant to enzyme digestion whereas CYP11B1 transcripts are digested into smaller fragments. The relative amount of CYP11B3 transcripts to the total amount of CYP11B1 transcripts from 1% agarose gels was computed.

Table 2. 11 Digestion buffer (15 μl)

Components	**Volume** (μl)
PCR product	10
10x Buffer J	1.5
BSA	0.15
dH_2O	2.35
Sma I enzyme	1

2.8 The methods for characterisation of StAR protein

Adult Wistar rats were killed by cervical dislocation. Adrenal tissues and testis were fixed in 10% neutral buffered formalin and embedded in paraffin for immunostaining uses. Microtome sections (5 μm) were mounted. In addition, adrenals from adult male CD mice were also collected. Samples, including heart, brain, kidney, gonads and liver were frozen immediately after dissection and used for Western blotting.

2.8.1 Immunostaining

The immunostaining for StAR protein was carried out using a similar protocol to that described in Chapter 2, but instead of using enzyme digestion, slides were heated with citrate buffer (0.01 M, pH 6.0) in the microwave oven using the intermittent heating method of two 10-minute cycles. Then, slides were incubated with normal donkey serum (Scottish Antibody Production Unit, Lanarkshire, UK) for 10 minutes.

Immunostaining was performed using a sheep antibody against a sequence of purified bovine StAR protein (AMQRALGILKDQEGWKKESRQANGDEV), diluted 1 in 100 in PBS buffer. Horse-radish-peroxidase (HRP) conjugated secondary antibody (Binding Site company, Birmingham, UK) (1 in 200 dilution) was used for StAR protein localisation. The immunostaining procedures for 3β-HSD and chromogranin A were described in Section 2.5.3.

2.8.2 Protein extraction and Western blotting

Whole or dissected tissues were homogenized at 4 °C in 5mM Tris-HCl buffer, pH7.4, containing 275mM sucrose. The homogenate was centrifuged at 680 x g for 10 minutes to remove debris. The supernant was centrifuged at 10,000 x g for 10 minutes to obtain the mitochondria pellet. Further centrifugation of the supernant at 105,000 x g for 1hour yielded the microsomal pellet. Both of the pellets were washed with the same buffer. Protein concentration of each sample was determined by Bradford method.

Proteins were analyzed on SDS-polyacrylamide mini-gel. Primary antibodies, StAR and 3β-HSD (1 in 10,000 and 1 in 5,000 diluted with 3% non-fat milk in PBS), and HRP-conjugated secondary antibodies, donkey-anti-sheep and anti-rabbit (1 in

100,000 and 1 in 50,000 dilution) were incubated. Proteins were detected by chemiluminescence (Pierce).

2.8.4 Reverse transcription-PCR amplification

RNA template was reverse transcribed into complementary DNA (cDNA) using Gene Amp kit (PE Biosystems, Cheshire, UK.). Template (10 ng) was hybridised at 25°C for 10 min. Reverse transcription was performed at 42°C for 12 min.

The 494 bp rat StAR cDNA was amplified using primers corresponding to nucleotide sequence from 321 bp to 815 bp. The PCR protocol comprised 1 min of denaturation at 94 °C, 1 min of annealing at 58 °C and 2 min of extension at 72 °C for 30 cycles.

Forward 5'-TACTCAACAACCAGGAAGGCTGG-3'
Reverse 5'-GTGATAAGACTTGGTTGATGATGGTC-3'

Chapter 3 Effects of dietary sodium chloride on steroidogenic enzymes in pregnant and fetal rat adrenal glands

3.1 Introduction

Changes of dietary sodium chloride (salt) intake during pregnancy can exert profound effects on the structure (Pike et al., 1966; Pohanka and Pike, 1970; Smiciklas et al., 1971) and function (Khokhar and Pike, 1973) of the maternal adrenal cortex. It is also known to affect the expression of genes involved in steroidogenesis, especially the CYP11B2 gene that encodes aldosterone synthase (Brochu et al., 1998; Malee and Mellon, 1991). Peak aldosterone secretory activity with adrenal hypertrophy and hyperplasia was observed at day 18 of pregnancy in rats fed a low sodium diet. Depletion of renin secretory granules in the renal juxtaglomerular cells is indicative of maximal activation of the renin-angiotensin system (Pike et al., 1966; Khokhar and Pike, 1973). Clearly, the maternal renin-angiotensin system is vital in regulating sodium homeostasis during pregnancy. However, in adrenalectomised rats, pregnancy proceeds normally and sodium continues to be retained. Plasma concentrations of aldosterone were only partly reduced after adrenalectomy whereas plasma values were negligible in a non-pregnant adrenalectomised control group (Churchill et al., 1981). This disparity of the effects of adrenalectomy might indicate an alternative fetal source of aldosterone.

In the fetal rat, adrenal development starts from around day 12 of gestation (E12) and its embryogenesis is completed by E16 (Josimovich et al., 1954; Roos, 1967). Although the zona glomerulosa (ZG) cells can be identified morphologically as early as E18, Mitani et al., reported that CYP11B2 remained undetectable until E20;

11β-hydroxylase, encoded by CYP11B1 gene, was seen from E18 in Sprague-Dawley rats (Mitani et al., 1997). Later, Wotus et al. (1998) found both CYP11B2 mRNA and protein expression at E18 and E19 and were able to show production of aldosterone by fetal adrenal tissue. They also described two distinct phases of ZG development. Firstly, the appearance of small clusters of CYP11B2-positive cells throughout the adrenal at E16. Then, secondly, cell differentiation and a change in the distribution of CYP11B2 expressing cells, which were localised exclusively in a sub-capsular region of the adrenal gland at E18 and E19.

It would appear therefore that the period around birth is a critical stage in the development of the rat adrenal gland in terms of both cell proliferation (Josimovich et al., 1954; Roos, 1967) as well as differentiation with the expression of CYP11B2 and CYP11B1, which are responsible for mineralocorticoid and glucocorticoid biosynthesis, respectively (Mitani et al., 1997; Wotus et al., 1998). On the basis of those studies, the primary focus of this chapter was to investigate whether dietary salt intake, a major factor regulating aldosterone synthesis in adults, has any influence on fetal adrenal function. Two experiments were carried out. In the first experiment, effects of dietary salt were compared with a particular emphasis on adrenal histology in non-pregnant female and pregnant rats and in fetal rats. In the second experiment, molecular techniques were used to investigate expression of CYP11B genes in maternal and fetal adrenal glands. Adrenal cortical functions at E20 and in maternal adrenal glands were compared after feeding high (3%), normal (0.25%) and low (0.03%) sodium diets throughout pregnancy. Immunohistochemical, Western blotting and competitive RT-PCR methods were used to examine adrenal gland morphology and expression of CYP11B genes. Radioimmunoassay was also used to measure plasma adrenal steroids.

3.2 Results

3.2.1 Characterisation of antibodies

The specificities of the CYP11B2, CYP11B1 and 3β-HSD antibodies were confirmed by immunohistochemistry (Fig. 3.1) and Western blotting (Fig. 3.2) in rat adrenal sections and protein extracts.

CYP11B2 was localised in the ZG (Fig.3.1A) exclusively whereas CYP11B1 was stained intensely in the ZF (Fig.3.1B). 3β-HSD was found in all cortical zones (Fig. 3.1C). There was no staining detected in the capsule or the medulla by CYP11B2, CYP11B1 and 3β-HSD antibodies, except that some cortical cells were positively stained in the medulla by CYP11B1 and 3β-HSD antibodies.

CYP11B2 and CYP11B1 recognized protein bands in mitochondrial pellets of the rat adrenal capsules and inner cortex with molecular sizes of ~48 kDa (Fig. 3.2A) and ~49 kDa (Fig. 3.2 B), respectively. After the membrane was incubated overnight with CYP11B2 antibody at 1 in 100 dilution, and the CYP11B1 antibody was applied for an hour at 1 in 3500 dilution on the next day, a doublet, which indicated a separation of CYP11B2 and CYP11B1, was seen in mitochondrial fractions from the low salt adrenal (Fig. 3.2C). The capsular fraction of the adrenal also showed a different size of band compared to the decapsulated adrenal cortex (Fig. 3.2C). Compared to the adult adrenals, fetal adrenal glands showed a very weak expression of CYP11B2 (Fig. 3.2D) and relatively high expression of CYP11B1 (Fig. 3.2E).

3.2.2 Organ weights and adrenal morphology changes in non-pregnant and pregnant rats

In non-pregnant rats, there were no significant differences in body weights, heart weights and adrenal weights among the three groups treated with various salt

Table 3. 1 Body weights and organ weights (mean ± S.E.) of non-pregnant female rats treated with various salt diets for 20 days

	High salt (n=7)	Normal salt (n=9)	Low salt (n=6)
Body weight (g)	271.4±9.2	265.3±8.5	263.5±12.9
Heart weight (g)	0.91±0.06	0.91±0.03	0.85±0.02
Heart wt / Body wt %	0.34±0.01	0.34±0.01	0.32±0.01
Kidney weight (g)	1.04 ± 0.05^{b}	0.84 ± 0.03^{a}	0.87 ± 0.04^{a}
Kidney wt / Body wt %	0.38 ± 0.01^{b}	0.31 ± 0.02^{a}	0.33 ± 0.01^{a}
Adrenal weight (mg)	50.64±2.94	45.50±1.83	48.00±2.50
Adrenal weight / Body wt %	0.019±0.002	0.017±0.001	0.018±0.001

n represents the number of the animals tested.

Data were analysed by Tukey's test. Values with the different superscripts are significantly different ($P<0.05$).

Table 3. 2 Body weights and organ weights (mean ± S.E.) of pregnant rats treated with various salt diets throughout pregnancy

	High salt (n=7)	Normal salt (n=7)	Low salt (n=6)
Body weight (g)	320.9±11.3	315.6±16.0	315.0±17.5
Heart weight (g)	0.90±0.03	0.91±0.04	0.95±0.07
Heart wt / Body wt %	0.28±0.01	0.29±0.01	0.30±0.01
Kidney weight (g)	0.87 ± 0.02^{b}	0.74 ± 0.04^{a}	0.76 ± 0.04^{ab}
Kidney wt / Body wt %	0.27 ± 0.01^{b}	0.24 ± 0.0^{a}	0.24 ± 0.01^{a}
Adrenal weight (mg)	49.83±1.94	43.00±2.99	45.42±3.72
Adrenal weight / Body wt %	0.016±0.001	0.014±0.001	0.015±0.001

n represents the number of the animals tested.

Data were analysed by Tukey's test. Values with the different superscripts are significantly different ($P<0.05$).

levels in their diets (Table 3.1). However, kidney weight was significantly increased by high salt dietary treatment compared to normal- and low-salt dietary treatments ($P<0.05$).

Similar results were observed in pregnant rats (Table 3.2). The body weights, heart weights and adrenal weights remained unchanged in pregnant rats. The relative kidney weight was elevated in high-salt-treated rats compared to normal rats. However, the increase was less in pregnant rats than in non-pregnant rats.

Salt treatment had a profound effect on the zonal width of the ZG. In both the non-pregnant (Fig. 3.3A) and the pregnant (Fig. 3.3B) rats, the greatest ZG width was revealed in the low-salt-treated rats. Low salt diet caused a 5.1% increase in the ZG width in non-pregnant rats and a 25.2% increase in pregnant rats compared to the rats treated with normal-salt diet. In contrast, high salt diet markedly decreased the ZG width in non-pregnant rats (26.7%) and in pregnant rats (27.3%) compared to the corresponding rats given normal salt diet.

Low-salt-diet-treated rats also showed a greater cell size of the ZG than those from high- or normal-salt-treated rats in both the non-pregnant (Fig. 3.3C) and pregnant (Fig. 3.3D) rats. Neither pregnancy nor various salt diets affected the ZF cell size. In addition, a coarsely vacuolar appearance was seen in the ZG in the low-salt-treated pregnant rats (Fig. 3.8C arrows), but not in the other animal group (Fig. 3.6A-C and 3.8A-B).

3.2.3 Expression of steroidogenic enzymes in non-pregnant and pregnant rat adrenal glands

3.2.3.1 Immunostaining results for 3β-HSD

In adult rats, 3β-HSD was expressed in all three cortical zones. The staining pattern and intensity for 3β-HSD were not affected by dietary salt (Fig. 3.4A-F), but pregnant rat adrenals had significantly higher 3β-HSD expression ($P<0.05$) than

non-pregnant rat adrenal glands in the ZG (Fig. 3.5A) and the ZF (Fig. 3.5B).

3.2.3.2 Immunostaining results for CYP11B2 and CYP11B1

A few cells were weakly stained in the adrenal ZG of the high salt intake rats (Fig. 3.6A and Fig. 3.8A). Most, but not all, ZG cells showed medium staining in the normal (Fig. 3.6B and Fig. 3.8B) and nearly all cells showed intense staining in the low salt groups (Fig. 3.6C and Fig. 3.8C). In adult adrenal glands isolated from non-pregnant and pregnant rats, a high salt diet significantly reduced ($P<0.002$) the ZG staining for CYP11B2 compared to adrenals from normal or low salt diet groups (Fig. 3.7A and Fig.3.9A).

CYP11B1 was stained throughout the ZF and ZR. The most intense staining was shown in the outer region of the ZF and the staining became weak in the ZR. There were no significant differences in the intensity of staining for CYP11B1 between non-pregnant and pregnant rats (Fig. 3.7B and Fig.3.9B). The staining of CYP11B1 in the ZF also showed no difference among the three dietary groups in non-pregnant (Fig. 3.6D-F and Fig. 3.7B) or pregnant rats (Fig. 3.8D-F and Fig.3.9B).

3.2.3.3 Western blotting results for CYP11B2 and CYP11B1

The expression of CYP11B2 was 5.8-fold and 2.8-fold higher in the adrenals of the low-salt-treated pregnant rats compared to the expression in the adrenals of the high- and normal-salt-treated rats (Fig. 3.10A and B).

The various salt treatments during pregnancy had no effect on the expression levels of CYP11B1 in pregnant rat adrenal glands (Fig. 3.10C and D).

3.2.3.4 Gene expression of CYP11B2 and CYP11B1

The abundances of CYP11B2 (Fig. 3.11A) and CYP11B1 (Fig. 3.11B) mRNA transcripts were determined by a competitive RT-PCR (c-RT-PCR) method. The CYP11B2 primers amplified a 297 bp fragment of the CYP11B2 mRNA. The CYP11B1 primers amplified a 312 bp fragment of the CYP11B1 mRNA. Amplification of the competitors using CYP11B2 and CYP11B1 primers showed no cross reaction between these two primers (Fig. 3.11C).

The levels of CYP11B2 mRNA (Fig. 3.12A) in pregnant rat adrenal glands were significantly elevated (5.4-fold) by low salt treatment compared with values from the normal sodium group (22.76 ± 7.68 attomole/μg RNA *vs* 4.15 ± 0.81 attomole/μg RNA). However, the expression of CYP11B2 mRNA in the adrenal glands of the high salt treated pregnant rats was reduced to barely detectable levels (<0.42 attomole/μg RNA). Even when the amounts of templates were increased to 1μg, CYP11B2 mRNA was often not measureable.

On the other hand, there was no effect on the expression of the adrenal CYP11B1 mRNA (Fig. 3.12B) in pregnant rats on the various dietary salt regimes.

3.2.4 Organ weights in fetal rats

Body weights of fetal rats were not affected by maternal sodium intake (Table 3.3). When organ weights expressed per 100 g body weight were compared, there was no difference observed in the relative heart weights of the fetal rats and no effects of low salt intake on kidney and relative adrenal weights. In contrast, the relative kidney and adrenal weights were significantly reduced by high salt diet ($P<0.05$). The decrease in fetal kidney weights contrasts with the effects on maternal kidney weights (Table 3.1 and 3.2). Various salt diets also had no apparent effect on the litter size and sex of the fetuses (Data not shown).

3.2.5 Plasma aldosterone and corticosterone levels in fetal rats

Plasma aldosterone and corticosterone levels were measured by radioimmunoassay kits according to the manufacturers' instruction (see Chapter 2). (Data were provided by Dr. Christopher Kenyon.) There was a 2.3-fold increase in plasma aldosterone levels of fetal rats given a low salt diet throughout pregnancy compared to the fetal rats given a normal salt diet (1005.0 ± 139.6 pmole/L vs 436.0 ± 46.4 pmole/L) (Fig. 3.13A). In contrast, there was no effect on the plasma aldosterone levels by high sodium diet (520.9 ± 69.5 pmole/L).

There was no obvious effect on the plasma corticosterone levels after various dietary sodium treatments throughout pregnancy, except modestly reduced levels in the high sodium group (High salt, $1.89 \times 10^5 \pm 1.70 \times 10^4$ pmole/L; normal salt, $2.26 \times 10^5 \pm 1.25 \times 10^4$ pmole/L; low salt, $2.18 \times 10^5 \pm 1.64 \times 10^4$ pmole/L) (Fig. 3.13B).

Table 3. 3 Body weights and organ weights (mean ± S.E.) of E20 rats prenatally treated with various salt diets throughout pregnancy

	High salt (n=7)	Normal salt (n=8)	Low salt (n=8)
Body weight (g)	3.963±0.037	4.081±0.049	3.976±0.035
Heart weight (mg)	20.02±0.27^{b}	21.11±0.29^{a}	20.68±0.28^{ab}
Heart wt / Body wt %	0.506±0.005	0.520±0.007	0.525±0.008
Kidney weight (mg)	16.24±0.17^{b}	17.89±0.30^{a}	17.45±0.17^{a}
Kidney wt / Body wt %	0.411±0.003^{b}	0.438±0.004^{a}	0.441±0.004^{a}
Adrenal weight (mg)	1.37±0.02^{b}	1.51±0.03^{a}	1.42±0.03^{b}
Adrenal weight / Body wt %	0.0348±0.001^{b}	0.037±0.001^{a}	0.036±0.00^{ab}

n represent the number of dam animals studied

Data were analysed by Tukey's test. Values with the different superscripts are significantly different ($P<0.05$).

3.2.6 Steroidogenic enzyme expression in fetal rat adrenal glands

3.2.6.1 Immunostaining results for 3β-HSD

The staining pattern for 3β-HSD in the fetal adrenals was similar to that in adult adrenals (Fig. 3.14A-C). However, the ZG showed weak staining for 3β-HSD in the fetal adrenals (Fig. 3.15A). The staining was mainly seen in the inner regions of cortex and was not affected by various maternal dietary salt intakes (Fig. 3.14A-C and Fig. 3.15B).

3.2.6.2 Immunostaining results for CYP11B2 and CYP11B1

The staining for CYP11B2 in fetal adrenal ZG was much weaker than in adult adrenals. In fetal adrenal glands, the ZG cells with closely packed nuclei were able to be distinguished from the ZF cells. However, only some of the cells in the outer region of the ZG were positive for CYP11B2 (Fig. 3.16A-C). CYP11B2 was nearly undetectable in the fetal adrenals of the high salt group. The staining intensity of CYP11B2 was significantly increased ($P<0.005$) in the low salt group compared with the normal and high salt groups (Fig. 3.17A).

The CYP11B1 staining found in the inner regions of fetal adrenal glands appeared to increase progressively from the high salt group to the normal salt to the low salt group although the differences were not significant (Fig. 3.16D-F and Fig. 3.17B).

3.2.6.3 Western blotting results for CYP11B2 and CYP11B1

The levels of adrenal CYP11B2 and CYP11B1 in fetal rats were analysed by immunoblotting. The expression levels of CYP11B2 were not detected in the fetal adrenal protein extracts as clearly as in the maternal adrenal extracts. This was confirmed using the CYP11B2 activity assay.

There were no obvious changes of the expression levels for either CYP11B2 (Fig.

3.18A and B) or CYP11B1 (Fig. 3.18C and D) among the groups treated with various dietary salt regimens by image analysis of the SDS-PAGE gel results. However, CYP11B2 did appear to be greater in adrenal tissues from both high and low sodium groups compared with normal diet group.

3.2.6.4 Gene expression of CYP11B2 and CYP11B1

The mRNA transcripts for CYP11B2 (Fig. 3.19A) in the adrenals of E20 rats were not changed by dietary treatments. The mRNA concentrations of CYP11B2 were lower in fetal adrenals of the normal and low salt groups than in their maternal adrenals (Fig. 3.12A). However, the mRNA expression of CYP11B2 was much higher in the fetal adrenals of the high salt group compared to that in the maternal adrenals (Fetal adrenal, 2.18±0.81 attomole/μg RNA; maternal adrenal, <0.42 attomole/μg RNA).

Dietary treatments had no effect on the mRNA transcripts of the CYP11B1 (Fig. 3.19B) in the adrenals of E20 rats. In addition, the transcript levels of CYP11B1 in fetal adrenals were similar to those in the maternal adrenals (Fig. 3.12B).

3.3 Discussion

Previous studies have shown that a salt-restricted diet induces hypertrophy and hyperplasia of the ZG of the adrenal cortex in both non-pregnant and pregnant rats (Pohanka and Pike, 1970). Ultrastructural changes including electron-dense inclusions in the mitochondria of the ZG cells, autophagic vacuoles and large cytoplasmic vacuoles reveal evidence of cellular degradation when rats receive either a low salt diet or have the stress of pregnancy. The combination of sodium restriction and pregnancy also results in the exhaustion of the ZG cells and the depletion of lipid droplets in the cells (Pohanka and Pike, 1970; Smiciklas et al., 1971). In this study, we also demonstrated that the low salt diet gives rise to hypertrophy of the ZG cells and

the expansion of ZG width. However, the adrenal glands of non-pregnant rats had no noticeable morphological differences from those of pregnant rats with the exception of the coarsely vacuolar appearance seen in the ZG in the low-salt-treated pregnant rats which is in agreement with a previous study (Pike and Gursky, 1970).

Body weights and organ weights were not changed in either non-pregnant or pregnant rats given a low salt diet. In this study, the low sodium level (0.03% of sodium or equal to 0.07% of sodium chloride) may be not as extreme as in some earlier experiments (0.03% of sodium chloride). Therefore, the low sodium diet does not affect maternal growth, litter size and pup weights. In contrast, renal growth was observed in high-salt-treated rats. Recently, Ying et al. (1998) demonstrated that a high level of dietary sodium caused renal growth in rats as a result of the modulation of transforming growth factor-β expression.

Elevated plasma aldosterone is proposed to be associated with increased plasma volume and sodium retention in rats during pregnancy (Schneider and Mulrow, 1973; Garland et al. 1987; Brochu et al., 1996). As a result, changes of adrenal steroidogenesis may play a key role in pregnant rats. Recently, Brochu et al. (1997) demonstrated that the augmented activity and mRNA levels of CYP11B2 in the ZG are responsible for the elevated plasma aldosterone levels during pregnancy. However, few comparisons of steroidogenic enzyme expression have been made between pregnant and non-pregnant rats. In this study, no significant differences of the staining intensity of CYP11B2 and CYP11B1 were observed between pregnant and non-pregnant rats. In both pregnant and non-pregnant rat adrenal glands, dietary sodium restriction caused an increase in CYP11B2 and had no effect on CYP11B1 assessed by immunohistochemistry. According to reports of others (Malee and Mellon, 1991; Brochu et al., 1998), CYP11B2 gene transcripts are regulated in the adrenals of the pregnant rats in a similar fashion to that in the non-pregnant rat adrenal although they have demonstrated a greater response to low salt treatment in pregnant adrenal glands when compared to non-pregnant adrenal glands.

On the contrary, a high salt diet appears to regulate the expression of CYP11B2 in

the opposite way. The expression of CYP11B2 mRNA in the adrenal glands of the high salt treated pregnant rats was dramatically reduced to barely detectable levels. The protein expression of CYP11B2 only reached half the expression level of the CYP11B2 in normal salt group. The expression of CYP11B2 was 5.8-fold lower in the adrenals of the high salt treated pregnant rats compared to the expression in the adrenals of the low salt treated rats. On the other hand, there was no effect on the expression of the adrenal CYP11B1 mRNA in the various dietary salt regimes in pregnant rats. This suggests that there is a profound effect on the expression of CYP11B2 and the corresponding gene, *CYP11B2*, in rats fed with high salt diet throughout pregnancy.

On the other hand, the staining pattern and intensity for 3β-HSD were not affected by dietary salt. However, the staining of 3β-HSD in both the ZG and the ZF cells was more intense in the pregnant rat adrenals than in the non-pregnant rat adrenals. Because 3β-HSD converts pregnenolone into progesterone, this implies that the progesterone synthesis is elevated during pregnancy. Progesterone is a vital hormone to maintain pregnancy. Whether the elevation of 3β-HSD is to sustain the pregnancy or to provide more substrates for adrenal steroidogenesis requires further investigation.

In fetal rat adrenals, it was expected that various dietary salt treatments in maternal rats might affect the regulation of aldosterone synthesis in a similar manner to that seen in adult rat adrenals. Low salt diet increases the plasma aldosterone levels and CYP11B2 expression levels compared to the normal salt diet in fetal rats similar to that observed in pregnant rats. However, competitive RT-PCR results did not show the same response to the dietary treatment. There are several possible explanations. Firstly, levels of CYP11B2 transcripts in fetal adrenals are relatively low compared to that observed in the adult adrenals though low salt diet amplifies the expression level in the fetal adrenal. Secondly, the results indicate that the diets may have no effect on CYP11B2 gene expression. This is in agreement with a previous study (Mellon et al., 1995), which suggested that the fetal adrenal might be transcriptionally quiescent *in*

vivo to dietary treatments. Thirdly, the elevated plasma aldosterone may be contributed from extra-adrenal sources. Although there is no direct evidence showing that maternal aldosterone can cross placenta, it has been shown that fetal injection of labelled progesterone produced small quantities of labelled aldosterone in the circulation of acutely adrenolectomised mothers (Milković et al., 1976; Klepac, 1979). Churchill et al. (1981) also demonstrated that significant levels of aldosterone were found in the blood of adrenalectomised pregnant rats whereas no dectectable aldosterone was present in the non-pregnant adrenalectomised female rats. Aldosterone can also be produced locally in the rat brain (Gomez-Sanchez et al., 1997; MacKenzie et al., 2000), heart (Silvestre et al., 1998) and blood vessels tissues (Takeda et al., 1996) through local RAS systems. Whether these tissues in fetal rats are capable of steroid production needs additional investigations. Furthermore, the elevated plasma aldosterone level may indicate an increased stability of aldosterone or a decreased metabolism of aldosterone. These possibilities cannot be excluded at present.

Especially noteworthy is that high salt diet may have different regulation of CYP11B2 and its gene in fetal rat adrenals. The levels of CYP11B2 mRNA transcripts were much higher in the fetal adrenals of the high salt group compared to the expression of CYP11B2 gene transcripts in the maternal adrenals that were markedly suppressed by high salt diet. CYP11B2 also appeared to be greater in adrenal tissues in the high sodium group compared with the normal diet group. Moreover, plasma aldosterone levels in fetal rats were maintained to similar levels as in fetal rats following maternal normal salt treatment. Consistent with previous results (Khokhar and Pike, 1973; Churchill et al., 1981; Wotus et al., 1998), the data provide direct evidence that CYP11B2 and its transcripts start to function in E20 fetal adrenal glands and are able to produce aldosterone. In addition, high salt diet may be not sufficient to suppress aldosterone secretion or CYP11B2 gene expression in fetal rat adrenals.

Furthermore, maternal dietary treatments had no effects on the plasma corticosterone levels and the expression of CYP11B1 and the corresponding CYP11B1 transcripts in the adrenals of E20 rats. However, it is of interest that the fetal adrenals have similar expression levels of CYP11B1 and its transcripts to the maternal adrenals. The present findings clearly support early studies (Dupouy et al., 1975; Dalle et al., 1978) that steroidogenesis, especially corticosterone biosynthesis, in fetal adrenal glands is highly active at the end of gestation.

The staining of 3β-HSD in fetal adrenal gland was not affected by the various maternal dietary salt intakes. The localisation was mainly in the inner cortex, including the ZF and the ZR with similar staining intensity. The ZG only showed very weak staining. The data are consistent with previous studies, which suggest that the low expression of 3β-HSD in the ZG of the fetal adrenal may be related to the low plasma aldosterone (Pignatelli et al., 1998) and angiotensin II (Dupont et al., 1991) levels in the newborn or fetal rats.

In conclusion, this is the first attempt to investigate the effects of high (3%), normal (0.3%) and low (0.03%) dietary sodium content on CYP11B2, CYP11B1 and 3β-HSD expression in adrenal glands obtained from pregnant rats and their 20-day old fetuses using various techniques. The results suggest that dietary sodium restriction may activate the RAS system that induces the expression of CYP11B2 in fetal adrenal glands. Whether the control is exerted by changes of maternal or the fetal RAS system is not clear although others have demonstrated that renin is expressed in the kidney of fetal lambs (Iwamoto and Rudolph, 1981; Siegel, 1981) and also in the adrenal tissue of the fetal mouse (Zemel et al., 1989; Jones et al., 1990). Whether the development of the fetal and pregnant rat adrenal gland are subject to the same regulatory processes as the normal adult female adrenal gland requires further investigation. The possible role of the local RAS systems in pregnant and fetal rat adrenals will be discussed in a later chapter.

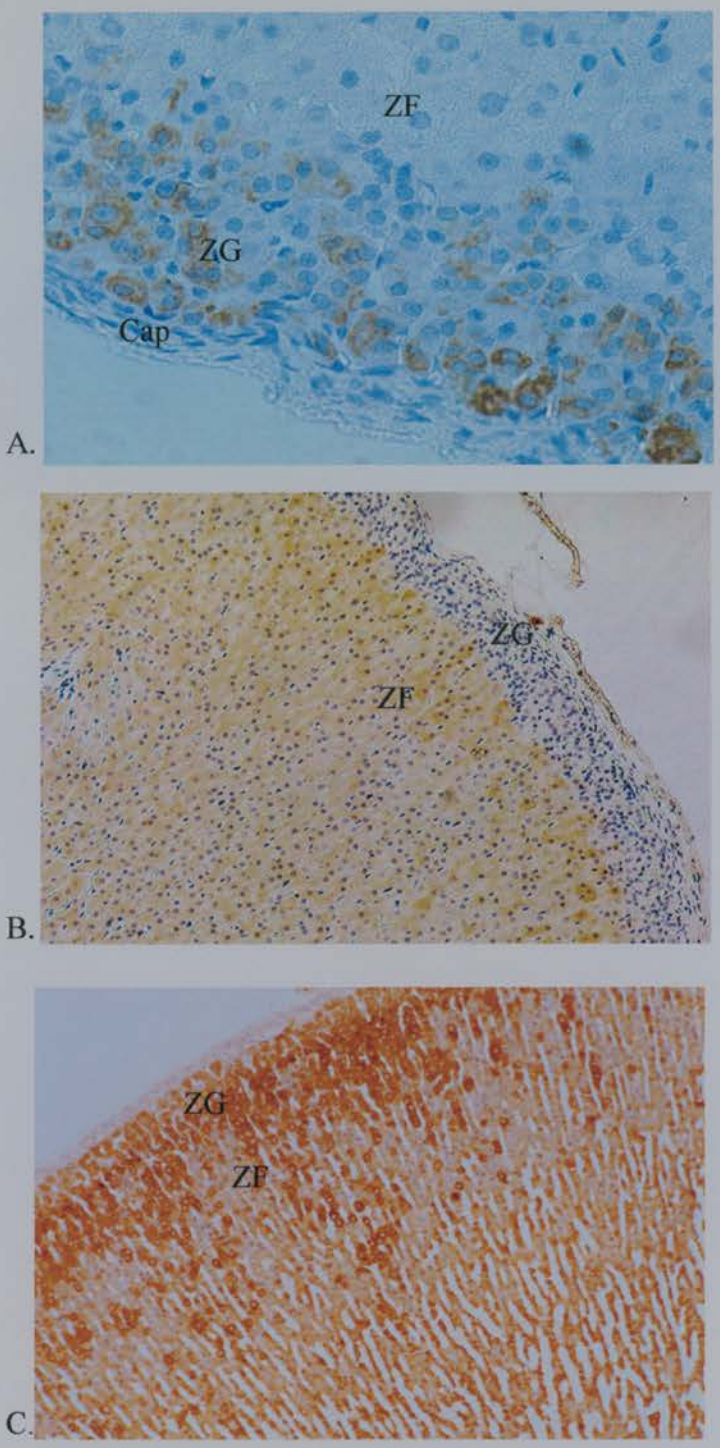


Fig. 3.1 Localisation of CYP11B2 (A), CYP11B1 (B) and 3β-HSD (C) antibodies in rat adrenal gland by immunohistochemical staining. The brown colour shows the positive staining. Nuclei (blue colour) were stained by haematoxylin. A, CYP11B2 monoclonal antibody localised in the ZG, exclusively (magnification, x400). B, CYP11B1 monoclonal antibody localised in the ZF (magnification, x100). C, 3β-HSD polyclonal antibody localised in all adrenal cortex (magnification, x100). Cap, ZG and ZF denote adrenal capsule, zona glomerulosa and zona fasciculata, respectively.

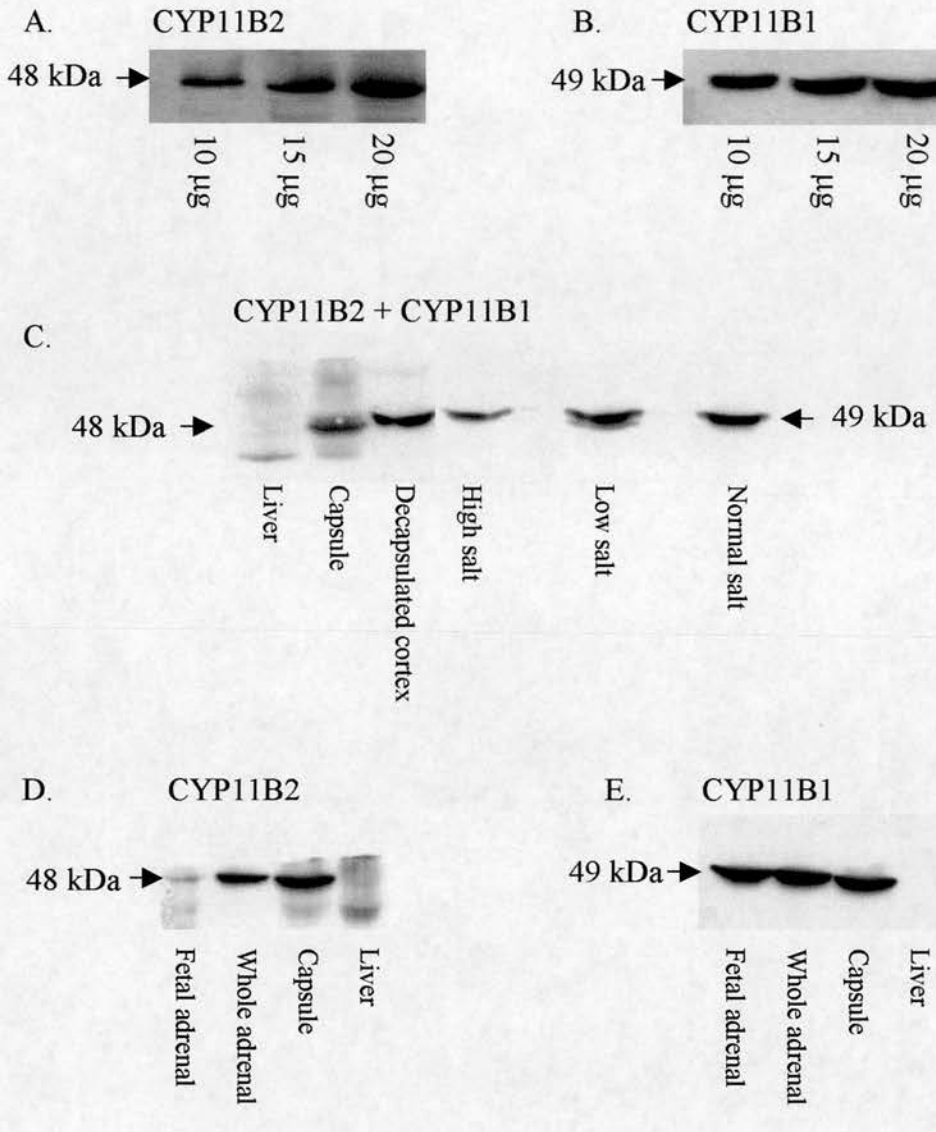
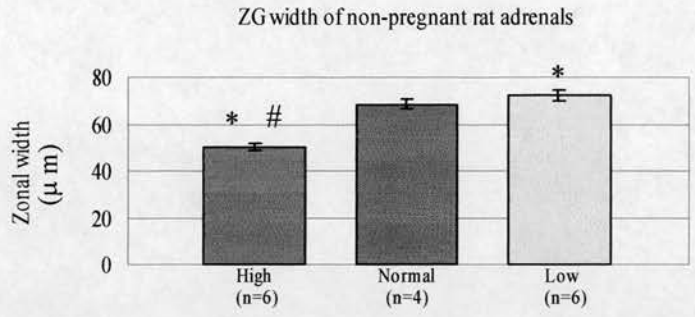
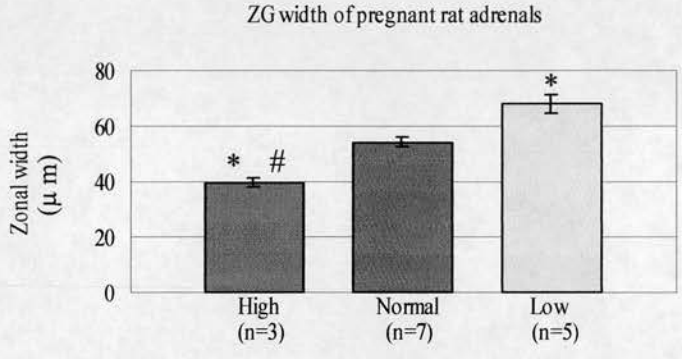


Fig. 3.2 Characterisation of CYP11B2 and CYP11B1 antibodies. A, CYP11B2 monoclonal antibody optimisation with various amounts of mitochondrial protein from adrenal capsules. B, CYP11B1 monoclonal antibody optimisation with various amounts of mitochondrial protein from decapsulated adrenal cortex . C, A membrane was incubated with both CYP11B2 and CYP11B1 antibodies. Mitochondrial pellets of adrenal samples were extracted from different salt treated pregnant rats. Mitochondrial pellets of the decapsulated or capsule portions and the liver were extracted from normal rats. D, Mitochondrial pellets from the adult adrenal capsules, whole adrenals, fetal adrenals and the liver were incubated with the CYP11B2 monoclonal antibody. E, Reblot of membrane shown in D with CYP11B1 monoclonal antibody. Each mitochondrial extract consisted of 20 µg protein/lane.

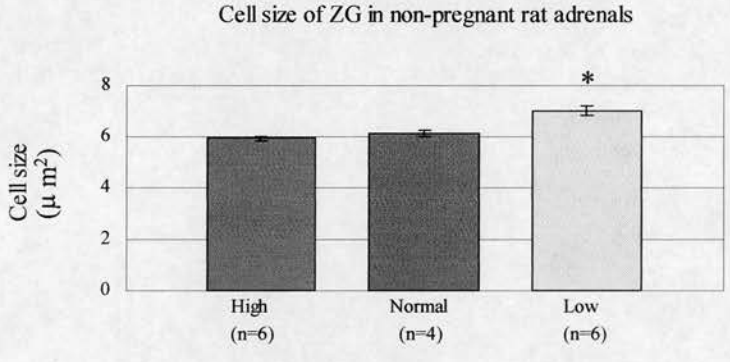
A.



B.



C.



D.

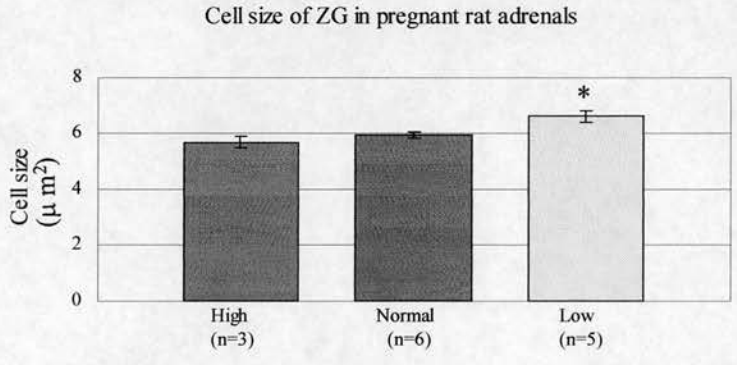


Fig. 3.3 Zonal widths (A and B) and cell size (C and D) of the zona glomerulosa in rat adrenal glands obtained from non-pregnant (A and C) and pregnant (B and D) rats treated with various salt diets for 20 days and throughout pregnancy, respectively. Zonal widths and cell size were measured at magnification of x400 and x100, respectively. Values are mean ± S.E. n represents the number of the animals tested. Data were analysed by the Tukey's test. * $P<0.05$ compared to normal-salt-diet group. # $P<0.05$ compared to low-salt-diet group.

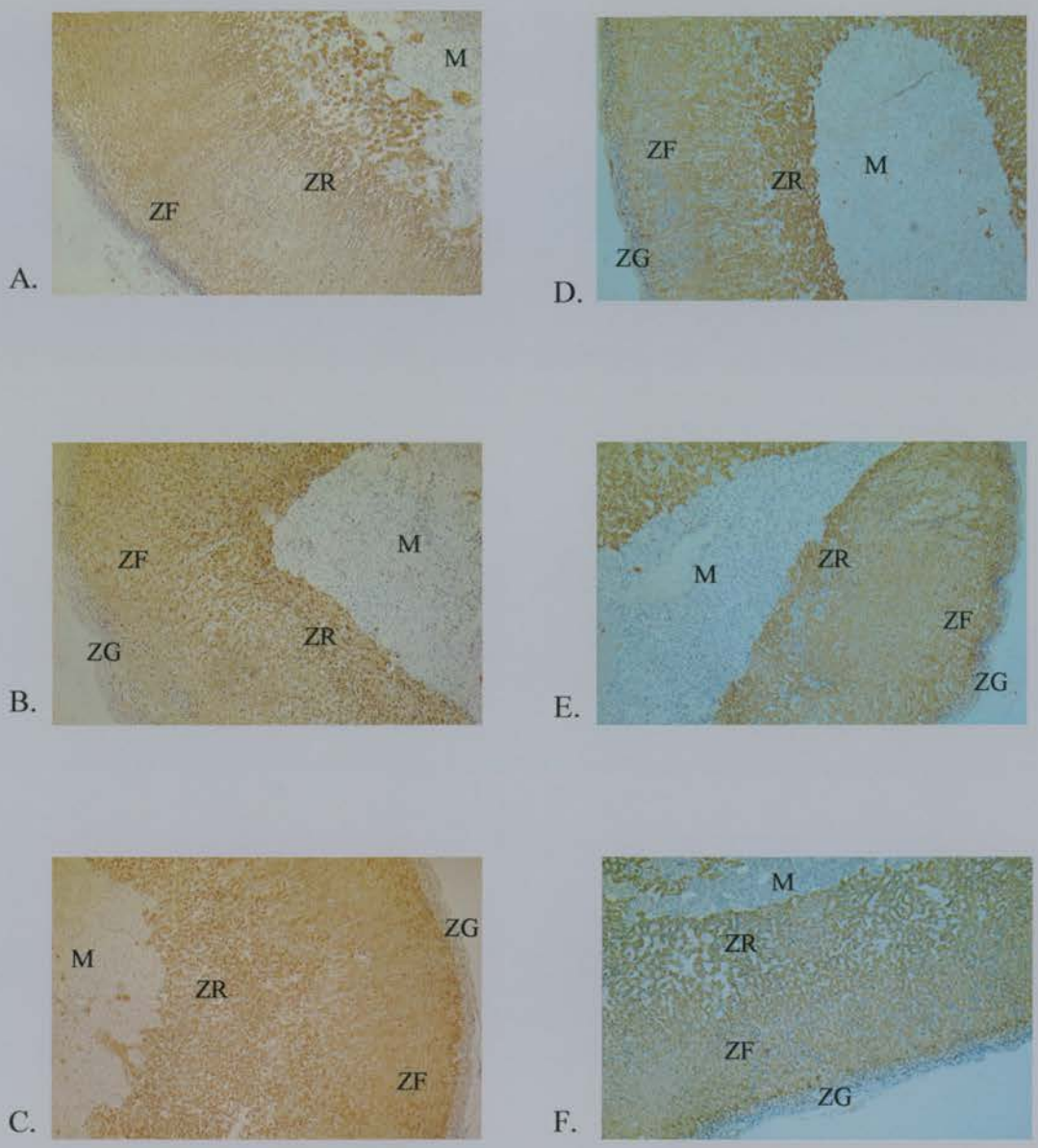
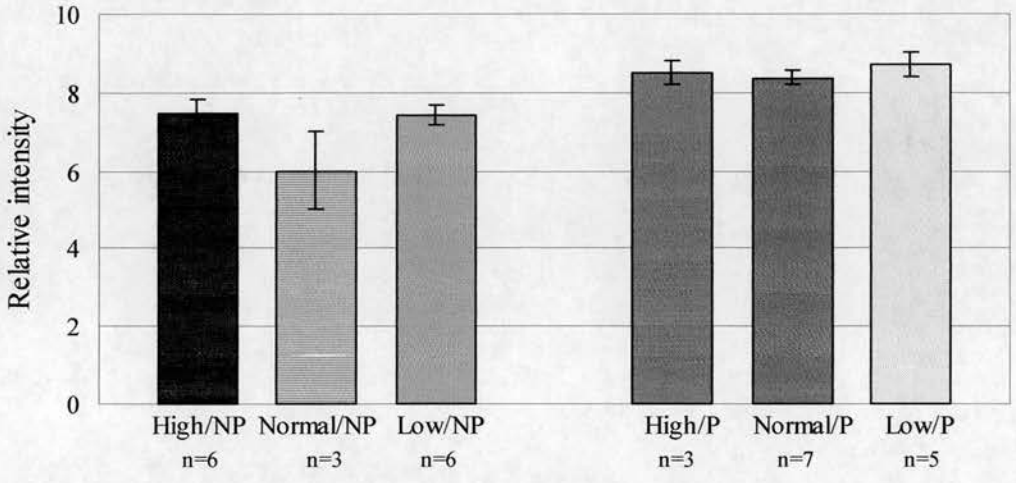


Fig. 3.4 Immunostaining results for 3β-HSD in rat adrenal glands obtained from non-pregnant (A-C) or pregnant (D-F) rats treated with various salt diets for 20 days or throughout pregnancy, respectively. A and D, Adrenal sections from rats fed a high salt diet. B and E, Adrenal sections from rats fed a normal salt diet. C and F, Adrenal sections from rats fed a low salt diet. ZG, ZF, ZR and M denote zona glomerulosa, zona fasciculata, zona reticularis and medulla, respectively. Magnification, x40.

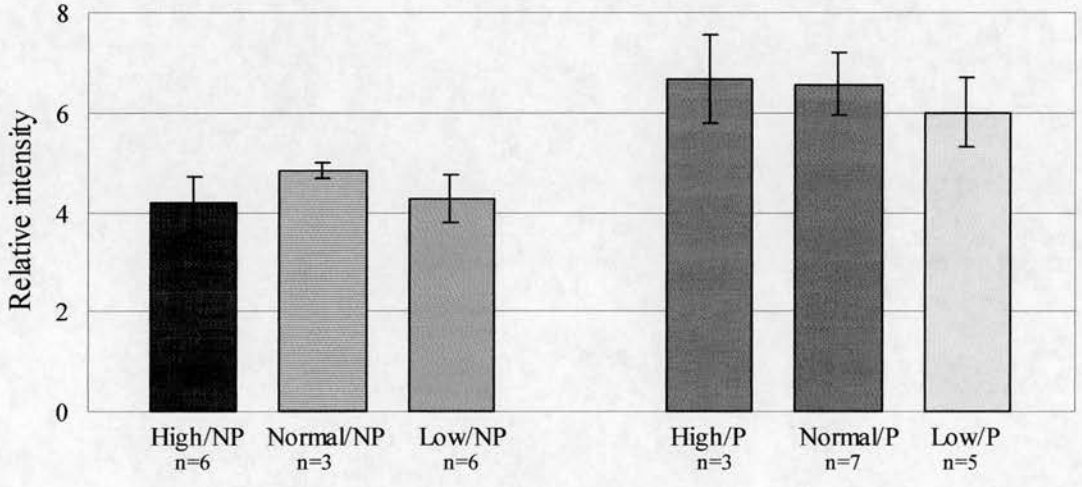
A. Immunostaining results for 3β-HSD in the ZG of adrenals in rats treated with various salt diets



(Various salt diets: P>0.05;

Non-pregnant *vs.* pregnant : P<0.05)

B. Immunostaining results for 3β-HSD in the ZF of adrenals in rats treated with various salt diets



(Various salt diets: P>0.05;

Non-pregnant *vs.* pregnant : P<0.05)

Fig. 3.5 Immunostaining results for 3β-HSD in rat adrenal glands obtained from non-pregnant (NP) or pregnant (P) rats treated with various salt diets for 20 days or throughout pregnancy, respectively. ZG and ZF denote adrenal zona glomerulosa and zona fasciculata, respectively. Values are mean ± S.E. n represents the number of animals studied. Data were analysed by two-way ANOVA and the Tukey's test.

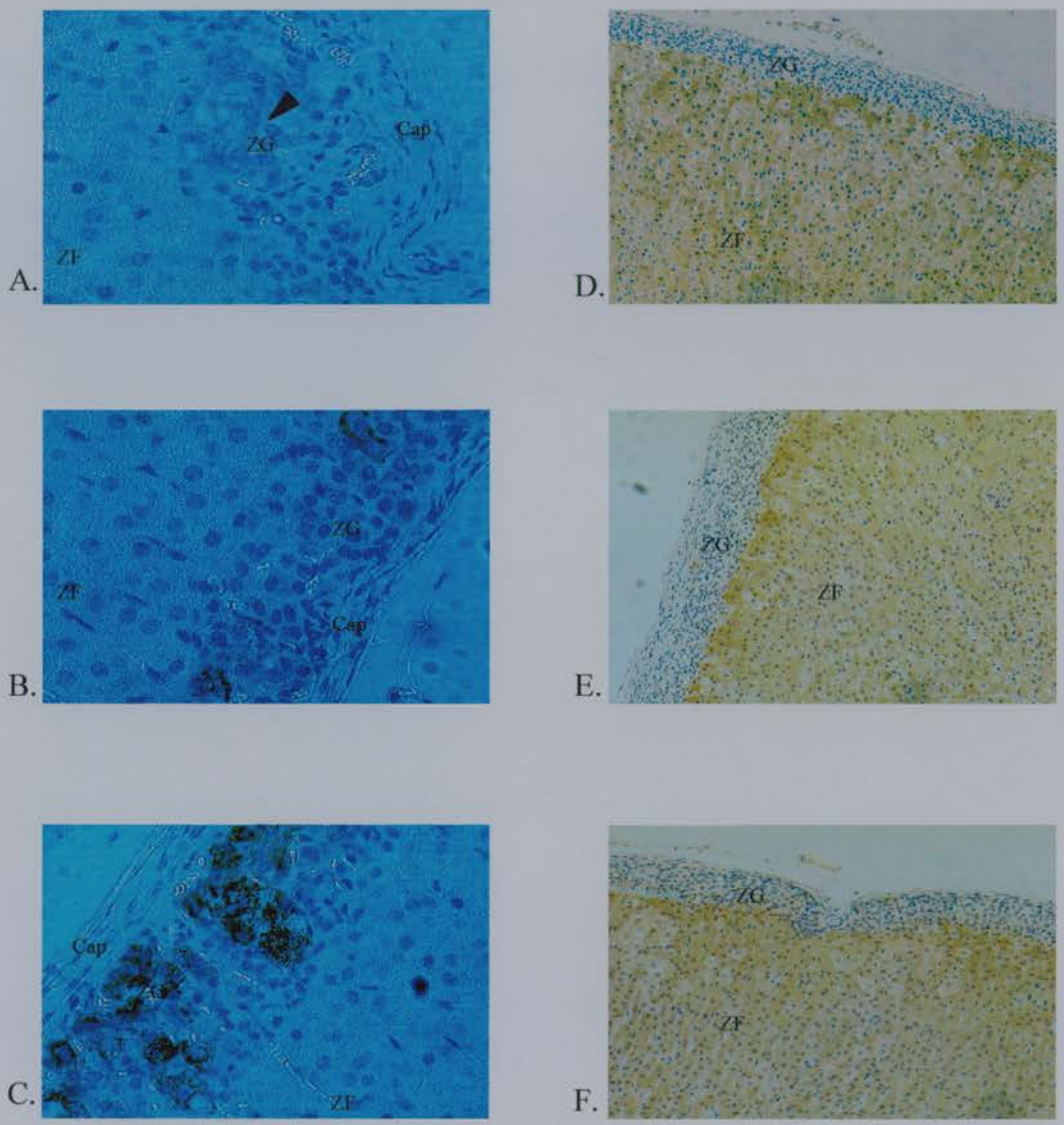
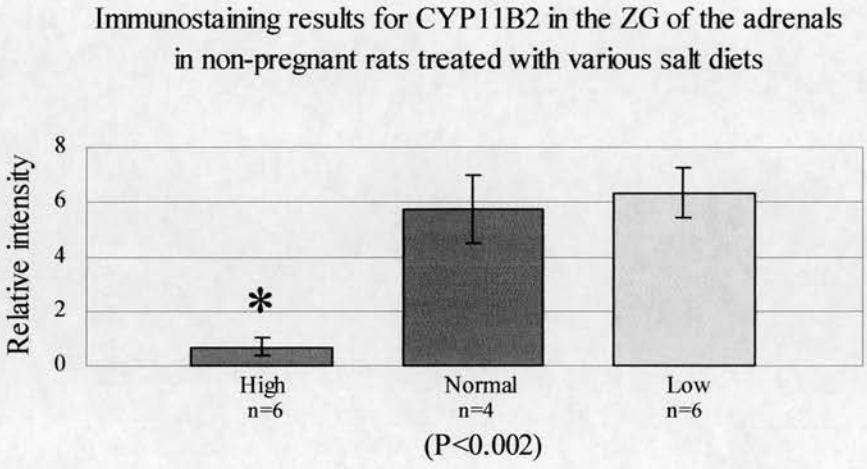


Fig. 3.6 Immunostaining results for CYP11B2 (A-C) and CYP11B1 (D-F) in rat adrenal glands obtained from non-pregnant rats treated with various salt diets for 20 days. A and D, Adrenal sections from rats fed a high salt diet. B and E, Adrenal sections from rats fed a normal salt diet. C and F, Adrenal sections from rats fed a low salt diet. Cap, ZG and ZF denote adrenal capsule, zona glomerulosa and zona fasciculata, respectively. A-C, Magnification, x400. D-F, Magnification, x100. Arrow head shows weak CYP11B2 staining in high-salt-treated adrenal section.

A.



B.

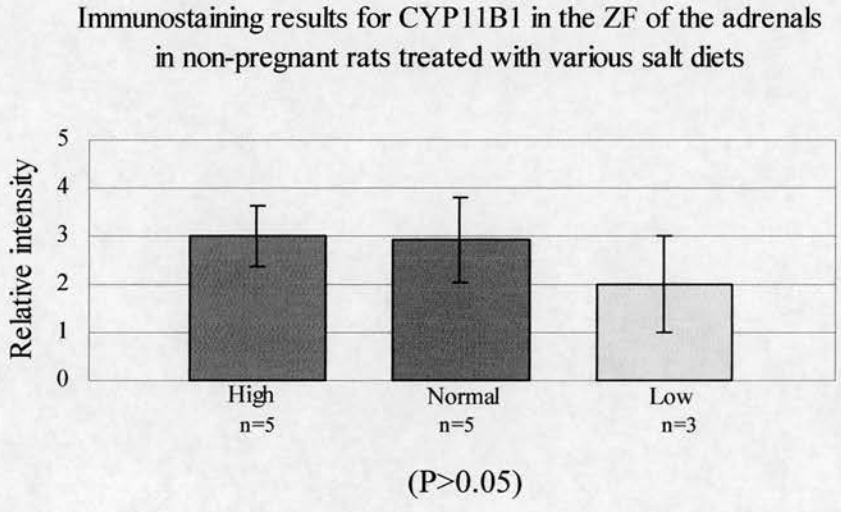


Fig. 3.7 Immunostaining results for CYP11B2 (A) and CYP11B1 (B) in rat adrenal glands obtained from non-pregnant rats treated with various salt diets for 20 days. ZG and ZF denote adrenal zona glomerulosa and zona fasciculata, respectively. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test. * $P<0.002$ compared to normal-salt-diet and low-salt-diet groups.

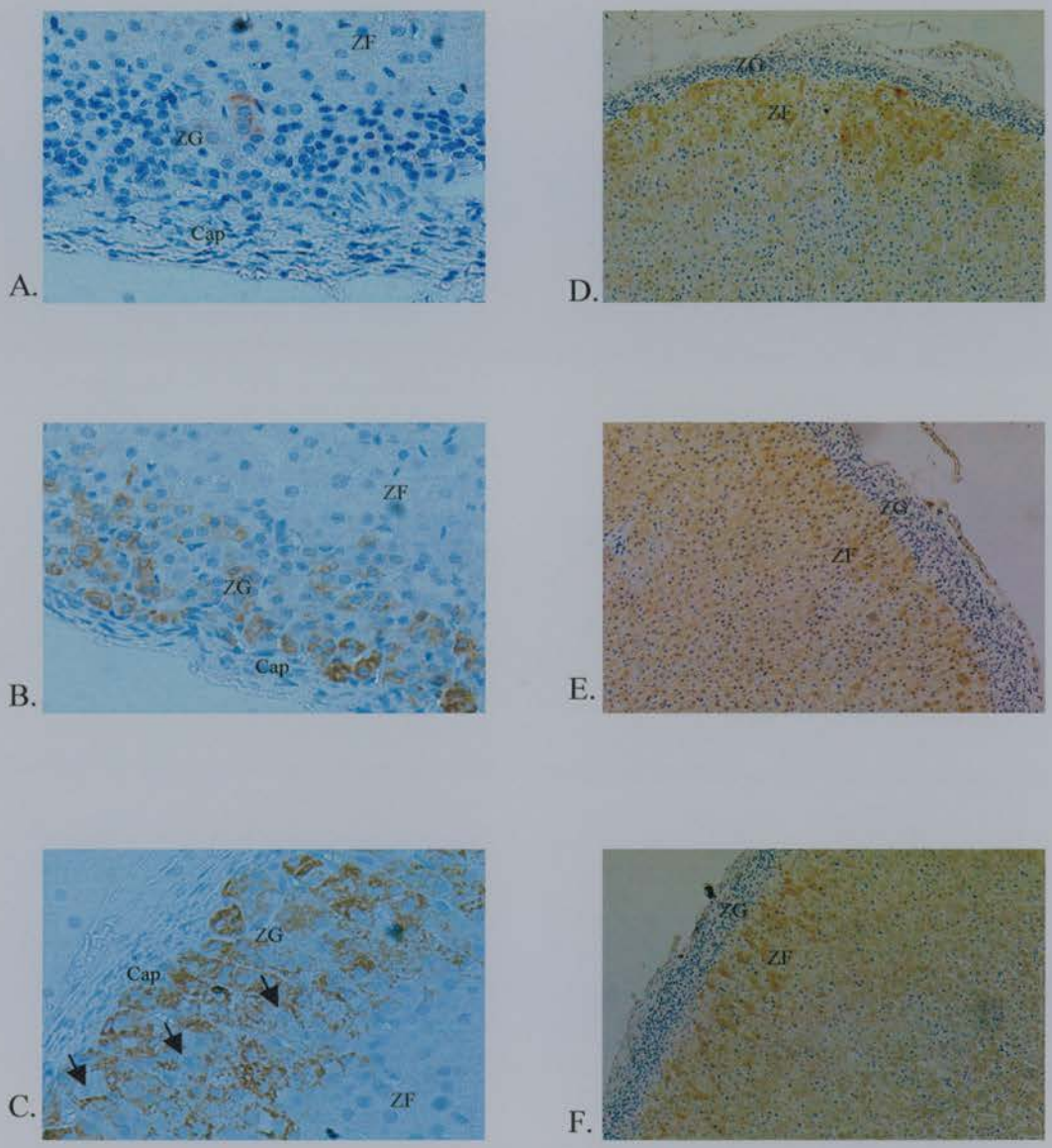
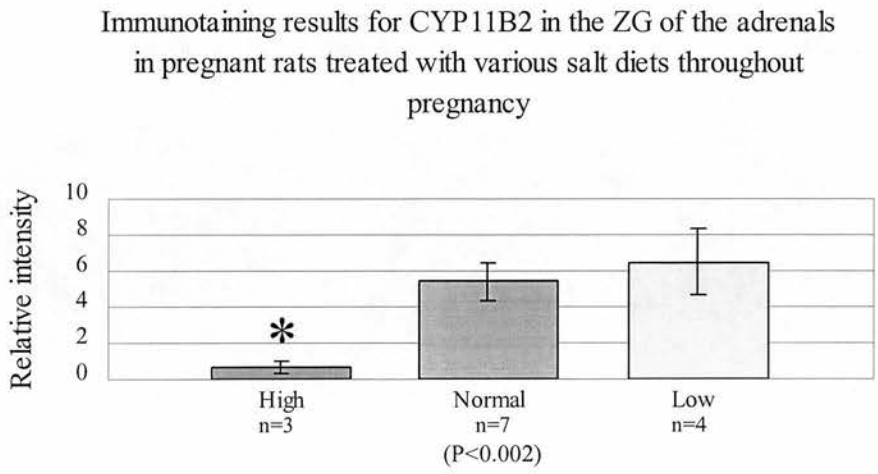


Fig. 3.8 Immunostaining results for CYP11B2 (A-C) and CYP11B1 (D-F) in rat adrenal glands obtained from pregnant rats treated with various salt diets for 20 days. A and D, Adrenal sections from rats fed a high-salt-diet. B and E, Adrenal sections from rats fed a normal-salt-diet. C and F, Adrenal sections from rats fed a low-salt-diet. Cap, ZG and ZF denote adrenal capsule, zona glomerulosa and zona fasciculata, respectively. A-C, Magnification, x400. D-F, Magnification, x100. Arrows show coarsely vacuolars in low salt pregnant adrenal section.

A.



B.

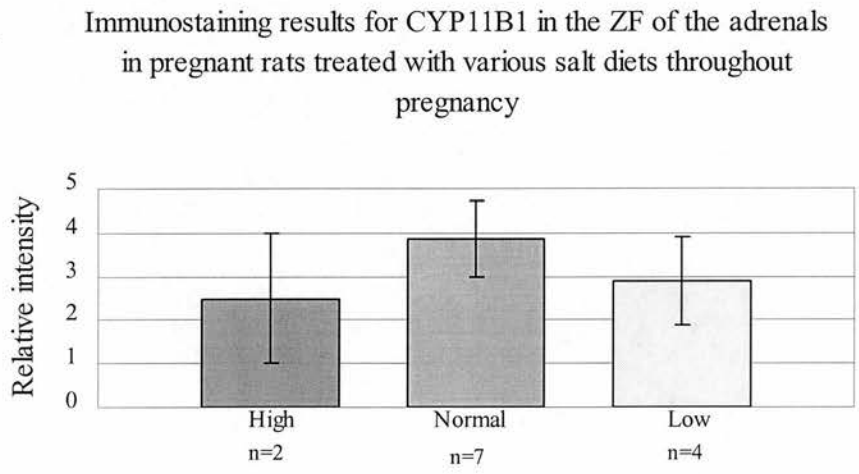


Fig. 3.9 Immunostaining results for CYP11B2 (A) and CYP11B1 (B) in rat adrenal glands obtained from pregnant rats treated with various salt diets for 20 days. In Fig. (A), data were analysed by Tucky's test. * $P<0.002$ compared to normal-salt-diet and low-salt-diet groups. Values are mean ± S.E. n represents the number of animals tested. In Fig (B), data are expressed as mean ± S.E. except that the data in high-salt-diet group (n=2) represent the range of the two determinations. ZG and ZF denote adrenal zona glomerulosa and zona fasciculata, respectively.

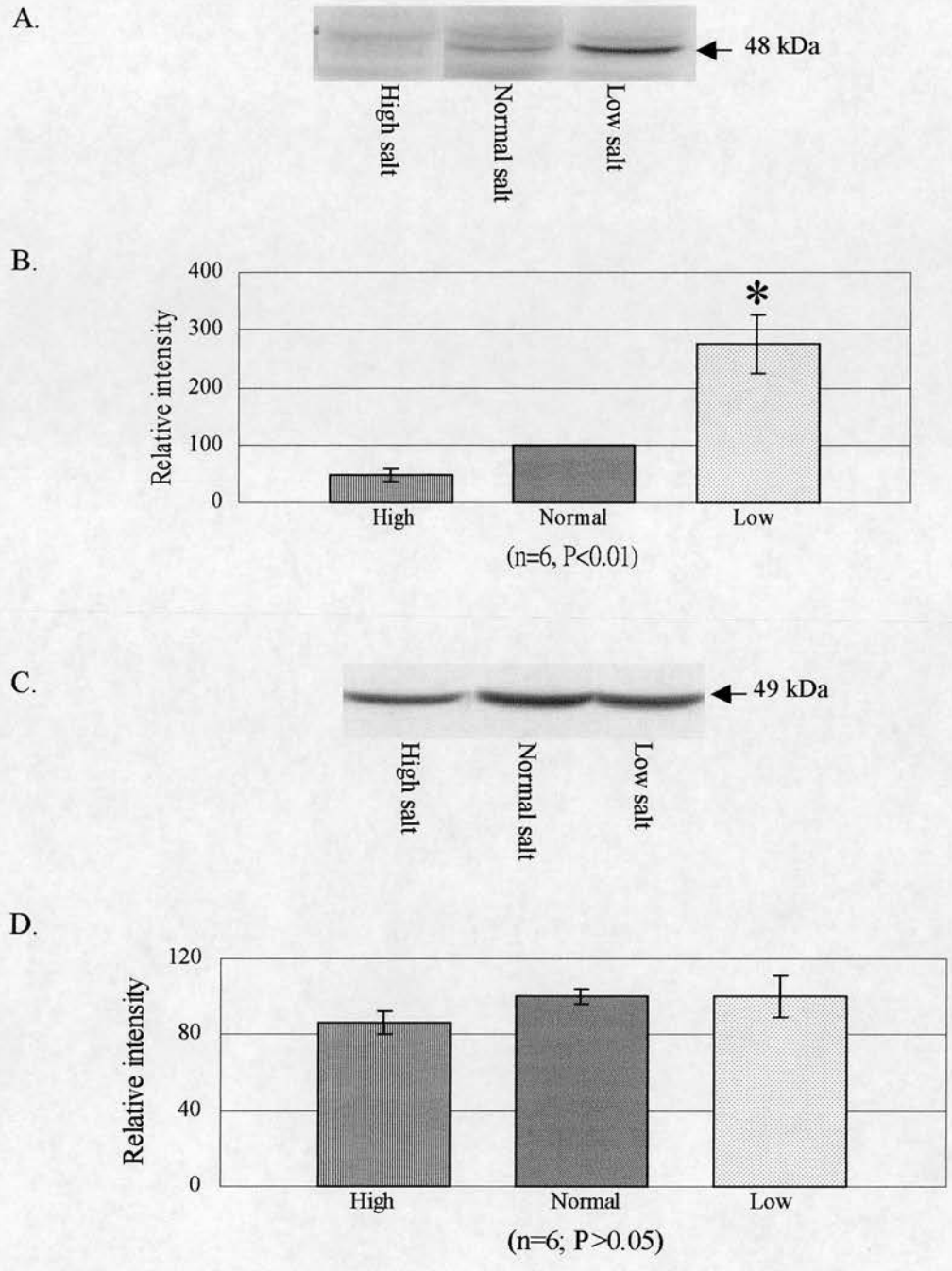
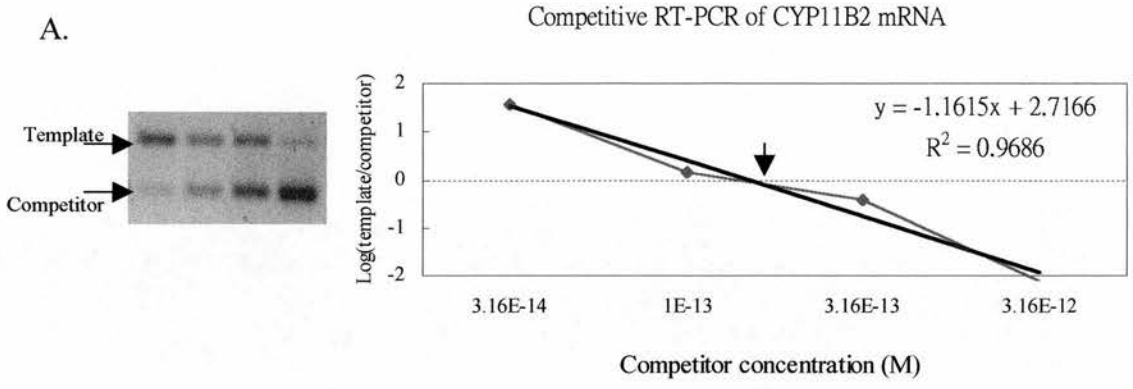
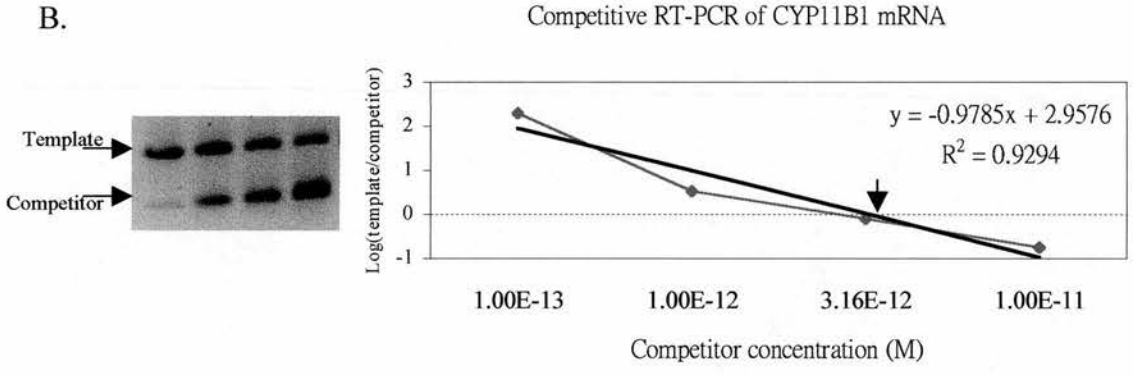


Fig. 3.10 Western blotting results for CYP11B2 (A and B) and CYP11B1 (C and D) of adrenal glands in pregnant rats treated with various salt diets throughout pregnancy. A and C, 10% SDS-PAGE gel results. B and D, Relative intensity of the proteins quantified from the gels. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test. * $P<0.01$ compared to normal-salt-diet and high-salt-diet groups.

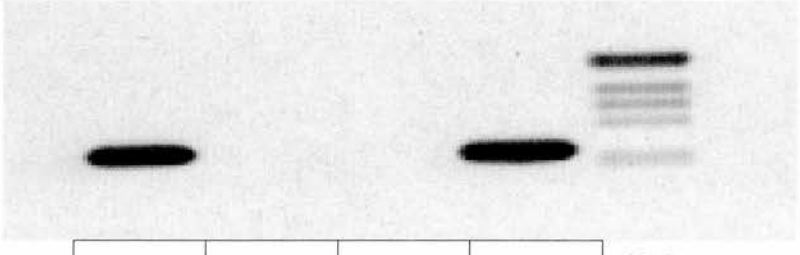
A.



B.



C.



Primer	CYP11B2	CYP11B1	CYP11B2	CYP11B1	Marker
Competitor	CYP11B2	CYP11B2	CYP11B1	CYP11B1	

Fig. 3.11 Expression of CYP11B2 (A) and CYP11B1 (B) mRNA using competitive RT-PCR in rat adrenal glands obtained from pregnant rats. There was no cross reaction between CYP11B2 and CYP11B1 primers (C). Three hundred nanograms and 5 nanograms of RNA templates (300ng and 5ng) were used for CYP11B2 and CYP11B1 analyses, respectively.

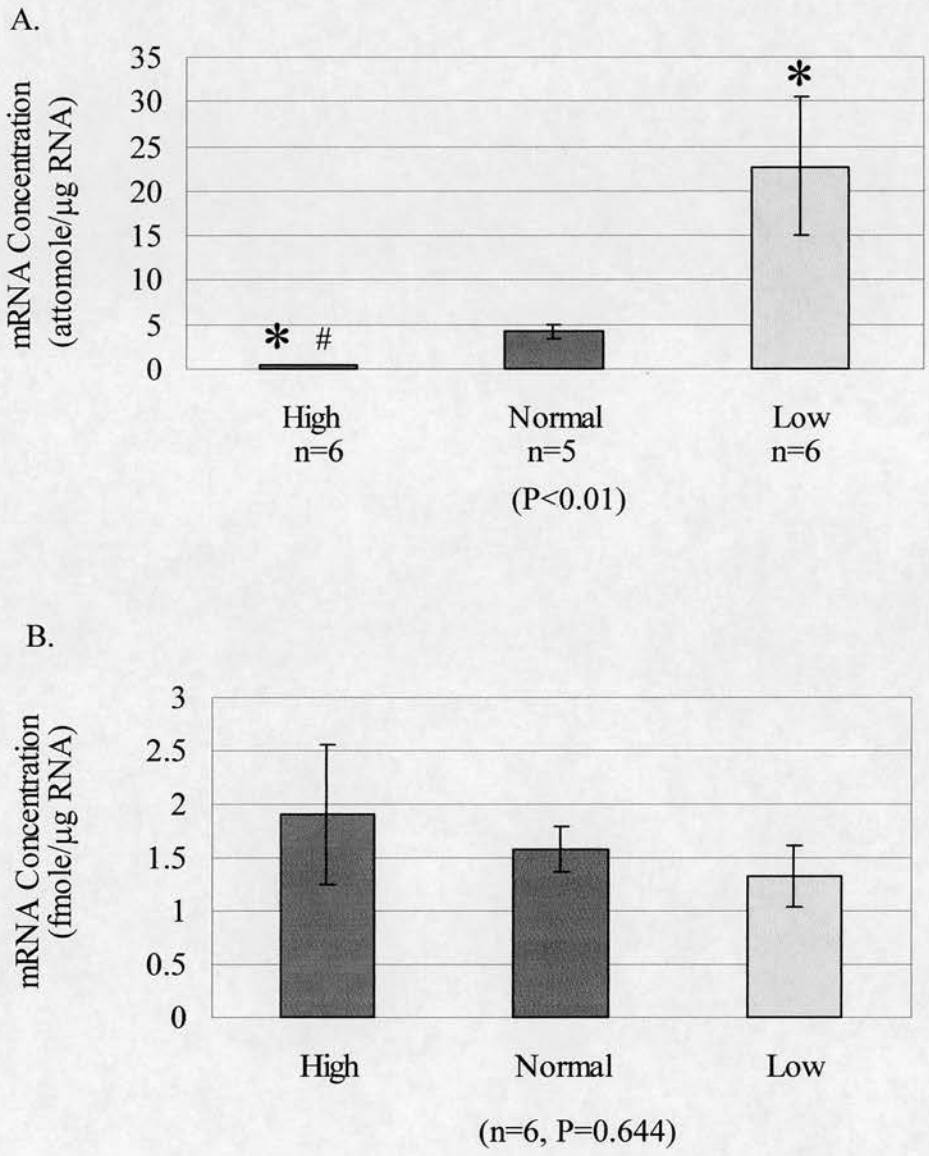
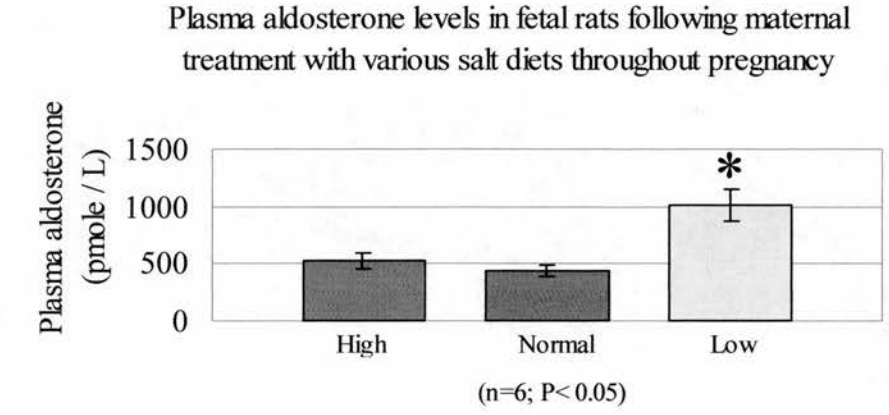


Fig. 3.12 Expression of CYP11B2 (A) and CYP11B1 (B) mRNA using competitive RT-PCR in rat adrenal glands obtained from pregnant rats treated with various salt diets throughout pregnancy. Three hundred nanograms and 5 nanograms of RNA templates (300ng and 5ng) were used for CYP11B2 and CYP11B1, respectively. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test. * $P<0.01$ compared to normal-salt-diet group. # $P<0.05$ compared to low-salt-diet group.

A.



B.

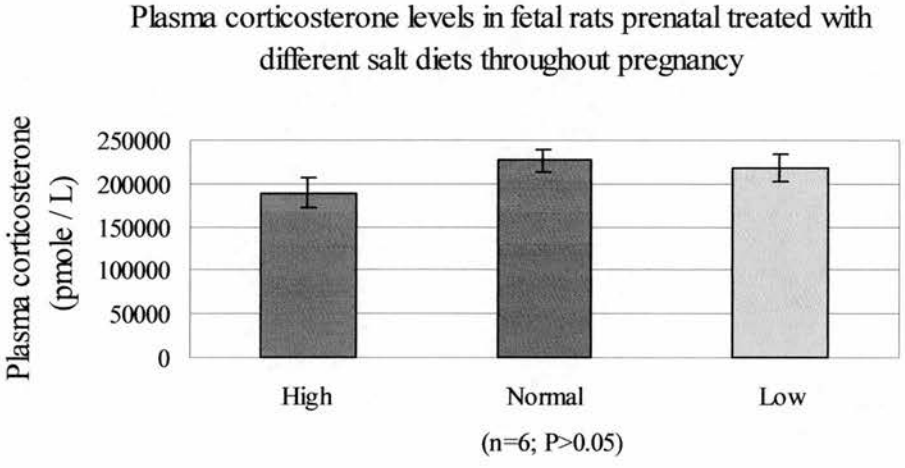
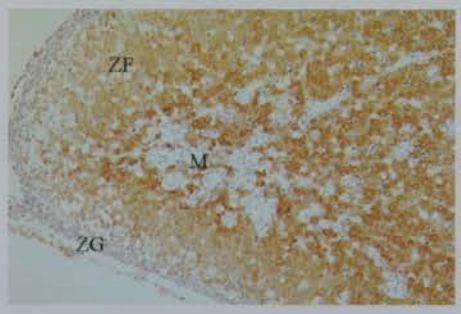
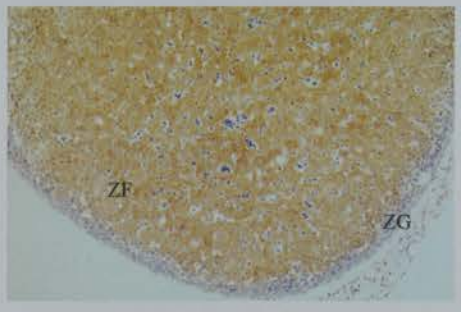


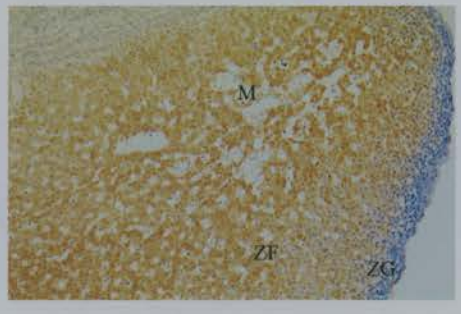
Fig. 3.13 Plasma aldosterone (A) and corticosterone (B) levels in rats at E20 stage following maternal treatment with various salt diets throughout pregnancy. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test. * $P<0.05$ compared to normal-salt-diet and high-salt-diet groups.



A.



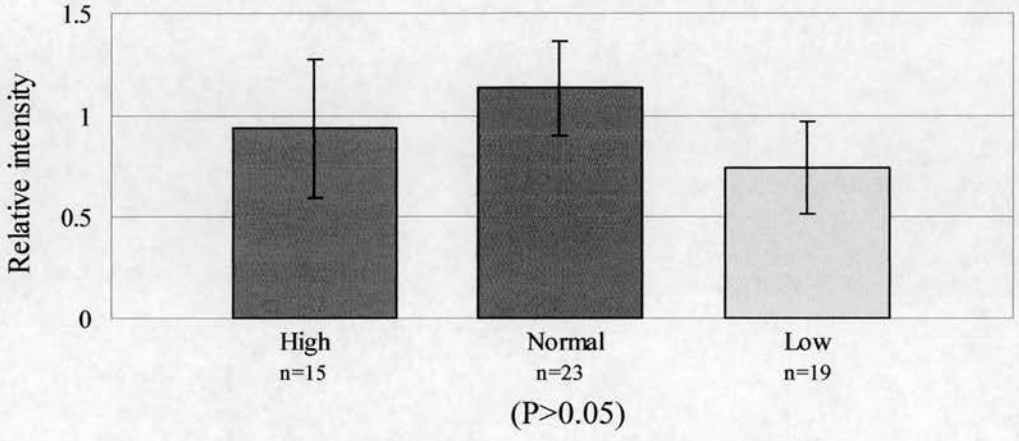
B.



C.

Fig. 3.14 Immunostaining results for 3β-HSD at E20 stage in rat adrenal glands following maternal treatment with various salt diets throughout pregnancy. A, An adrenal section from a E20 rat following maternal treatment with high-salt-diet throughout pregnancy. B, An adrenal section from a E20 rat following maternal treatment with normal salt diet throughout pregnancy. C, An adrenal section from a E20 rat following maternal treatment with low salt diet throughout pregnancy. ZG, ZF and M denote zona glomerulosa, zona fasciculata and medulla, respectively. A-C, Magnification, x100.

A. Immunostaining results for 3β-HSD in the ZG of the adrenals in fetal rats following maternal treatment with various salt diets throughout pregnancy



B. Immunostaining results for 3β-HSD in the ZF of the adrenals in fetal rats following maternal treatment with various salt diets throughout pregnancy

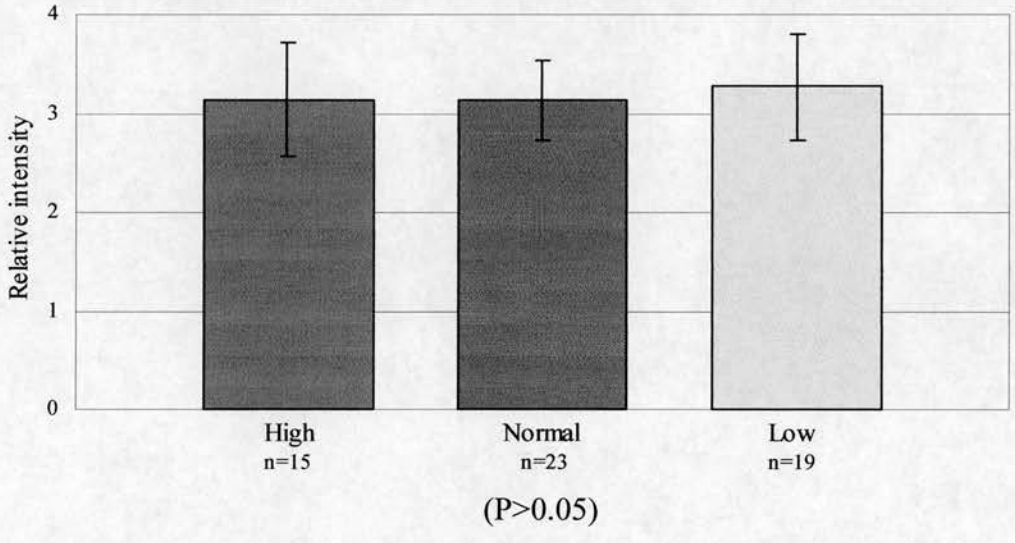


Fig. 3.15 Immunostaining results for 3β-HSD at E20 stage rat adrenal glands following maternal treatment with various salt diets throughout pregnancy. A, Immunostaining results for 3β-HSD in the ZG of the adrenal gland. B, Immunostaining results for 3β-HSD in the ZF of the adrenal gland. ZG and ZF denote zona glomerulosa and zona fasciculata, respectively. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test.

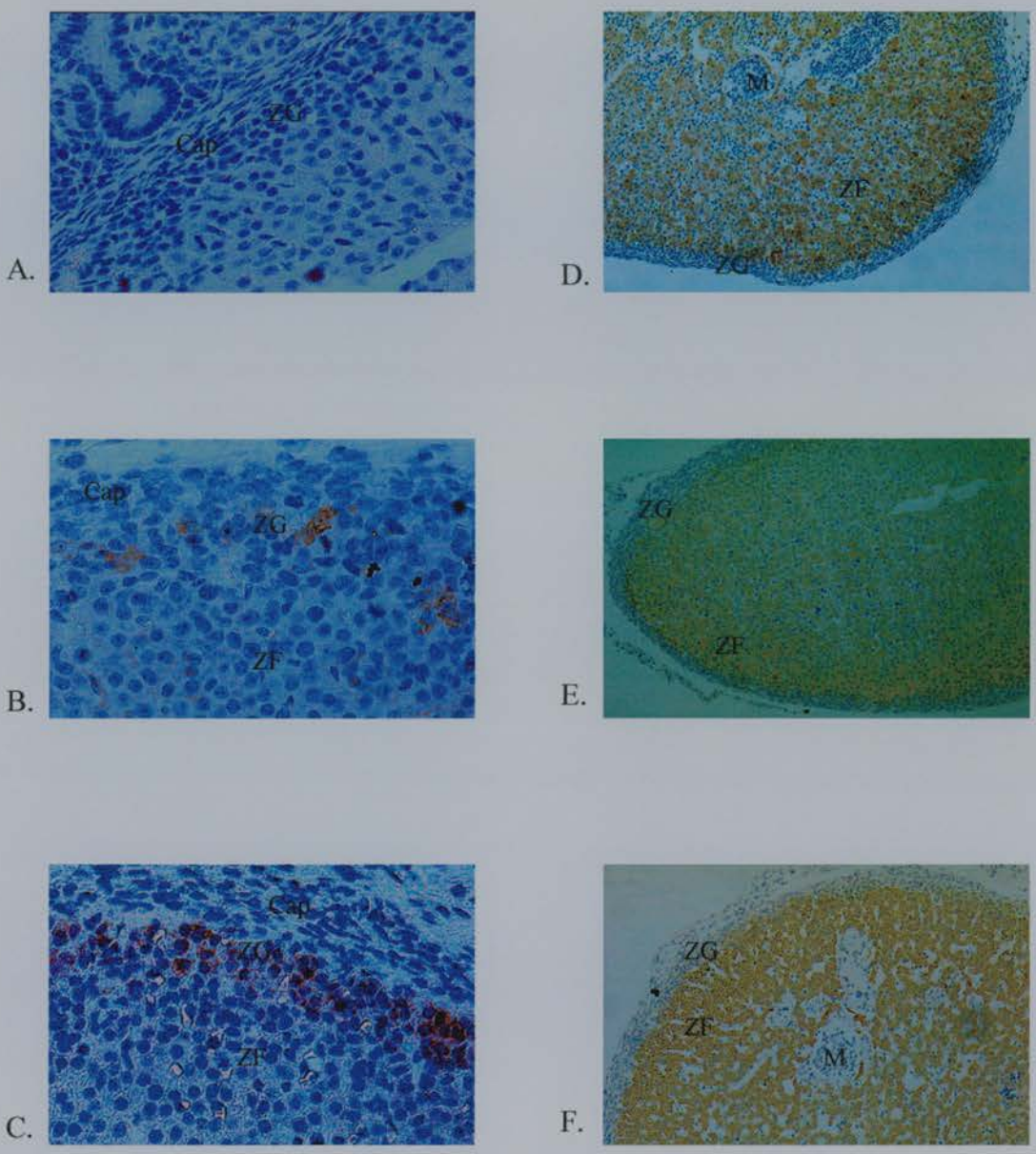
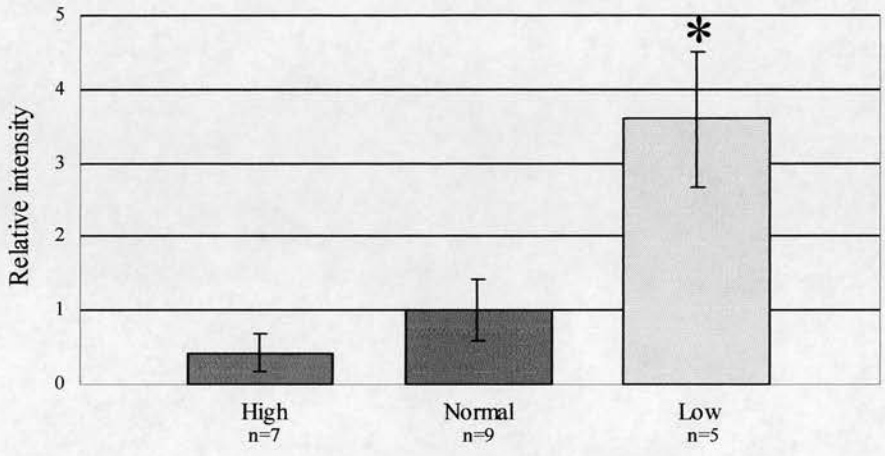


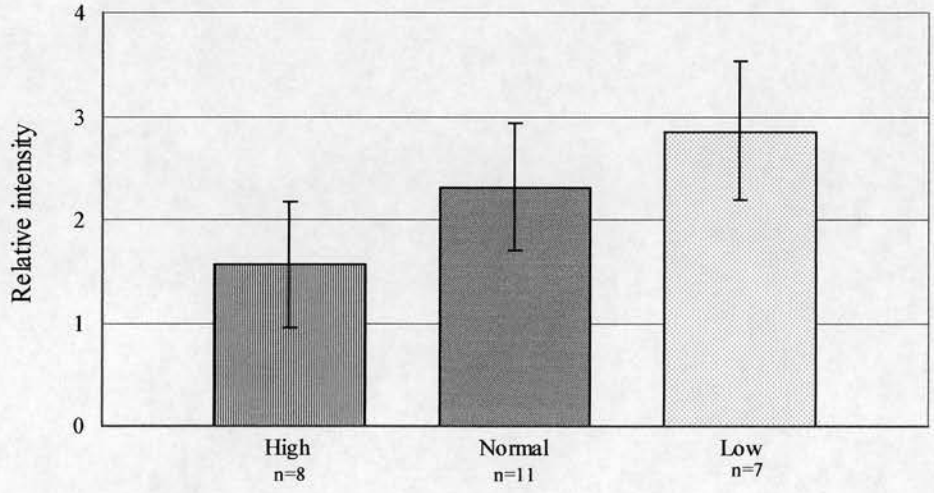
Fig. 3.16 Immunostaining results for CYP11B2 (A-C) and CYP11B1 (D-F) of adrenal glands in E20 rats following maternal treatment with various salt diets throughout pregnancy. A and D, Adrenal sections from E20 rats following maternal treatment with high salt diet throughout pregnancy. B and E, Adrenal sections from E20 rats following maternal treatment with normal-salt-diet throughout pregnancy. C and F, Adrenal sections from E20 rats following maternal treatment with low-salt-diet throughout pregnancy. Cap, ZG and ZF denote adrenal capsule, zona glomerulosa and zona fasciculata, respectively. A-C, Magnification, x400. D-F, Magnification, x100.

A. Immunostaining results for CYP11B2 in the ZG of the adrenals in fetal rats following maternal treatment with various salt diets throughout pregnancy



(P<0.005)

B. Immunostaining results for CYP11B1 in the ZF of the adrenals in fetal rats following maternal treatment with various salt diets throughout pregnancy



(P>0.05)

Fig. 3.17 Immunostaining results for CYP11B2 (A) and CYP11B1 (B) of adrenal glands in E20 rats following maternal treatment with various salt diets throughout pregnancy. ZG and ZF denote adrenal zona glomerulosa and zona fasciculata, respectively. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test. * P<0.005 compared to normal-salt-diet and high-salt-diet groups.

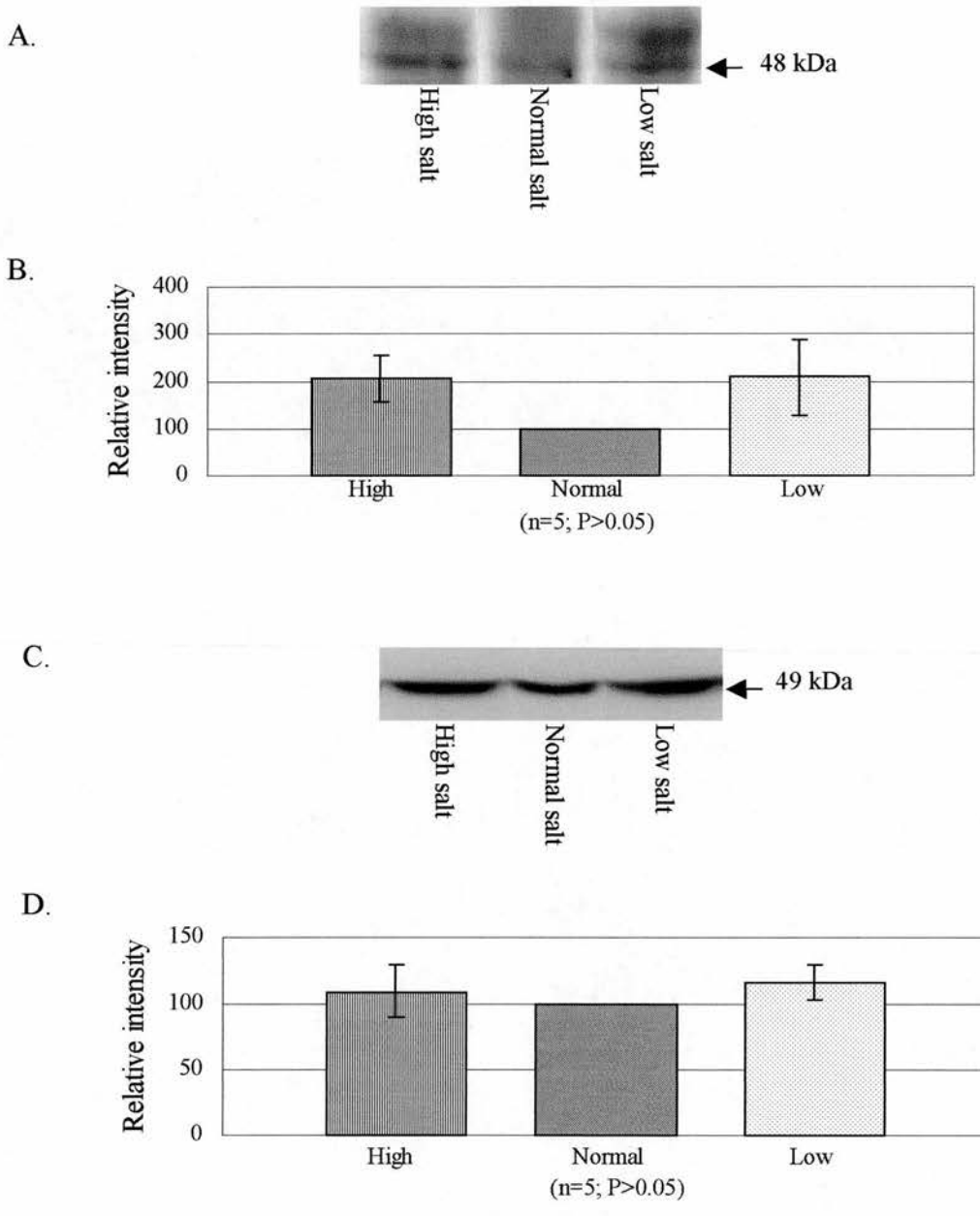
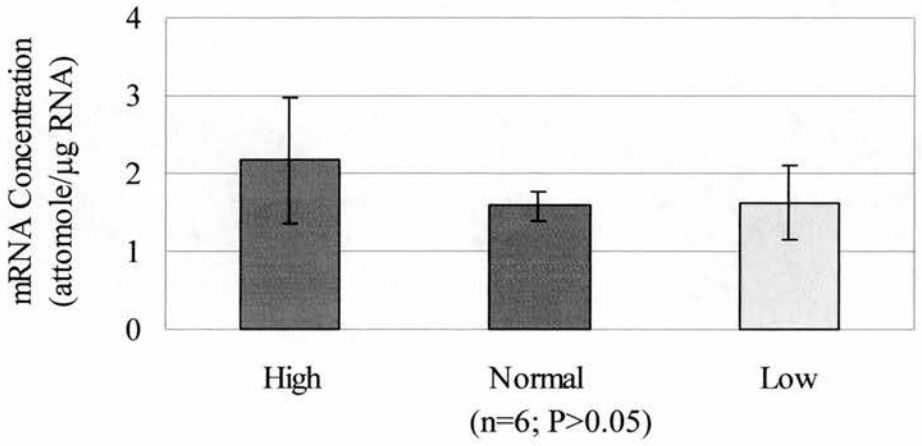


Fig. 3.18 Western blot results for CYP11B2 (A and B) and CYP11B1 (C and D) in adrenal glands at E20 rats following maternal treatment with various salt diets throughout pregnancy. A and C, 10% SDS-PAGE gel results. B and D, Relative intensity of the proteins quantified from the gels. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test.

A.



B

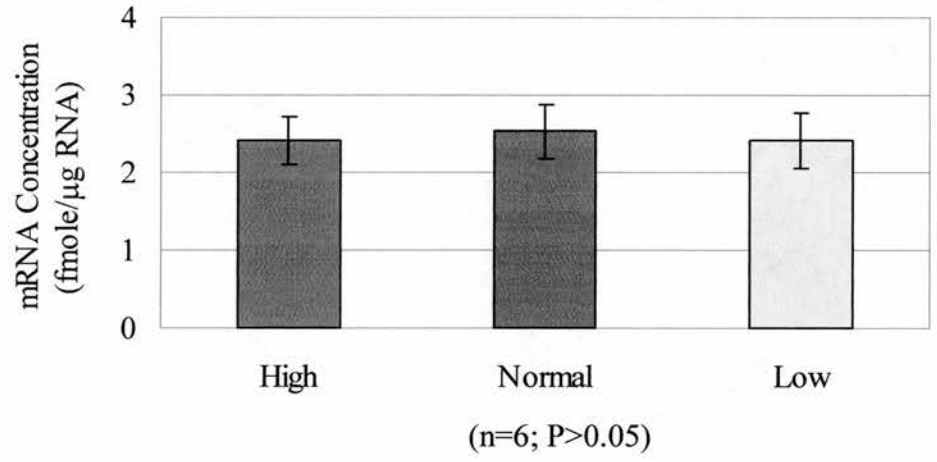


Fig. 3.19 Expression of CYP11B2 (A) and CYP11B1 (B) mRNA using competitive RT-PCR at E20 stage rat adrenal glands following maternal treatment with various salt diets throughout pregnancy. Three hundred nanograms and 5 nanograms of RNA templates (300ng and 5ng) were used for CYP11B2 and CYP11B1, respectively. Values are mean ± S.E. n represents the number of animals tested. Data were analysed by Tukey's test.

Chapter 4 Effects of dexamethasone on steroidogenic enzyme expression in fetal and pregnant rat adrenal glands

4.1 Introduction

The control of the fetal hypothalamo-pituitary-adrenal (HPA) axis has been the subject of intense investigation. The factors influencing the differentiation and secretory activity of the fetal adrenal glands have been studied using various experimental approaches. It has been postulated that maternal adrenocorticosteroids cross the placenta in the prenatal period to regulate ACTH secretion by the fetal hypophysis (Paul and D'Angelo, 1972). Treatments designed to prevent or diminish maternal glucocorticoid hormone synthesis; such as adrenalectomy (D'Angelo et al., 1973; Machin et al., 1995) or hypophysectomy (Paul and D'Angelo, 1972) have the effect of promoting fetal adrenal steroid synthesis. In contrast, stimulation of endogenous maternal corticosterone synthesis by implantation of ACTH-secreting-tumour cells (Milković et al., 1968; Milković et al., 1973) or injection with synthetic glucocorticoid hormones (Klepac and Milković, 1979) has the opposite effect causing the suppression of fetal adrenal growth and steroidogenesis.

Alteration of fetal steroidogenesis manifested by maternal treatments has been studied morphologically and functionally. Pregnant rats implanted with transplantable ACTH-secreting-tumour cells had high levels of plasma ACTH, which gave rise to a suppressed fetal adrenal size. The cells in the inner cortical zones remained small without signs of secretory activity and characteristic adrenocortical arrangement (Milković and Domac, 1973). Conversely, adrenals of fetuses injected with ACTH or from adrenalectomised mothers showed more abundant larger cortical cells full of lipid droplets with abundant mitochondria and smooth endoplasmic reticulum compared to those fetal adrenals from the control pregnant rats (Yamamoto et al., 1986; Machin et al., 1995). Fetal steroidogenesis has also been assessed by monitoring *in vitro* and *in vivo* conversion of radiolabelled precursors to a variety of

products. In the adrenals of rat fetuses from dexamethasone treated adrenalectomised mothers and from the ACTH-tumour-bearing mothers, the conversion of ^{14}C-progesterone to deoxycorticosterone (DOC), corticosterone, 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) was inhibited during the final days of intrauterine development. However, the production of 18-hydroxy-corticosterone (18-OH-B) and aldosterone was not affected (Klepac, 1976; Klepac et al., 1977; Klepac et al., 1978; Klepac, 1981). On the other hand, fetuses injected with metopirone, an inhibitor of 11β- and 18-steroid hydroxylases, resulted in a decrease in the synthesis of corticosterone, 18-OH-DOC and 18-OH-B in fetal adrenals (Klepac et al., 1975). Administration of ACTH directly to fetuses through the uterine wall increased the conversion of progesterone to the above-mentioned steroids (Klepac and Milković, 1975; Sugihara et al., 1982).

As described in the introduction and Chapter 3, the adrenal zonal specific cells are possibly identifiable between day 16 and day 20 of gestation. Studies of the ontogeny of corticosterone biosynthesis indicated that production of corticosterone begins around embryonic day 16 and the fetal adrenals become sensitive to ACTH stimulation around that time (Roos, 1967; Kalavsky, 1971; Milković et al., 1973; Yamamoto et al., 1983; Yamamoto et al., 1986). On the basis of these studies, the fetal HPA axis regulation seems to appear between day 16 and day 18 of gestation. Aldosterone synthesis and the development of the ZG is a little later, but is clearly seen from day 18 onwards (Milković et al., 1976; Churchill et al., 1981), though Mitani et al. (1997) reported that CYP11B2 remained undetectable immunohistochemically until E20 (Mitani et al., 1997).

During the developing stages of fetal steroidogenesis, maternal steroids cross the placental barrier, thereby modulating the fetal HPA axis. It is also apparent that the process is bi-directional. In pregnant adrenalectomised rats, the maternal plasma corticosterone level can be maintained as high as that observed in intact pregnant rats after 18 days of gestation (Dupouy et al., 1975). Significant levels of maternal hormone and radioactive products have been identified in the maternal circulation after adrenalectomy following fetal injection of radiolabelled steroid precursors

(Milković et al., 1973; Milković et al., 1976). Moreover, it was confirmed that the increase in maternal plasma corticosterone concentration was inhibited 2 hours later in adrenalectomised mothers whose fetuses were injected with metopirone immediately after the operation (Milković et al., 1975).

It is also clear that, as with adult adrenals, fetal steroids in the adrenal cortex influence the development of chromaffin cells. Chromaffin cells are seen between embryonic day 17 and day 18 by immunohistochemical methods (Josimovich et al., 1954; Roffi, 1968) and assays of adrenal catecholamine contents (Seidl and Unsicker, 1989; Kennedy and Ziegler, 2000). These developments suggest that fetal catecholamine secretion or the related enzyme activity may be intimately linked to fetal adrenocortical activity. Therefore, treatments designed to influence fetal steroidogenesis may also affect fetal chromaffin tissues. In the present study, we have investigated the effects of maternal dexamethasone treatment. The main aim of this study was firstly to examine whether a long-term, modest dose of glucocorticoid hormone would have effects similar to those achieved with short-term (less than 7 days) and higher dose treatment reported in previous studies. A second aim was to assess whether the expression of CYP11B1 and CYP11B2 transcripts in the developing adrenals was affected by the maternal treatment. A third aim was to investigate whether dexamethasone treatment induced changes in the development of the adrenal cortex and medulla that may initiate programming of adult onset disease (see Chapter 5).

4.2 Results

4.2.1 Effects of maternal dexamethasone treatment on adrenal morphology changes and the steroidogenic enzymes in fetal rats

4.2.1.1 Organ weights

Fetal rats treated with dexamethasone throughout gestation (called DEX-treated in the following sections) exhibited growth retardation (Table 4.1). The body weights were significantly reduced by DEX-treatment compared to the control animals.

DEX-treated fetal rats also had lower organ weights and relative organ weights, except for the relative heart weights, compared to the saline-treated fetuses (called saline-treated in the following sections) ($P<0.05$).

Table 4. 1 Body weights and organ weights (mean ± S.E.) of E20 rats following prenatal treatment with saline or dexamethasone throughout pregnancy

	Saline (n=3)	**Dexamethasone (n=4)**
Body weight (g)	3.715±0.049[1]	3.494±0.085[1]*
Heart weight (mg)	25.14±0.55	22.81±0.65*
Heart wt / Body wt %	0.677±0.012	0.658±0.014
Kidney weight (mg)	14.37±0.37	12.57±0.48*
Kidney wt / Body wt %	0.386±0.008	0.357±0.007*
Adrenal weight (mg)	1.06±0.05 (n=2)	0.83±0.04 (n=2)

n represents the number of pregnant rats tested.

[1]The number of fetuses in saline and dexamethasone groups is between 48 and 51. Data were analysed by Student's t-test.

*: Significantly different from saline group ($P<0.05$).

4.2.1.2 Adrenal cell size

There was no difference in fetal adrenal ZF cell size between the two experimental groups (Fig. 4.1A). In contrast, the size of the chromaffin cells was significantly increased in DEX-treated fetal adrenals compared to the saline-treated adrenals at a magnification of x400 (89.06 μm^2 *vs.* 75.62 μm^2; $P<0.05$) (Fig. 4.1B). There was a 17.8% increase of the medullary cell size.

4.2.1.3 Serial adrenal sections stained by various antibodies

To investigate further the morphological changes, serial sections of adrenal glands were stained by antibodies, which recognise specific zonal markers of the adrenal cortex or medulla. The brown staining achieved with the CYP11B2 antibody was seen in the ZG with low staining intensity of the saline-treated fetal adrenal (Fig. 4.2A), but clear staining was exhibited under the capsule of DEX-treated fetal adrenals (Fig. 4.2E). Most cells were positive for CYP11B1, except for those in the outer ZG and chromaffin-positive cells. The saline-treated adrenals showed weak staining between the edge of the ZG and the ZF with increasing intensity towards the inner cortex (Fig. 4.2B), while the DEX-treated adrenal showed homogeneous staining for CYP11B1 (Fig. 4.2F). 3β-HSD was localised in all cortical cells and the non-steroidogenic cells, which localised in the central area of the adrenal, exhibited clear negative staining (Fig. 4.2D and H). Chromogranin A antibody was used as a specific marker for medullary cells (Fig. 4.2C and G). Comparing the serial staining results, the most obvious difference between the saline- and DEX-treated adrenals was the area in the centre of the gland. DEX-treated adrenals showed a broad area of medullary cells. In contrast, saline-treated adrenals showed a few scattered medullary cells. Different staining intensities for each antibody between the control group (Fig 4.2 A-D) and experimental group (Fig. 4.2 E-H) are discussed in a later section (Section 4.3.1.4).

4.2.1.4 Immunostaining results

DEX-treated fetuses appeared to show higher relative adrenal staining intensity for CYP11B2 compared to those of saline-treated animals (Fig. 4.3 A). In contrast, the staining intensity for CYP11B1 was slightly reduced in the DEX-treated group compared to the control group (Fig. 4.3 B). Neither of the differences, however, reached statistical significance.

4.2.1.5 Western blotting results

Due to the low protein levels of the CYP11B2 and the low sensitivity of the antibody, it was difficult to observe clear 48 kDa bands in fetal adrenal samples (Fig. 4.4A). In contrast, CYP11B1 was highly expressed at this stage (Fig. 4.4C). According to the Western blotting results, no changes in CYP11B2 or CYP11B1 were seen when the prenatal treated saline and DEX groups were compared (Fig. 4.4B and D).

4.2.1.6 Gene expression for CYP11B2 and CYP11B1

The abundance of CYP11B2 transcripts appeared to be elevated in DEX-treated fetal adrenals compared to the saline-treated adrenals (Fig. 4.5A). There was, however, a lack of statistically significant differences. DEX-treatment throughout pregnancy markedly reduced the level of CYP11B1 transcripts in fetal adrenals (Fig. 4.5B).

4.2.2 Effects of dexamethasone on the CYP11B enzymes in pregnant rats

4.2.2.1 Immunostaining results

In pregnant rats, the staining intensity for CYP11B2 was not affected by DEX-treatment (Fig. 4.6A, Fig 4.7 A and C). On the other hand, the staining of CYP11B1 in ZF was markedly reduced in the DEX-treated pregnant rats (Fig. 4.6B, Fig. 4.7 B and D). The maternal adrenal micrographs for CYP11B2 and CYP11B1 staining were shown in Fig. 4.7.

4.2.2.2 Western blotting results

Maternal DEX-treatment led to a marked increase (2.8- fold) in the relative abundance of CYP11B2 in the adrenals of the pregnant rats compared to the saline controls (Fig. 4.8A and B). In contrast, the expression for CYP11B1 in pregnant rat adrenals was suppressed to a level of 42% by DEX-treatment compared to the saline treatment (Fig. 4.8C and D).

4.2.2.3 Gene expression of CYP11B2 and CYP11B1

The expression of the CYP11B2 transcripts appeared to be modestly increased in the adrenals of the DEX-treated pregnant rats compared to the saline controls (Fig. 4.9A). Contrasting with the pattern observed in CYP11B2 transcripts, maternal DEX-treatment caused a dramatic reduction in CYP11B1 expression (Fig. 4.9B) since similar CYP11B1 transcript levels were seen with 150 nanograms of RNA templates from adrenal samples of the DEX-treated rats when compared to 5 nanograms of RNA template in control adrenal samples.

4.3 Discussion

Numerous studies have indicated that prenatal exposure to glucocorticoids causes intrauterine growth retardation in humans and animals (Paul and D'Angelo, 1972; D'Angelo et al., 1973; Klepac and Milković, 1979; Reinisch et al., 1978; Benediktsson et al., 1993). Dexamethasone, an effective synthetic glucocorticoid, has been used in the clinical treatment of glucocorticoid excess for years. The most profound effects of DEX adminstration during gestation are low birth weights and low adrenal weights with the subsequent development of hypertension and insulin resistance in the adult offspring (see Chapter 5). In this study, a slight decrease in heart weight and markedly reduced birth, kidney and adrenal weights were observed in fetal rats exposed to DEX *in utero*. This study, in agreement with previous studies, shows the signs of the intrauterine growth retardation produced by the administration of DEX.

A high dose of DEX-treatment (0.3 mg/kg body weight/day) to pregnant rats during the last 5 days of gestation resulted in atrophic changes, including the reduction of cell volume and cell number, in the adrenal cortex of the fetal rats (Hristić et al., 1997). In our study, no difference in cell size was observed in ZF cells of the adrenal glands from the control and maternal DEX-treated fetal rats. This is

likely to be due to the modest DEX dosing treatment (100 μg/kg body weight/day) was applied in the present study. Also, it may be as a result of the inhibition of fetal ACTH secretion by DEX, which could result in the suppression of steroid output and cell proliferation rather than affecting the adrenocortical cell size. However, the cell number or proliferation activity of the adrenal cortex was not examined. In this study, the protein levels of CYP11B1 in fetal adrenals detected by immunostaining and Western blotting appeared to be slightly reduced by the maternal DEX-treatment compared to the control rats. More obviously, the level of the CYP11B1 mRNA in the adrenals of DEX-treated fetal rats was lowered to 50% of the expression level in the saline-treated fetal rat adrenals ($P<0.05$). Although the plasma corticosterone levels were not measured in this study, the present data are suggestive that the corticosterone production could potentially be inhibited in the adrenals of fetal rats treated *in utero* with DEX throughout gestation.

In the present study, there were no apparent effects on the protein and gene expression of CYP11B2 in fetal adrenals following prenatal DEX-treatment although the immunostaining and cRT-PCR results showed a tendency for CYP11B2 expression to be higher in DEX-treated fetal adrenals. According to the morphological studies, Hristić et al. (1997) reported both the volume and the number of ZG cells were markedly reduced in the adrenals of rats with prenatal dexamethasone treatment. Others failed to demonstrate the morphological changes in the ZG either in the adrenals of fetuses from ACTH-tumour-bearing (Milković and Domac, 1973) or adrenalectomised-treated (Machin et al., 1995) mothers. However, Sugihara et al. (1977) showed that both the ZG and ZF cells of fetal adrenals became remarkably hyperaemic to exogenous ACTH injection (Sugihara et al., 1977).

On the other hand, it is worthy of note that the changes of CYP11B2 and CYP11B1 and their transcripts in fetal adrenals were in a similar fashion to those observed in the pregnant mothers with the exception of the more profound effects seen in the DEX-treated mothers. These results are inconsistent with previous studies. DEX reduced markedly the amount of CYP11B1 mRNA without affecting CYP11B2 mRNA levels in normal, non-pregnant rats, however, there was little effect on the

abundance of the CYP11B2 and CYP11B1 mRNA in the fetal adrenals (Malee and Mellon, 1991). Consequently, it was suggested that fetal adrenal CYP11B2 and CYP11B1 might be transcriptionally quiescent *in vivo* to the maternal dexamethasone treatment although changes in plasma and adrenal corticosterone levels were clearly observed in fetal rats following prenatal treatment with dexamethasone (20 μg/daily) for one week (Malee and Mellon, 1991; Mellon et al., 1995). The divergent results for the protein and gene expression of the CYP11B2 and CYP11B1 may result from the various doses and the duration of the dexamethasone treatment used in the different studies. In the present study, pregnant rats were injected with a modest dose of dexamethasone (100 μg/kg body weight/day) throughout pregnancy compared with 20 μg/daily injection for a week in the study of Malee and Mellon (1991) or 0.3 mg/kg body weight/day during last 5 days of gestation in Hristić's experiments (Hristić et al. 1997).

It is also interesting that the expression of the CYP11B2 protein and gene transcripts showed great responses to dexamethasone in pregnant rat adrenal glands. One possible explanation may be the increased relative amounts of CYP11B2 due to the atrophy of the adrenal gland in DEX-treated rats compared to the saline-treated animals. Alternatively, exogenous dexamethasone may modulate angiotensin II receptors in the ZG of the adrenal gland. Recently, it has been demonstrated that glucocorticoid receptors and angiotensin II type I receptor (AT1) expression, but not angiotensin II type II receptor (AT2), were elevated in sheep and rat adrenal glands exposed to maternal malnutrition during pregnancy (Bertram et al., 2001; Whorwood et al., 2001). It has also been demonstrated in vascular smooth muscle cells that dexamethasone induces expression of the AT1 receptor gene and results in an increase in the number of the AT1 receptors (Sato et al., 1994). This may give rise to the increase of CYP11B2 expression. Furthermore, in the present study, the pregnant rats were treated with dexamethasone or saline throughout gestation. It has been known that the last few days of gestation may be a crucial window for fetal development. However, a modest dose of dexamethasone with long-term treatment may exert more profound effects *via* alterations of the glucocorticoid and/or mineralocorticoid

receptor gene expression.

It is remarkable that the medullary cell size was significantly increased in DEX-treated fetal adrenal glands. In addition, the maternal DEX-treated adrenals showed a more extensive array of medullary cells, which was confirmed by the serial staining for chromogranin A and 3β-HSD, compared to the saline-treated fetal adrenals. When pregnant rats were given a single injection of dexamethasone (1.5 mg/kg body weight) on day 16 of gestation, the medullary volume and the number of chromaffin cells were significantly reduced in the fetal adrenals. However, during the second neonatal week, the mitotic index of chromaffin cells reached higher values in comparison to control animals (Manojlivić et al., 1998). It has been reported that glucocorticoid receptors appear in chromaffin tissue on day 17 of gestation and the activity of fetal phenylethanolamine N-methyltransferase (PNMT) enzyme required to convert noradrenaline into adrenaline in chromaffin cells also starts to be initiated at this stage. Therefore, it is possible that glucocorticoids are essential to trigger the differentiation of sympathoadrenal precursors in endocrine chromaffin cells (Seidl and Unsicker, 1989; Kennedy and Ziegler, 2000). Lack of glucocorticoid receptors in the adrenal medulla impairs the development of chromaffin cells in fetal adrenal glands (Cole et al., 1995) with loss of PNMT gene expression (Cole et al., 1995; Finotto et al., 1999). On the other hand, chronic dexamethasone exposure of rat fetuses enhances both PNMT activity and adrenaline contents in the fetal adrenal, heart and head tissues in the later stage of gestation (Kennedy and Ziegler, 2000). However, injection of ACTH in fetuses did not result in an increase of fetal medullary PNMT activity despite leading to fetal adrenal hypertrophy and increases in fetal plasma and adrenal corticosterone concentrations. This suggests that corticosterone may be essential in maintaining the activity of PNMT, but that increases in corticosterone do not necessarily result in an increase in PNMT (Milković et al., 1974). It is interesting therefore that, in the present experiments, a greater cell size and a more extensive area of the adrenal medulla was seen in fetal rats prenatally exposed to dexamethasone. This perhaps resulted from the modification of the differentiation of the medullary cells, especially the PNMT-containing cells associated with adrenaline secretion.

In conclusion, long-term dexamethasone exposure throughout gestation causes significant changes in the expression of CYP11B1 in the adrenals of pregnant rats. Modest increases of CYP11B2 mRNA expression and significantly increased levels of CYP11B2 are also shown in pregnant rat adrenal glands. In the adrenals of the fetal rats prenatally treated with dexamethasone throughout gestation, gene and protein expression for CYP11B2 and CYP11B1 appeared to be regulated in a similar fashion to those in the maternal adrenals. In addition, the morphology of the fetal adrenal medulla is also affected by chronic DEX-treatment *in utero*. Whether these changes in the fetal adrenals are sustained and programmed in the same way in later postnatal and adult life will merit further investigation.

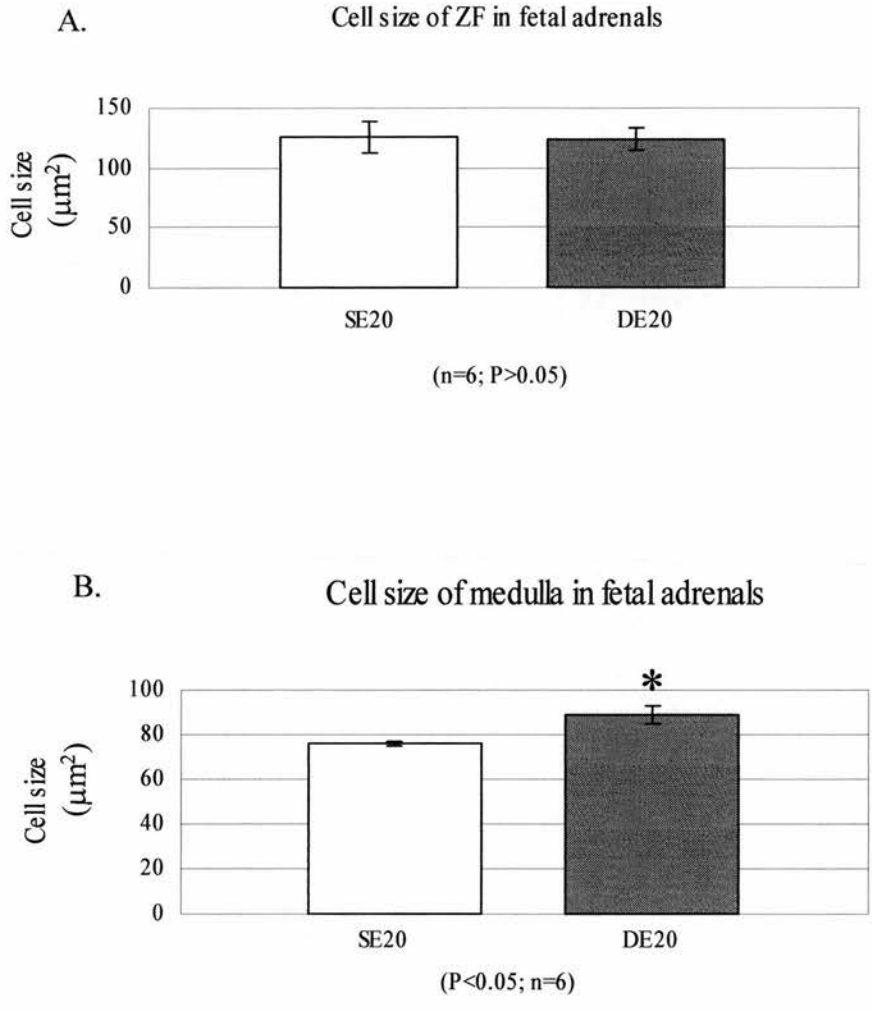


Fig. 4.1 Cell size of zona fasciculata (ZF)(A) and medulla (B) in the adrenals of prenatal saline (SE20)- or dexamethasone (DE20)-treated 20-day rat embryos. Values are mean±S.E. n represents the number of the animal tested. Data were analysed by the Student's t-test. *P<0.05

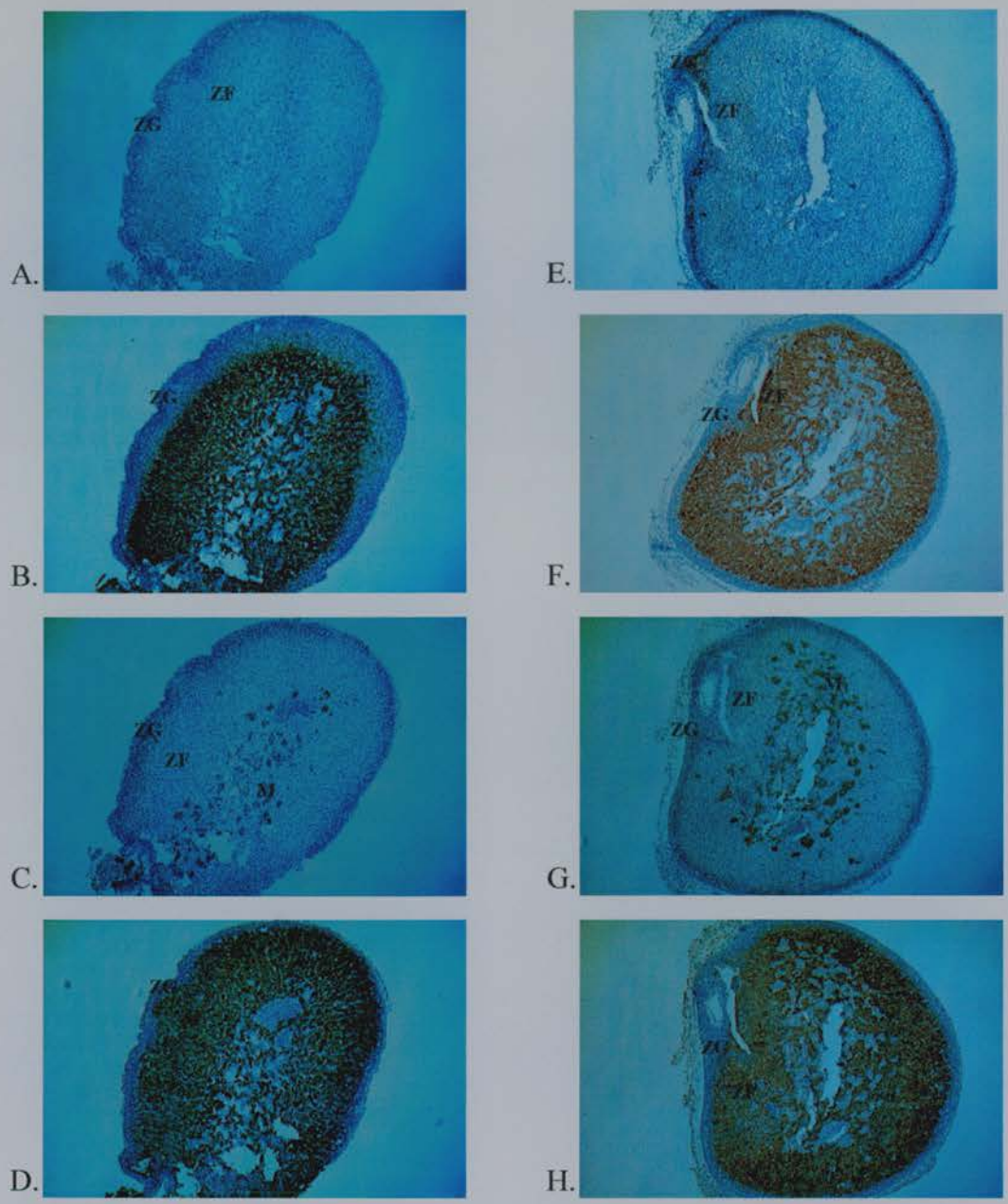
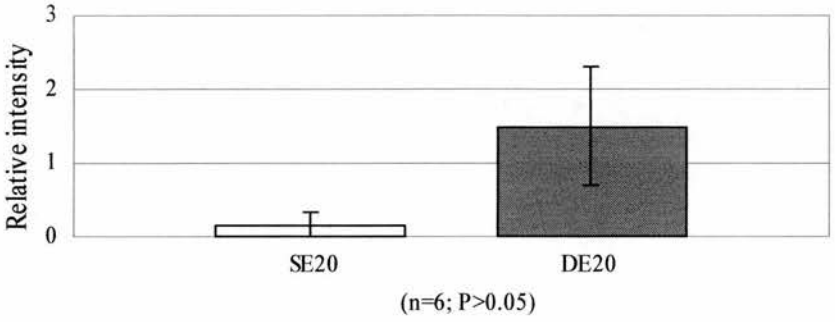


Fig. 4.2 Serial staining of various antibodies in the adrenals of prenatal saline- (A-D) or dexamethasone- (E-H) treated 20-day rat embryos. A and E; CYP11B2, B and F; CYP11B1, C and G; Chromogranin A, D and H; 3 β-HSD. ZG, ZF and M denote zona glomerulosa, zona fasciculata and medulla, respectively. Magnification: 50X.

A.

Immunostaining results CYP11B2 in the ZG of fetal adrenals



B.

Immunostaining results of CYP11B1 in the ZF of fetal adrenals

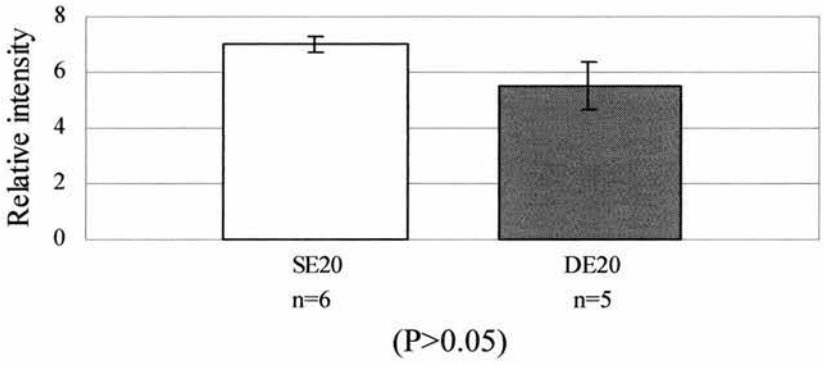


Fig. 4.3 Immunostaining results for CYP11B2 (A) and CYP11B1 (B) in the adrenals of prenatal saline (SE20)- or dexamethasone (DE20)- treated 20-day rat embryos. Values are mean±S.E. n represents the number of the animal tested. Data were analysed by the Student's t-test.

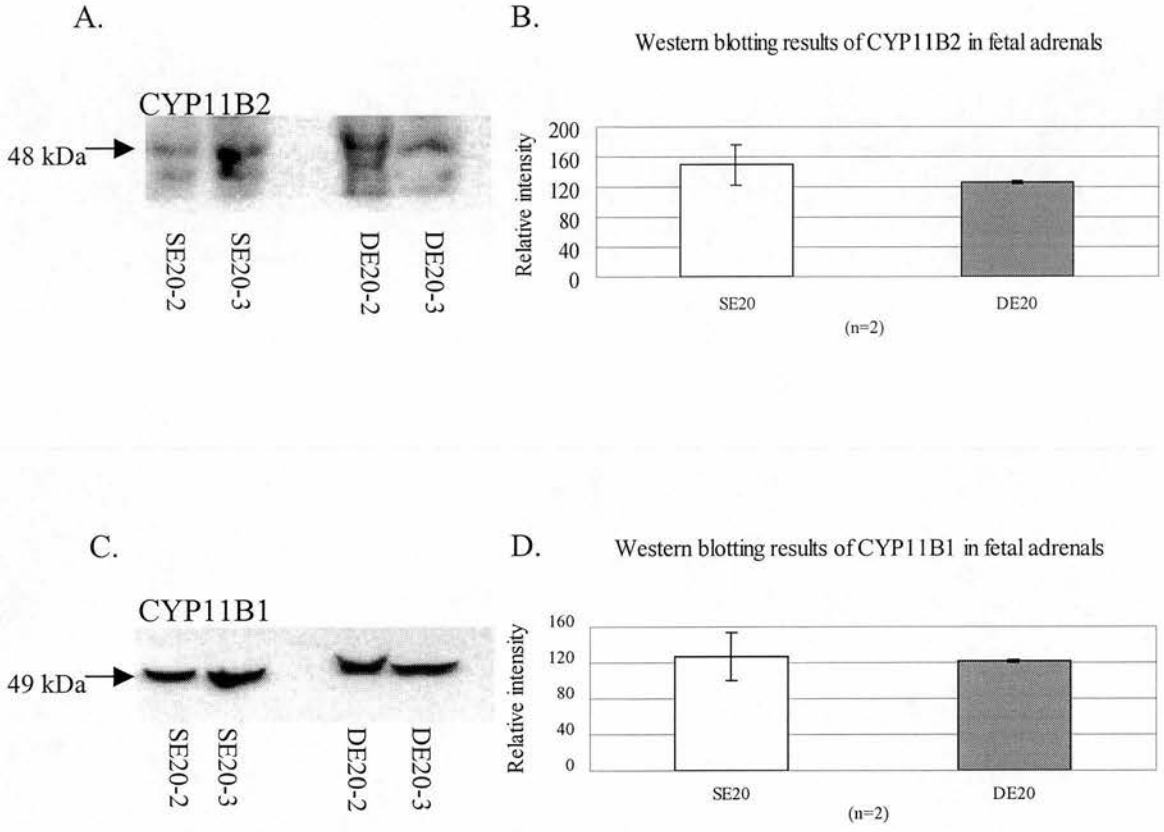


Fig. 4.4 Western blotting results for CYP11B2 (A and B) and CYP11B1 (C and D) staining in the adrenals of prenatal saline (SE20-X)- or dexamethasone (DE20-X)-treated 20-day rat embryos. A and C, 10% SDS-PAGE gel results. B and D, Relative intensity of the proteins quantified from the gels. Values are the range of the two determinations. n represents the number of animals tested. X represents an individual animal from different pregnant rats.

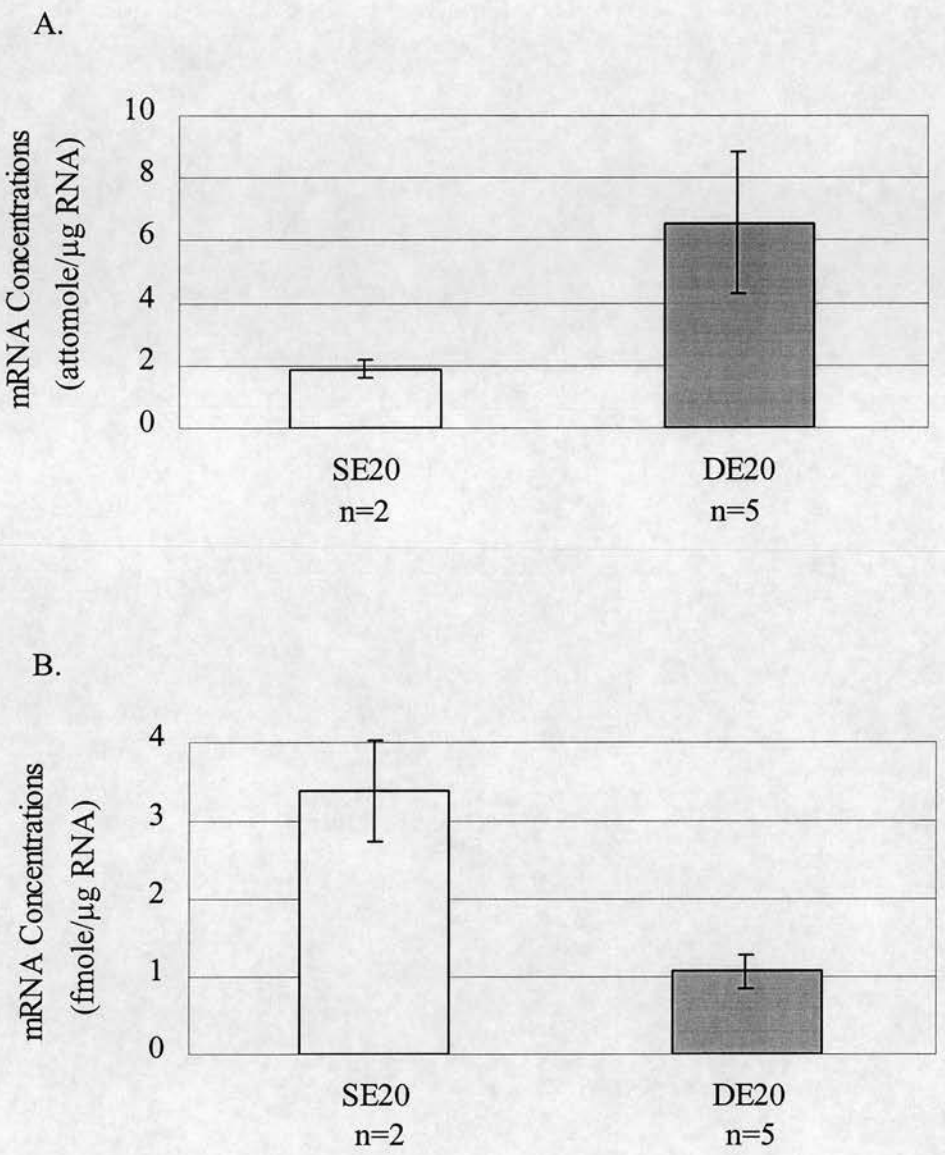
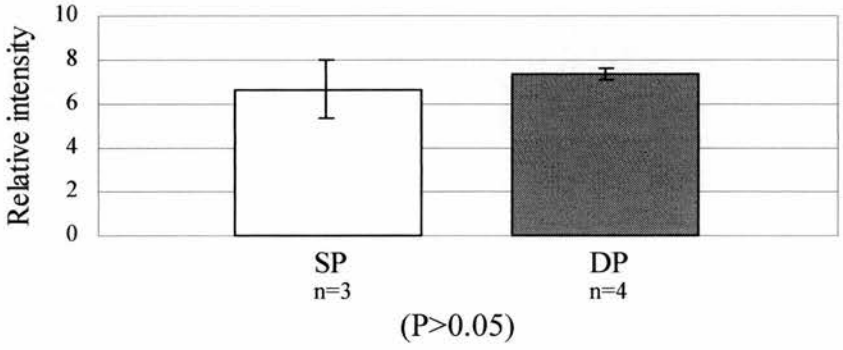


Fig. 4.5 Transcript levels of CYP11B2 (A) and CYP11B1 (B) using cRT-PCR in the adrenals of prenatal saline (SE20)- or dexamethasone (DE20)-treated 20-day rat embryos. n represents the number of animals tested. Data are expressed as mean±S.E. in the dexamethasone-treated groups and the data in saline-treated groups (n=2) represent the range of the two determinations.

A. Immunostaining results of CYP11B2 in the ZG of pregnant rat adrenals



B. Immunostaining results of CYP11B1 in the ZF of pregnant rat adrenals

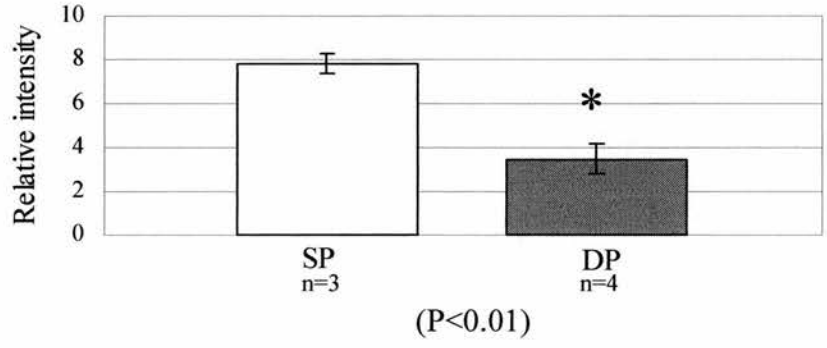


Fig. 4.6 Immunostaining results for CYP11B2 (A) and CYP11B1 (B) in the adrenals of pregnant rats treated with saline (SP) or dexamethasone (DP) throughout the gestation. Values are mean±S.E. n represents the number of animals tested. Data were analysed by the Student's t-test. *P<0.01

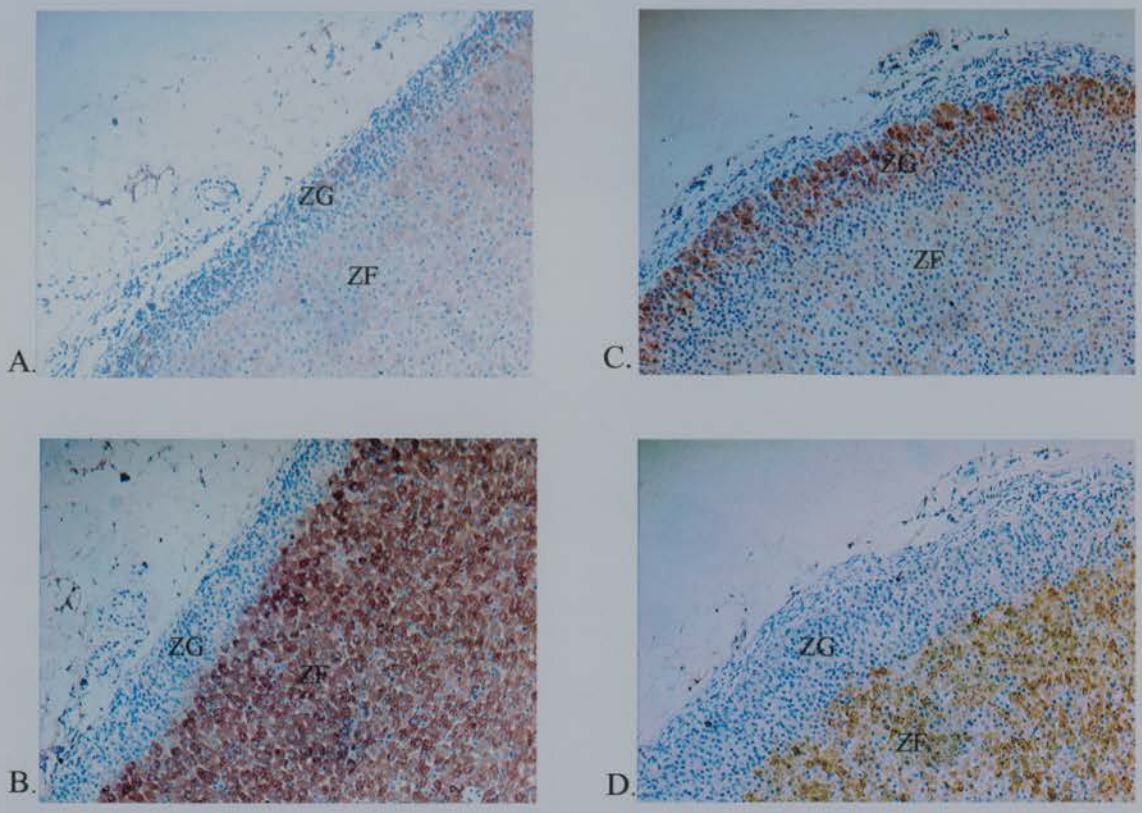


Fig. 4.7 Immunostaining for CYP11B2 (A and C) and CYP11B1 (B and D) in rat adrenal glands obtained from pregnant rats treated with saline (A and B) or dexamethasone C and D) throughout pregnancy. ZG and ZF denote zona glomerulosa and zona fasciculata, respectively. Magnification: 100X.

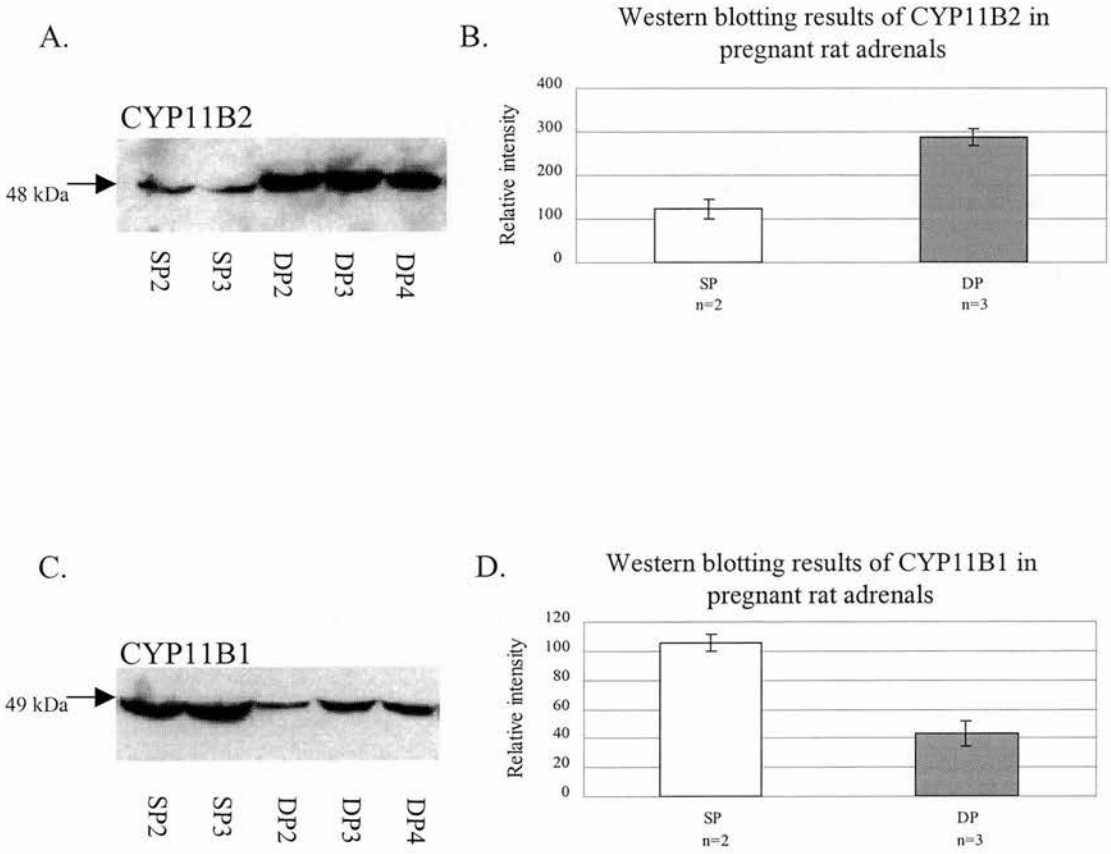
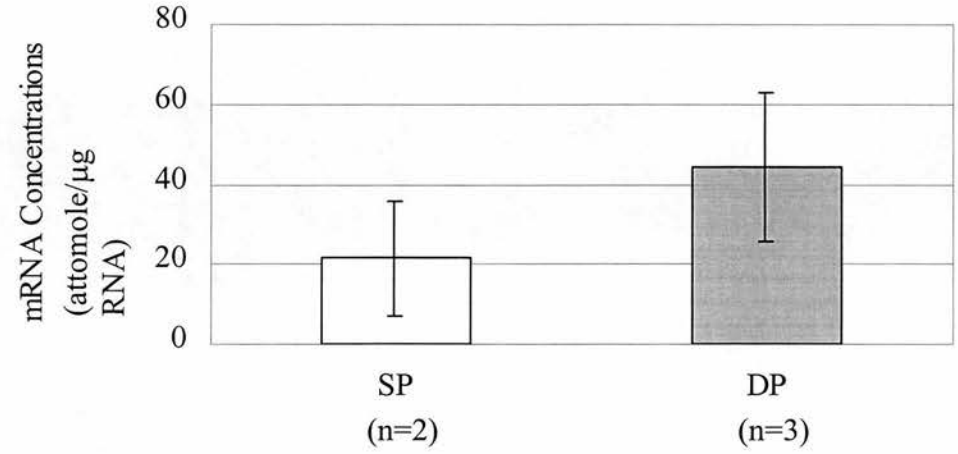


Fig. 4.8 Western blotting results for CYP11B2 (A and B) and CYP11B1 (C and D) in the adrenals of pregnant rats treated with saline (SP) or dexamethasone (DP) throughout the gestation. A and C, 10% SDS-PAGE gel results. B and D, Relative intensity of the proteins quantified from the gels. n represents the number of animals tested. Data are expressed as mean±S.E. in the dexamethasone-treated groups and the data in saline-treated groups (n=2)represent the range of the two determinations.

A.



B.

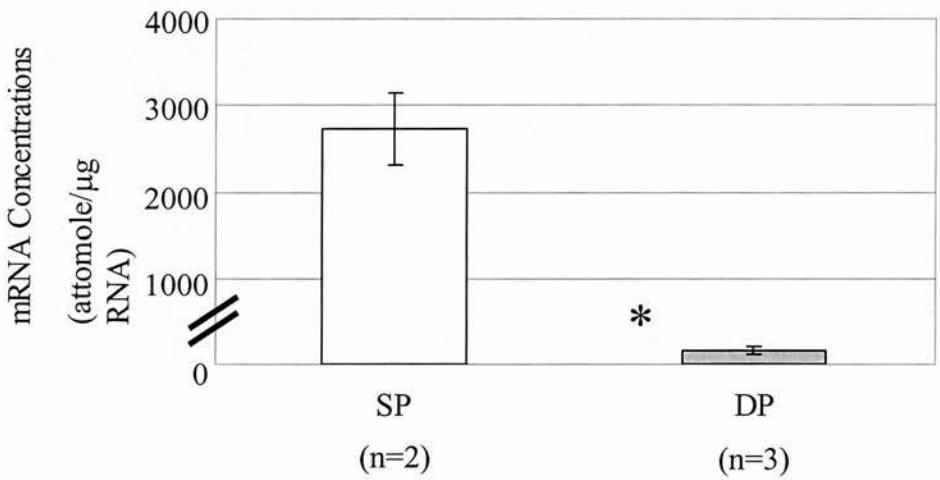


Fig. 4.9 Transcript levels of the CYP11B2 (A) and CYP11B1 (B) using cRT-PCR in the adrenals of pregnant rats treated with saline (SP) or dexamethasone (DP) throughout the gestation. n represents the number of animals tested. Data are expressed as mean±S.E. in the dexamethasone-treated groups and the data in saline-treated groups (n=2) represent the range of the two determinations.

Chapter 5 Effects of dexamethasone treatment on adrenal morphology and CYP11B enzyme expression in the postnatal rat offspring

5.1 Introduction

It has been extensively reported that birth weight is related to the nature of the intrauterine environment. Epidemiological studies have suggested that low birth weight is strongly associated with the incidences of ischaemic heart disease (Barker et al., 1989), hypertension (Barker et al., 1990), cardiovascular (Barker, et al., 1993a) and metabolic disorders (Barker, et al., 1993b), and, for instance syndrome X, a combination of non-insulin-dependent diabetes, hypertension and hyperlipidaemia, in the adult. Clinical and experimental investigations have identified a number of factors that cause intrauterine growth retardation, including undernutrition (Barker et al., 1993c), stress and glucocorticoid exposure (Levitt et al., 1996; Welberg et al., 2001). The adaptation to such intrauterine environments in fetuses may change the concentrations of fetal and placental hormones and result in persistent changes of the physiological function and disease in adult life (Barker et al., 1993b; Clark, 1998).

In rodents, many studies are demonstrative that offspring exposed to synthetic glucocorticoids during gestation show reduced birth weight (Blackburn et al., 1965; Reinisch et al., 1978; Benediktsson et al., 1993) which may also lead to increased blood pressure in the later adult life after the last exposure to glucocorticoid *in utero* (Benediktsson et al., 1993; Levitt et al., 1996). It has been suggested that fetal growth is directly correlated with the activity of the placental 11β-hydroxysteroid dehydrogenase (11β-OHSD2), which converts excess maternal active corticosterone to inactive 11-dehydrocorticosterone and protects the fetus from exposure to the

excess glucocorticoids *in utero*. In contrast, there is an inverse correlation between 11β-OHSD2 activity and placenta weight in fetus with dexamethasone treatment throughout pregnancy (Benediktsson et al., 1993). The disproportion between birth weight and placenta size of the fetus has been linked with high blood pressure in adult life in humans (Barker et al., 1990). It has also been found that dexamethasone treatment during the last week of pregnancy diminishes the expression levels of the glucocorticoid and mineralocorticoid receptors in specific brain areas, e.g. hippocampus in the adult offspring in the rat. This may account for the high blood pressure in the adult offspring after intrauterine dexamethasone treatment (Levitt et al., 1996).

Treatment of pregnant rats with glucocorticoid hormone or by feeding a low protein diet has life long effects on the offspring (Benediktsson et al., 1993; Langley-Evans et al., 1996). The programmed effects of both these treatments are associated with dysregulation of the HPA axis (Langley-Evans et al., 1996; Levitt et al., 1996). More recently, Lindsay et al. observed increased plasma aldosterone concentrations in adult rats previously exposed to dexamethasone *in utero*. In addition, chronic and acute effects of angiotensin II (AII) elevated the blood pressure response more in the dexamethasone-treated group than in the control group (Lindsay et al., unpublished data). The results suggested that prenatal exposure to dexamethasone might have a programming effect on hypertension in later adult life mediated by modification of the renin-angiotensin system. In view of these observations and of the effects described in Chapter 4 of maternal dexamethasone on fetal adrenal development, it is possible that programmed dysregulation of the HPA axis in the adult offspring is due to abnormal growth and differentiation of the adrenal gland at a critical stage of fetal adrenal development. The hypothesis that effects of prenatal dexamethasone on fetal adrenal function persist into adulthood has been tested in the present chapter.

The protocol for these studies was similar to that described in the previous chapter except that tissues erere sampled at various intervals after birth when dexamethasone treatment has stopped. As before, adrenal expression of CYP11B2 and CYP11B1

proteins and mRNA transcripts were measured by western blotting and competitive RT-PCR. Preliminary adrenal morphologies from saline and dexamethasone-treated rats were examined using various immunostaining techniques. Samples were obtained from adult rats as well as from neonates. Measurements of enzyme expression in neonatal samples is complicated by the presence of CYP11B3. As indicated in the Introduction (Chapter 1), CYP11B3 is one of two other genes located adjacent to the CYP11B2 and CYP11B1 locus on rat chromosome 7. It is highly homologous with CYP11B1 and has similar biological and immunological properties which make it difficult to identify. A method for distinguishing CYP11B1 and CYP11B3 mRNA transcripts is described that confirmed which CYP11B3 is not expressed in adult adrenal glands.

5.2 Results

5.2.1 Effects of dexamethasone on body weights and organ weights

DEX-treatment throughout gestation resulted in a trend for lower body weights and organ weights in the offspring at 7 days, but without any significant differences compared to the 7-day-old control rats (Table 5.1). However, the prenatally DEX-treated offspring had a higher relative heart weight when compared to the control rats ($P<0.05$).

DEX-exposure during pregnancy also caused growth retardation, including lower body weights and organ weights, in 28-day-old offspring ($P<0.05$) (Table 5.2). However, the relative organ weights were significantly increased in the offspring with DEX-treatment *in utero* compared to the control 28-day-old offspring ($P<0.05$).

In 4-month-old offspring, prenatal DEX-treated rats showed a trend for lower body weights and organ weights compared to the saline-treated control rats though the differences did not reach statistically significant levels (Table 5.3).

Table 5. 1 Body weights and organ weights (mean ± S.E.) of 7-day old rats following prenatal treatment with saline or dexamethasone throughout pregnancy

	Saline (n=3)	Dexamethasone (n=4)
Body weight (g)	14.5±0.6	13.0±1.0*
Heart weight (mg)	105±3	101±5
Heart wt / Body wt %	0.74±0.02	0.80±0.03*
Kidney weight (mg)	90.9±3.7	82.6±5.3
Kidney wt / Body wt %	0.64±0.02	0.65±0.01
Adrenal weight (mg)	2.68±0.28	2.12±0.24
Adrenal wt (mg) / Body wt %	19.5±2.5	16.0±1.0

Data were analysed by the Student's t-test.

*: Significantly different from saline group ($P<0.05$).

n represents the number of dam animals

Table 5. 2 Body weights and organ weights (mean ± S.E.) of 28-day old rats following prenatal treatment with saline or dexamethasone throughout pregnancy

	Saline (n=3)	Dexamethasone (n=4)
Body weight (g)	110.8±4.9	77.8±4.2*
Heart weight (mg)	569.1±21.9	468.9±18.3*
Heart wt / Body wt %	0.52±0.02	0.59±0.02*
Kidney weight (mg)	577.8±23.2	445.1±19.5*
Kidney wt / Body wt %	0.52±0.01	0.56±0.01*
Adrenal weight (mg)	16.97±1.04	14.29±1.23
Adrenal wt (mg) / Body wt %	15.5±0.9	18.6±0.7*

Data were analysed by the Student's t-test.

*: Significantly different from saline group ($P<0.05$).

n represents the number of dam animals

Table 5. 3 Body weights and organ weights (mean ± S.E.) of 4-month-old rats following prenatal treatment with saline or dexamethasone throughout pregnancy

	Saline (n=5/m=8)	**Dexamethasone (n=5/m=5)**
Body wt. (g)	557±22	513±25
Heart wt. (g)	1.70±0.09	1.48±0.08
Heart wt/ Body wt %	0.31±0.02	0.29±0.02
Kidney wt. (g)	1.71±0.05	1.52±0.05
Kidney wt/ Body wt %	0.31±0.01	0.30±0.01
Adrenal wt. (mg)	39.3±2.1	33.0±3.1
Adrenal wt. (mg) / Body wt %	7.20±0.63	6.58±0.90

Data were analysed by the Student's t-test.

n numbers represent the number of dam animals treated and examined.

m numbers represent the number of offspring examined.

5.2.2 Immunostaining results

DEX-treatment increased the relative staining intensity for CYP11B2 in adrenals of 7-day-old offspring compared with the age-matched control rats (Fig. 5.1A). There was no effect on the adrenal staining intensity for CYP11B1 in the DEX-treated group compared to the control group (Fig. 5.1B).

No obvious effects on the adrenal staining intensity for CYP11B2 (Fig. 5.2A) and CYP11B1 (Fig. 5.2B) were found in 28-day-old offspring following prenatal treatment with DEX compared to the saline group.

5.2.3 Western blotting results

Prenatal saline- or DEX-treatment had no significant effect on the protein expression of CYP11B2 (Fig. 5.3A and B) and CYP11B1 (Fig. 5.3C and D) in 7-day-old rat adrenal glands although DEX-treatment tended to reduce (by 20% and 30%) in the enzyme expression of CYP11B2 and CYP11B1, respectively.

There was an apparent 20% reduction in the mean enzyme expression of adrenal CYP11B2 (Fig. 5.4A and B) in the 28-day-old offspring with DEX-treatment *in utero* compared to the control saline-treated offspring. However, the difference was not significant. Prenatal saline- or DEX-treatment had similar expression levels of adrenal CYP11B1 (Fig. 5.4C and D). It is worthy of note that there was an unknown band observed in some adrenal extracts which showed a similar molecular weight to CYP11B1 when the membrane was reapplied with the CYP11B1 antibody after stripping off the original CYP11B2 signal (Fig. 5.4C).

There was a 2.1-fold increase in the enzyme expression of adrenal CYP11B2 (Fig. 5.5A and B) in the 4-month-old offspring with DEX-treatment *in utero* compared to the control offspring. However, the difference was of borderline significance (P=0.052). DEX-treatment *in utero* also resulted in an apparent increase (20%) in the expression of adrenal CYP11B1 (Fig. 5.5C and D).

5.2.4 Gene expression for CYP11B2 and CYP11B1

The abundance of the adrenal CYP11B2 gene transcripts appeared to be slightly elevated in prenatally DEX-treated adrenals compared to the saline-treated adrenals in the 7-day-old rats (DEX, 18.61 ± 0.46 attomole/μg *vs.* saline, 10.92 ± 6.06 attomole/μg) (Fig. 5.6A). On the other hand, DEX-treatment throughout pregnancy did not alter the expression of CYP11B1 gene in adrenals of the 7-day-old offspring (DEX, 327.6 ± 39.2 attomole/μg *vs.* saline, 280.5 ± 90.1 attomole/μg) (Fig. 5.6B).

In adrenals of the 28-day-old rat offspring, prenatal DEX-treatment did not affect the expression of adrenal CYP11B2 gene transcripts (DEX, 14.0 ± 1.7 attomole/μg *vs.* saline, 15.0 ± 2.9x10^{-13} attomole/μg; P>0.5) (Fig. 5.10A). The expression levels of adrenal CYP11B1 gene transcripts were apparently 1.6-fold higher in prenatally DEX-treated adrenals than in the saline-treated adrenals (DEX, 2.1 ± 0.2 fmole/μg *vs.* saline, 1.3 ± 0.3 fmole/μg) (Fig. 5.10B). However, there was no significant difference (P>0.1).

The abundance of adrenal CYP11B2 gene transcripts appeared to be elevated (1.4-fold) in the adrenals of the 4-month-old offspring with DEX-treatment throughout pregnancy compared to the adrenals in the saline group (DEX, 3.11 ± 0.65 attomole/μg *vs.* saline, 2.20 ± 0.84 attomole/μg) (Fig. 5.13A) though there was no significant difference. Intrauterine DEX-treatment throughout pregnancy also resulted in a higher expression of CYP11B1 gene in adrenals of the 4-month-old offspring compared to the saline control animals (DEX, 0.92 ± 0.48 fmole/μg *vs.* saline, 0.25 ± 0.16 fmole/μg) (Fig. 5.13B). However, the difference was not significant because of the large variation within the experimental groups.

5.2.5 Detection of gene expression for CYP11B3

According to previous unpublished data (Communicated by Dr. C.J. Kenyon), CYP11B1 primers used in this experiment could amplify both CYP11B1 and CYP11B3 gene transcripts. However, when the RT-PCR products were digested with *Sma*I and were separated by 1% agarose gel electrophoresis, CYP11B1 gene transcripts were digested into a 220 bp and a 92 bp fragments. In contrast, the RT-PCR products of CYP11B3 gene transcripts were protected from *Sma*I digestion.

To determine whether the CYP11B3 gene was expressed in various rat adrenal specimens, RNA templates of adrenals from rats at different stages of development were amplified using CYP11B1 primers. Adrenal RNA templates were amplified from pregnant rats, E20 embryos, 7-day, 28-day and 4-month-old rats. In 7-day and

28-day-old rat adrenals, both CYP11B1 and CYP11B3 gene transcripts were detected. However, the adrenals of E20 embryos appeared to lack CYP11B3 gene transcripts (Fig. 5.7A). Neither saline nor DEX-treated pregnant rats and their 4-month-old offspring beared CYP11B3 gene transcripts (Fig. 5.7B and C).

To investigate if saline- or DEX-treatment throughout pregnancy would affect the expression levels of CYP11B3 in 7- and 28-day-old rat offspring, the relative intensities of the CYP11B3 bands, which were protected from the *Sma*I digestion, were quantified by AIDA software. The signals were calculated as the percentage of the total expression levels which were amplified by the CYP11B1 primers. In prenatally saline- and DEX-treated adrenals of the 7-day-old rats, CYP11B3 expression levels were only 10% and 7.5% of the total CYP11B1 expression levels, respectively (Fig. 5.8A and B). There was no correlation between CYP11B1 or CYP11B3 and total CYP11B1/CYP11B3 gene transcripts.

The abundance of the CYP11B3 gene was slightly reduced in prenatally DEX-treated adrenals compared to the saline-treated adrenals in the 7-day-old rats (DEX, 24.2 ± 2.9 attomole/μg *vs.* saline, 29.2 ± 9.4 attomole/μg) (Fig. 5.9A). Taking into consideration the co-expression of CYP11B3 and CYP11B1 gene transcripts amplified by CYP11B1 primers, the actual expression levels of CYP11B1 gene in the saline-treated adrenals were 251.3 ± 80.8 attomole/μg and the levels in the DEX-treated adrenals were 303.4 ± 36.3 attomole/μg (Fig. 5.9B) whereas the total expression levels of CYP11B1 were 280.5 ± 90.1 attomole/μg (saline) and 327.6 ± 39.2 attomole/μg (DEX), respectively (Fig. 5.6B).

The expression levels of total CYP11B1, including the expression of CYP11B1 and CYP11B3, contained 20% and 38% of CYP11B3 in prenatally saline- and DEX-treated adrenals of the 28-day-old rat offspring, respectively (Fig. 5.11A and B). The abundance of the CYP11B3 gene was moderately increased in prenatally DEX-treated adrenals compared to the saline-treated adrenals in the 28-day-old rats (DEX, 0.79 ± 0.11 fmole/μg *vs.* saline, 0.31 ± 0.21 fmole/μg; $P>0.05$) (Fig. 5.12A).

The actual expression levels of CYP11B1 gene in the saline-treated adrenals were 0.96 ± 0.06 fmole/μg and the levels in the DEX-treated adrenals were 1.29 ± 0.15 fmole/μg ($P>0.05$) (Fig. 5.12B) whereas the total expression levels of CYP11B1 were 1.3 ± 0.3 fmole/μg (saline) and 2.1 ± 0.2 fmole/μg (DEX) (Fig. 5.10B).

5.2.6 Effects of dexamethasone on morphological changes using serial staining by various antibodies

To assess the morphological changes of the adrenal gland, serial sections of adrenal glands were stained using various antibodies, which recognise specific cells of the adrenal cortex or medulla. The CYP11B2 staining was restricted to the ZG whereas CYP11B1 was detected in the ZF and the ZR (Fig. 5.14 and 5.15, A and E). 3β-HSD was localised over the entire cortex (Fig. 5.14 and 5.15D and H). Some 3β-HSD-positive cells were scattered in the medulla in both the saline and DEX-treated adrenals (Fig. 5.15D and H). Medullary cells could be distinguished by chromogranin A antibody (Fig. 5.14 and 5.15 C and F).

In the adrenal of the 7-day-old offspring, clear brown positive staining for CYP11B2 was shown under the capsule in the adrenals from the DEX-treated group (Fig. 5.14E) whereas low staining intensity was detected in control animals (Fig. 5.14A). On the other hand, the adrenal in the saline-treated rat showed weak staining for the CYP11B1 (Fig. 5.14B), while the DEX-treated adrenal showed intense staining between the edge of the ZG and the ZF with decreasing intensity towards the inner cortex (Fig. 5.14F). Comparison of the adrenal morphology and serial staining results in this preliminary study, showed that the most obvious difference between the saline- and DEX-treated adrenals of the 7-day-old offspring was in the central area of the adrenal gland. DEX-treated adrenals had a broad area of medullary cells and had more medullary cells, which were heavily stained by chromogranin A. In contrast, most medullary cells in the control adrenal showed weak chromagranin A staining (Fig. 5.14 C and F).

The positive staining for CYP11B2 was also clearly shown beneath the capsule and restricted to the ZG (Fig. 5.15A and E) and the staining of CYP11B1 was intense in the adrenals of the 28-day-old offspring (Fig. 5.15B and F). In addition, some adrenal cortical cells were also stained in the medulla in contact with chromaffin cells forming islets in the adrenals from both saline- and DEX-treated rats (Fig. 5.15B and F).

When the cortical areas of adrenal glands were carefully examined, a group of cells was seen apparently cortical cells, because they were positive for 3β-HSD staining (Fig. 5.16D). However, they were negative for CYP11B2, CYP11B1 and chromogranin A staining, respectively (Fig. 5.16A-C). Comparison of the adrenal cortical cell morphology in the 28-day-old rats treated with DEX *in utero* and in the saline-treated control rats, the adrenal sections of prenatally saline-treated rats showed homogenous staining for CYP11B1 in the ZF whereas the ZG was easily distinguished by negative staining (Fig. 5.17A). On the other hand, unusual zonation between the ZG and the ZF was observed in all adrenal sections from the prenatally DEX-treated rats (Fig. 5.17B-F). In these adrenal sections, CYP11B2 staining was still restricted to the ZG region, which was about 3 to 5 layers of cells. CYP11B1 was located in most of the cortical area. However, a broad area that was negative for both CYP11B2 and CYP11B1 was also observed in these sections (Fig. 5.17B-F).

To further investigate the morphological changes of the prenatally DEX-treated adrenal in the 28-day-old rat offspring, more serial sections of one adrenal gland randomly selected from each group were stained to examine the morphological differences. Several features were observed in the earlier sections of the DEX-treated adrenal gland in the 28-day-old rat offspring. Firstly, the medulla had an eccentric morphology (Fig. 5.16E-H). An irregular border between the medulla and the ZR was found unexpectedly in the adrenal from one of the DEX-treated offspring (Fig. 5.15F). Secondly, the medullary cells invaded the cortex. Rays of chromaffin tissue traversed the entire adrenal cortex. Thirdly, chromaffin cells were protruding into the ZG, often in subcapsularly (Fig. 5.16G). This was further confirmed by chromogranin A

staining (Fig. 5.15G) which identified the medullary cells. In contrast, few medullary cells occasionally showed in the ZR of adrenal sections of the 28-day-old saline-treated rats (Fig. 5.15C). In addition, cortical cells were also found intermingled with medullary cells (Fig. 5.16F and H).

5.3 Discussion

A wide range of studies, including the data presented in Chapter 4, have demonstrated that glucocorticoid treatment during pregnancy can lead to intrauterine growth retardation in man and experimental animals (Blackburn et al., 1965; Reinisch et al., 1978; Benediktsson et al., 1993; Levitt et al., 1996). Moreover, the impaired growth and development have been associated with increased risks for the later development of clinical diseases (Barker et al., 1989; Barker et al., 1990; Barker, et al., 1993a; Barker, et al., 1993b). In the present experiments, we tested the hypothesis that DEX exposure *in utero* may contribute to the postnatal growth failure and the changes of the expression levels of steroidogenic enzymes and their gene transcripts. The body weights and organ weights continued to be lower in prenatally DEX-treated rat offspring than in saline-treated rat offspring at the neonatal and adult stages. This observation is consistent with other animal studies which showed experimentally induced intrauterine growth retardation due to malnutrition (Langley and Jackson, 1994), stress (Pellegrini et al., 1998) or prenatal DEX exposure (Levitt et al., 1996; Welberg et al., 2001) resulting in postnatal growth failure without any signs of catch-up growth, and especially lighter body weight compared to control animals. It was reported recently that maternal DEX treatment regulated the HPA axis in a sex-specific manner in adult offspring of guinea pigs (Liu et al., 2001). The authors suggested that the increase in adrenal-to-body weight in the adult female offspring with antenatal DEX treatment was likely to be due to the increased cortical mass accompanied by the increased plasma cortisol levels, which indicated an increase in the HPA axis activity. In contrast, male adult offspring with intrauterine DEX treatment showed no significant changes of adrenal weight, however, marked reduction of the brain weight and the decrease of plasma cortisol levels were observed

(Liu et al., 2001). In the present study, it is possible that the lower adrenal weight of male adult offspring (4-month-old) with intrauterine DEX treatment results from the altered feedback regulation of the HPA axis.

Another interesting observation relates to the morphological changes deeper within the adrenal gland, especially in the adrenal medulla. Adrenals from the 7-day-old offspring with maternal DEX treatment throughout pregnancy had a broad area of medullary cells. More profound effects were found in the 28-day-old rats with the same prenatal treatment. Chromaffin tissues traversed the adrenal cortex and also protruded into the subcapsular areas. Although considerable research has been devoted to the effects of glucocorticoids on the enzyme activity in the adrenal medulla (Cole et al., 1995; Finotto et al., 1999; Kennedy and Ziegler, 2000), rather less attention has been paid to the morphological changes in the adrenal medulla. Some studies demonstrated that chromaffin tissues were also interspersed with cortical cells in normal rats, but less intensely than appeared in the present studies(Gallo-Payet et al., 1987; Bornstein et al., 1991; Bornstein and Ehrhart-Bornstein, 1992; Bornstein et al., 1994). Gallo-Payet et al. described that rays of chromaffin tissues were seen over four or five consecutive 5-μm sections in the rat adrenal gland (Gallo-Payet et al., 1987). However, the cord-like structure of the adrenal medulla from the 28-day-old offspring exposed to DEX throughout gestation was observed over 30 consecutive 4-μm sections whereas no particular feature was seen in the adrenal from the control rat. Such marked morphological changes of the adrenal medulla in neonatal and postnatal rat offspring exposed to intrauterine DEX was somewhat unexpected. Although there is no direct evidence to explain why the DEX exposure *in utero* leads to the morphological changes of the medulla at the postnatal stages in rats, it is likely to assume that the glucocorticoids may trigger the differentiation of sympathoadrenal precursors to endocrine chromaffin cells (Seidl and Unsicker, 1989) or that the phenomenon resulted from the direct effect of prolonged DEX treatment on adrenal tissues (Klepac, 1976; Barlow et al., 1979; Huss et al., 1996). Lack of glucocorticoid receptors (GR) in the adrenal medulla in GR-deficient mice impairs the development of chromoaffin cells in the adrenal glands, especially the loss of the expression of the

phenylethanolamine N-methyltransferase (PNMT) gene in chromaffin cells (Finotto et al., 1999). Whether the regulation of the glucocorticoid receptor expression in the medulla is altered by prolonged intrauterine DEX treatment remains to be determined.

In addition, scattered cortical cells, identified by 3β-HSD staining, were also found in the adrenal medulla of rats exposed to maternal saline and DEX treatments. This observation is consistent with other studies in mammals (Gallo-Payet et al., 1987; Bornstein et al., 1991; Bornstein and Ehrhart-Bornstein, 1992; Bornstein et al., 1994). The close arrangement of cortical and medullary cells is suggestive that functionally relevant intra-adrenal paracrine factors interact between these two distinct endocrine tissues.

Administration of ACTH or dexamethasone to rats can restore the activity of the PNMT enzyme that converts noradrenaline into adrenaline in the chromaffin cell, and the effect is diminished by hypophysectomy (Wurtman and Axelrod, 1966; Roffi, 1968). Also, catecholamines and other medullary products may also exert a regulatory effect on aldosterone secretion (De Léan et al., 1984; Racz et al., 1984; Pratt et al., 1985). Taken together, the morphological changes of the adrenal medulla may affect the paracrine regulation of the cortex mediated by chromaffin cells. Moreover, an ultrastructural study clearly demonstrated an exocytotic process from a chromaffin cell to a neighbouring cortical cell (Bornstein and Ehrhart-Bornstein, 1992).

Another prominent effect of the prenatal DEX exposure on adrenal morphology of the 28-day-old rats was the surface area of the adrenal cortex. A group of cells appeared to be cortical cells and this was confirmed by 3β-HSD staining. However, these cells were lacking in CYP11B2 and CYP11B1 expression. The features of these cells are similar to the stem cells that were identified by Mitani et al. (1994). These authors reported a cell layer located between the ZG and the ZF without CYP11B2 and CYP11B1. These cells were capable of proliferation and migration into the ZF. In addition, the cell number of the unstained cells was rapidly decreased when the rats were given a low sodium diet. It is likely that prenatal DEX exposure may affect the

proliferation of the stem cells and result in the change of the progenitor cell zone of the adrenal cortex.

CYP11B3 has been cloned from a rat genomic library (Mukai et al., 1993). Expression of the CYP11B3 gene could result in the formation of CYP11B3 mRNA encoding a protein of 498 amino acids. CYP11B3 closely resembles CYP11B1 in nucleotide (96% identical) and amino acid (94% identical) sequences. However, it was first reported that the CYP11B3 mRNA could not be detected in the adrenal even by the RT-PCR (Nomura et al., 1993). Later, others found that CYP11B3 mRNA was expressed in various rat brain tissues and in neonatal (Zhou et al., 1995) and early postnatal (8-32 days old) rat adrenal glands (Mellon et al., 1995), but was not expressed during fetal development or in the adult rat adrenal. In the present experiment, the CYP11B1 primers, amplified a 312 bp fragment corresponding to position 528-840 of CYP11B1 (Oaks and Raff, 1995), also amplified CYP11B3 transcripts. However, the RT-PCR products of CYP11B1 were digested into 92 bp and 220 bp fragments using *Sma*I enzyme (CCCGGG site). In contrast, CYP11B3 transcripts are protected against the enzyme digestion. To examine the expression levels of CYP11B3 in rat adrenals, different developmental stages of rats were tested. In agreement with other studies (Mellon et al., 1995; Zhou et al, 1995), the CYP11B3 transcripts were found in 7-day- and 28-day-old rat adrenals, but not in fetal, 4-month-old and pregnant rat adrenals. The relative amount of CYP11B3 mRNA was higher in 28-day-old than in 7-day-old rat adrenal. Mellon et al., (1995) suggested that CYP11B3 gene expression in the newborn rat adrenal was turned on between the 6th and 8th day of life.

When the CYP11B3 enzyme was expressed in MA-10 or COS-7 cells, the enzyme converted deoxycorticosterone to corticosterone and 18-hydroxydeoxycorticosterone, but not aldosterone (Mellon et al., 1995; Zhou et al., 1995). Furthermore, the zonal distribution of CYP11B3 was also restricted to the ZF and ZR. These results demonstrate that the function of CYP11B3 enzyme is more similar to CYP11B1. In addition, the regulation of CYP11B3 gene in the adrenal gland by ACTH is sex-dependent. When rats were treated with ACTH, the accumulation of CYP11B3

mRNA decreased only in adrenals from male rats and not from female rats (Mellon et al., 1995). In the present experiments, no obvious effect of the prenatal DEX treatment on the amount of the CYP11B3 transcripts was observed in the male offspring.

It is worthy of note that there is a doublet observed in some adrenal extracts of 28-day-old rats that is recognised by CYP11B1 antibody after stripping off the original CYP11B2 signal. Whether the extra band observed corresponds to the CYP11B3 enzyme remains to be determined. Furthermore, the physiological function of the CYP11B3 enzyme is still unclear and also why the rat adrenal expresses CYP11B3 gene only for a limited time. Whether the existence of this enzyme plays a role in steroidogenesis during development needs further investigation.

In the studies reported in Chapter 4, the protein and gene expression of the CYP11B2 were slightly increased and CYP11B1 were decreased in adrenals of the fetal rats prenatally treated with DEX throughout gestation. In the present experiments, the expression of the adrenal steroidogenic enzymes was investigated at three separate postnatal stages (7-day, 28-day and 4-month-old) in rat offspring treated with DEX *in utero*. Although there were no significant changes of the protein and gene expression of the CYP11B2 and CYP11B1 in adrenals of rat offspring, all the differences became more obvious in the 4-month-old offspring. For instance, the protein levels of CYP11B2 and CYP11B1 were slightly reduced in DEX-treated rats at 7-day and 28-day-old offspring whereas CYP11B2 had nearly 2-fold and CYP11B1 had 20% increases in the 4-month-old offspring. The CYP11B2 mRNA was raised by 40% in 4-month-old DEX-treated offspring compared to the age-matched control offspring. It is also interesting that the differences of the CYP11B1 expression levels between DEX-treated offspring and saline-treated control offspring appeared to increase progressively from 7-day-old offspring to 4-month-old offspring although the differences were not significant. It is also notable for the levels of CYP11B mRNA among the three various developmental stages. Both CYP11B2 and CYP11B1 gene transcripts were lower in the 4-month-old offspring compared to 7-day- and 28-day-old offspring. However, if the existence of CYP11B3 transcripts was

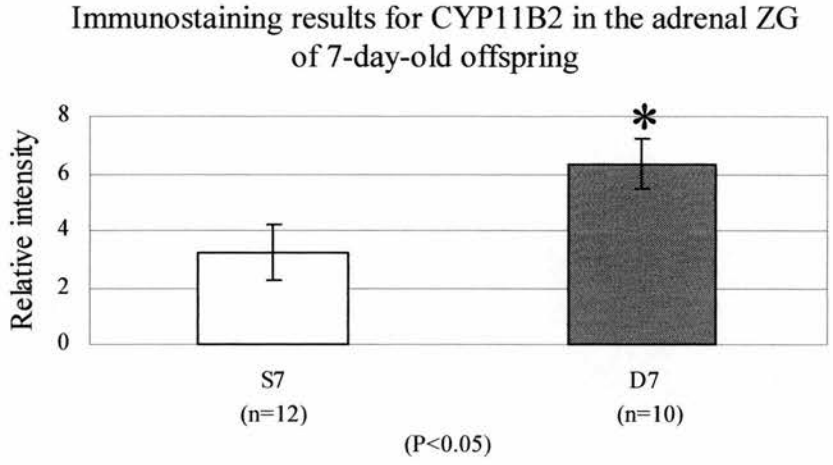
considered, the adrenal levels of CYP11B1 transcripts in 4-month-old offspring were close to those of the 7-day- and 28-day-old offspring.

Taking all these data into account, the lack of significant changes in the protein and gene expression of the steroidogeneic enzymes in 7-day and 28-day-old offspring might be due to a hyporesponsive period in rats. The hyporesponsive period is associated with decreased ACTH secretion that has been attributed to increased sensitivity of the neonatal pituitary to glucocorticoid feedback or to increased expression of a corticotropin release-inhibiting factor during the early postnatal stages (Schapiro, 1962; Sapolsky and Meany, 1986; Walker et al., 1986). In addition, it was demonstrated that the levels of angiotensin II receptors in the adrenal ZG were decreased from very high levels at birth to adult levels during the early postnatal period (Jones et al., 1989). Also, the sensitivity of aldosterone responses to angiotensin II was reduced in 7-day-old rats (Feuillan and Aguilera, 1996). These previous studies may indicate that the higher levels of the CYP11B2 and CYP11B1 mRNA are necessary to compensate for different feedback regulation of the RAS and HPA axis in 7-day- and 28-day-old offspring compared to the adult offspring. The existence of CYP11B3 gene transcripts at the specific period may also have some physiological significance. Another explanation of the negligible changes in steroidogenic enzyme expression is possibly because no further treatment was given to offspring after delivery. When female adult offspring prenatally treated with DEX were given chronic and acute AII injection, blood pressure and plasma aldosterone levels were elevated in the DEX-treated group compared to the control group (communicated by Dr. C.J. Kenyon). Therefore, the changes of steroidogenic enzyme expression and the programming effect on hypertension in later adult life may be manifested by AII, ACTH or other stress stimulations.

It has been shown that both maternal malnutrition (Ozanne et al., 1996) and DEX treatment (Benediktsson et al., 1993; Lindsay et al., 1996) result in impaired glucose intolerance, insulin resistance and hypertension in adult offspring in rats. Recently, Bertram et al. (2001) demonstrated that protein restriction during pregnancy programmed a tissue-specific increase in glucocorticoid receptors in the adrenal gland

and kidney whereas the expression of the 11β-HSD-2 enzyme was decreased in these two organs during adult life. Treatment of pregnant rats with DEX programmed decreased expression of GR in the hippocampal nuclei that mediate the central control of the HPA axis activity (Levitt et al., 1996). On the other hand, Whorwood et al. (2001) also reported that angiotensin II type I receptor (AT1), expression, but not AT2, was elevated in adrenal glands and kidneys of neonatal sheep exposed to maternal malnutrition during early to mid-gestation. These studies suggest that treatment of pregnant rats with DEX possibly programmes hyperactivity of the HPA axis through the increased GR expression in key peripheral glucocorticoid target tissues and diminished GR expression in the brain. In addition, 11β-HSD-2 enzyme expression in the adrenal and kidney is a key factor to protect the mineralocorticoid receptor from access by glucocorticoid. Furthermore, attenuation of the AT1 receptors and the RAS system in the adrenal and kidney by DEX may be a potential mechanism involved in the programming effects. Although in this study, there were no significant changes of the adrenal steroidogeneic enzyme expression, the morphological changes of the adrenal medulla may indicate another possible mechanism underlying the programming of adult disease by maternal glucocorticoid treatment.

A.



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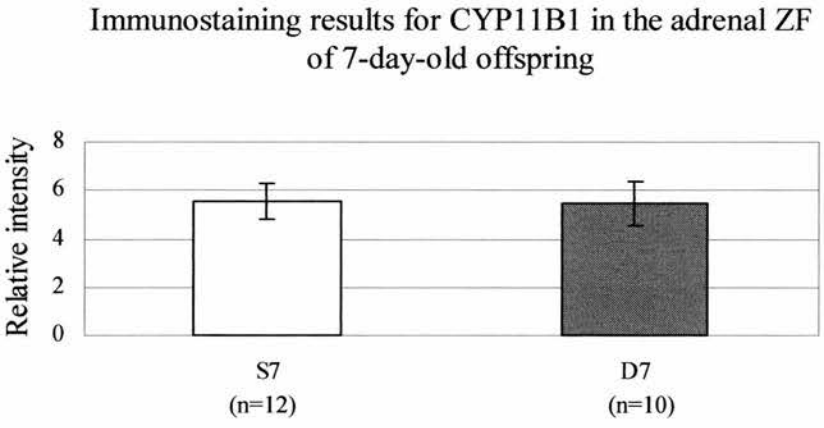
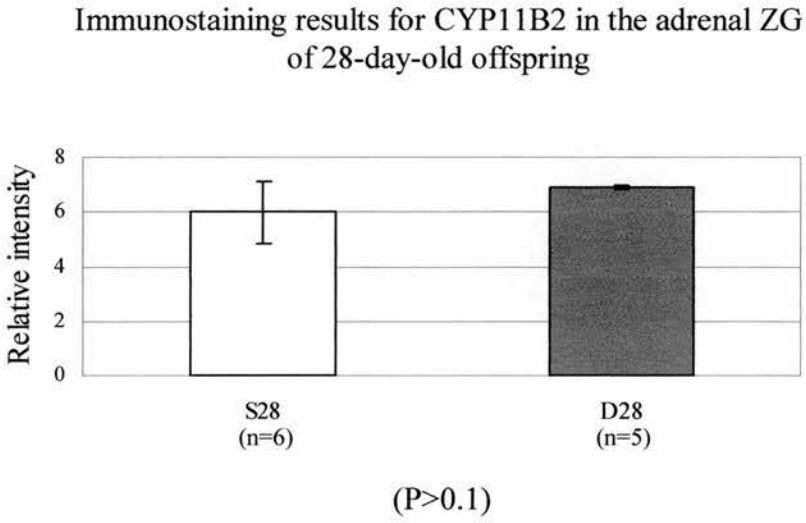


Fig. 5.1 Immunostaining results for CYP11B2 (A) and CYP11B1 (B) staining in the adrenal glands of prenatally saline- (S7) or dexamethasone- (D7) treated 7-day-old rat offspring. Values are mean±S.E. n represents the number of the animals tested. ZG and ZF denote zona glomerulosa, zona fasciculata. Data were analysed by the Student's t-test. * denotes statistical differences (P<0.05).

A.



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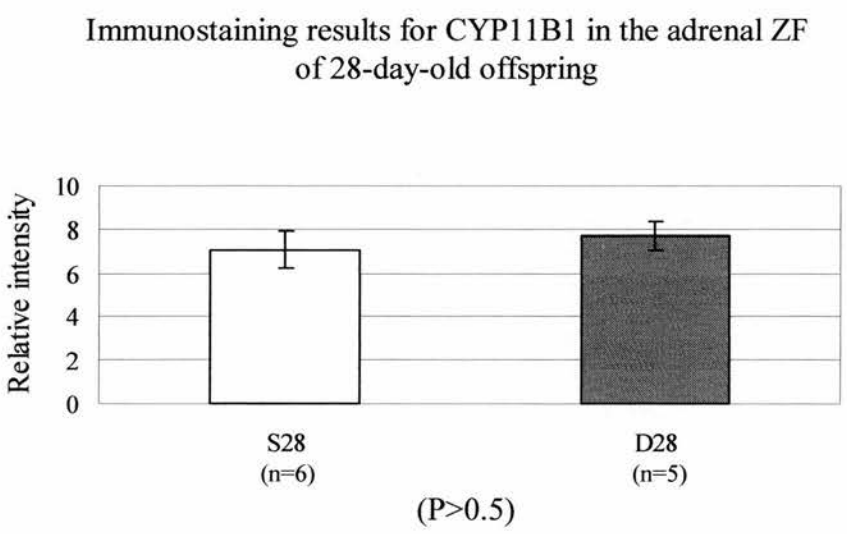


Fig. 5.2 Immunostaining results for CYP11B2 (A) and CYP11B1 (B) staining in the adrenal glands of prenatally saline- (S28) or dexamethasone- (D28) treated 28-day-old rat offspring. Values are mean±S.E. n represents the number of animals tested. ZG and ZF denote zona glomerulosa, zona fasciculata. Data were analysed by the Student's t-test.

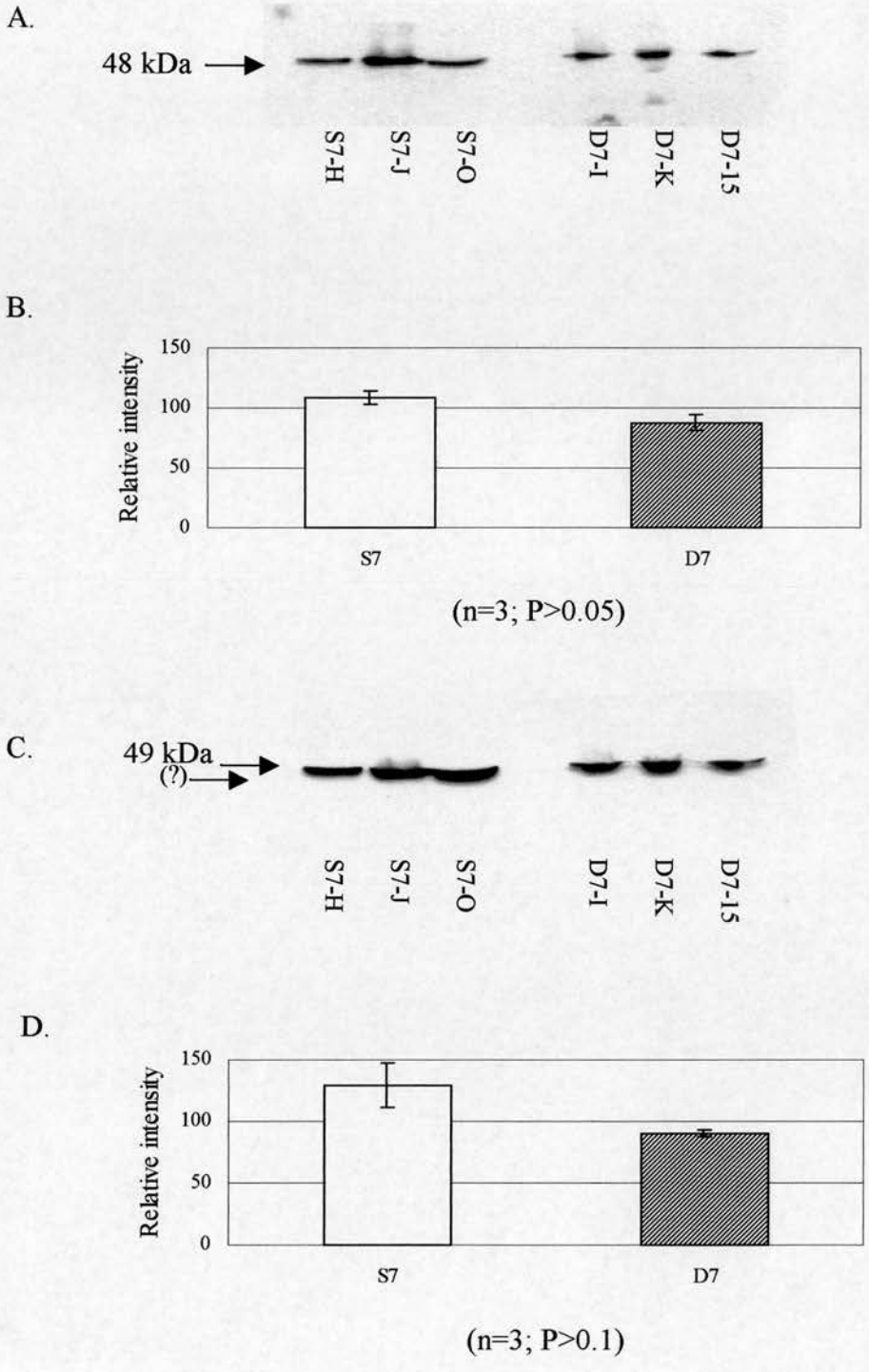


Fig. 5.3 Western blotting results for CYP11B2 (A and B) and CYP11B1 (C and D) in the adrenal glands of prenatally saline- (S7) or dexamethasone- (D7) treated 7-day-old rat offspring. A and C: 10% SDS-PAGE gel results, B and D: Relative intensity of the proteins quantified from the gels. Values are mean±S.E. n represents the number of the animals tested. Data were analysed by the Student's t-test.

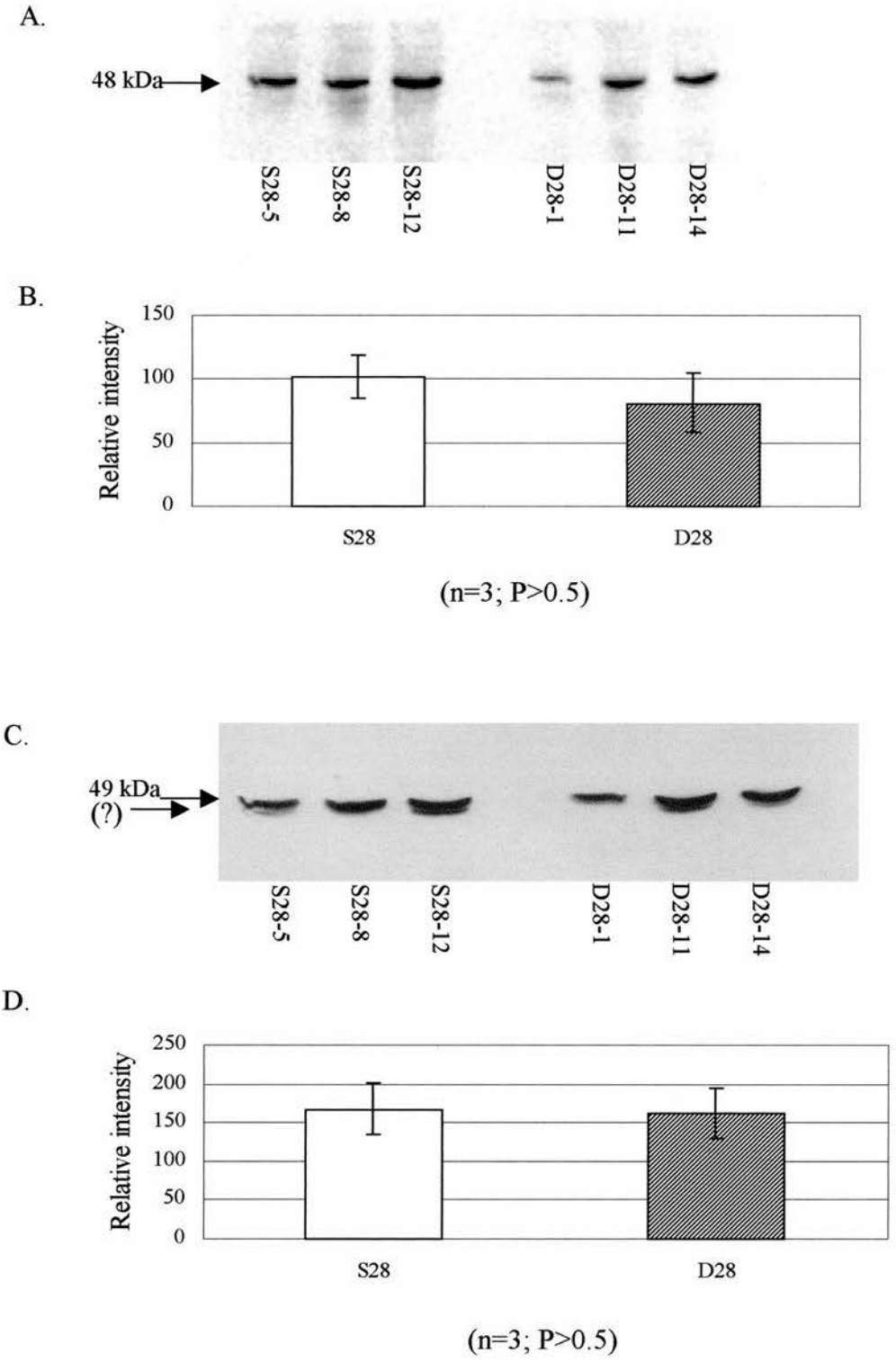


Fig. 5.4 Western blotting results of CYP11B2 (A and B) and CYP11B1 (C and D) in the adrenal glands of prenatally saline- (28) or dexamethasone- (D28) treated 28-day-old rat offspring. A and C, 10% SDS-PAGE gel results. B and D, Relative intensity of the proteins quantified from the gels. Values are mean±S.E. n represents the number of animals tested. Data were analysed by Student's t-test.

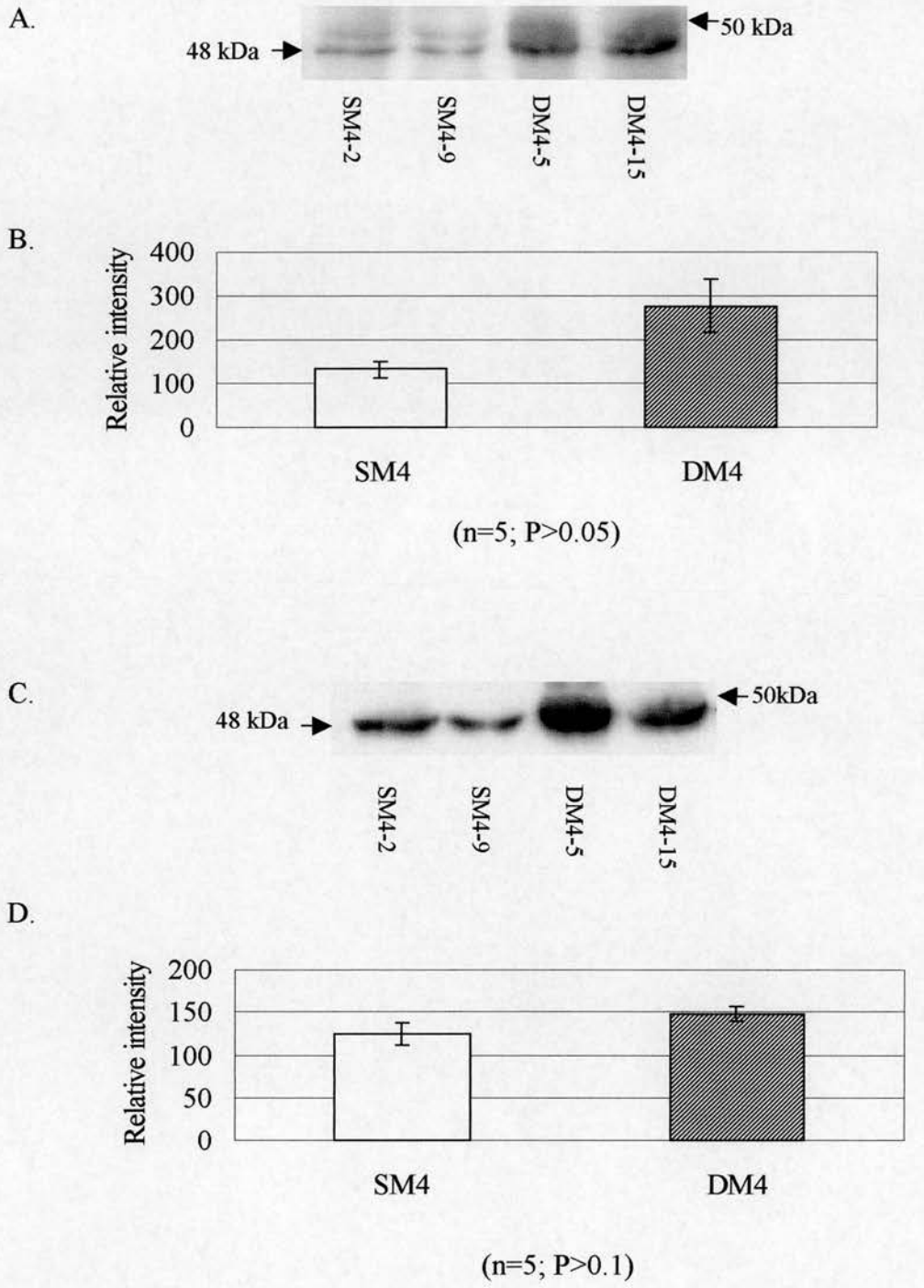
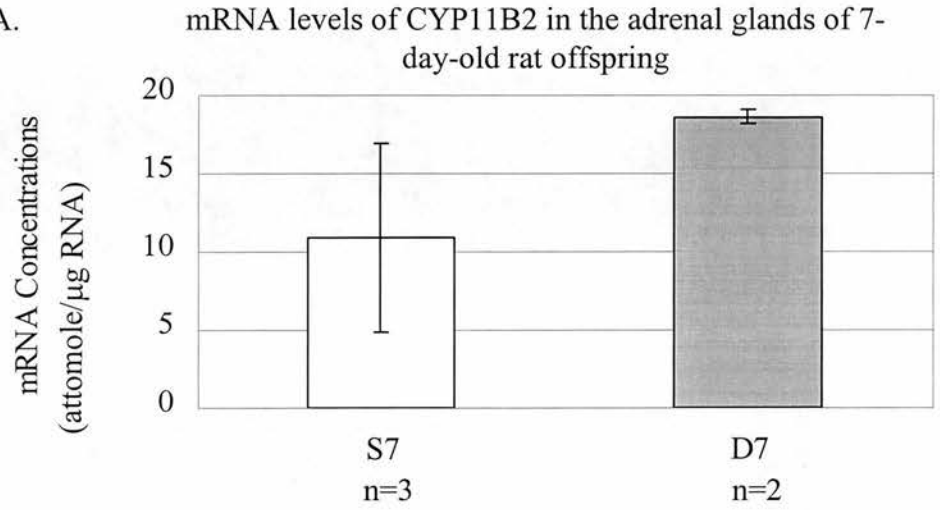


Fig. 5.5 Western blotting results of CYP11B2 (A and B) and CYP11B1 (C and D) in the adrenal glands of prenatal saline (SM4-X) or dexamethasone (DM4-X) treated 4-month-old rats. A and C, 10% SDS-PAGE gel results. B and D, Relative intensity of the proteins quantified from the gels. Values are mean±S.E. n represents the number of the animals tested. X represents an individual animal tested. Data were analysed by the Student's t-test.

A.



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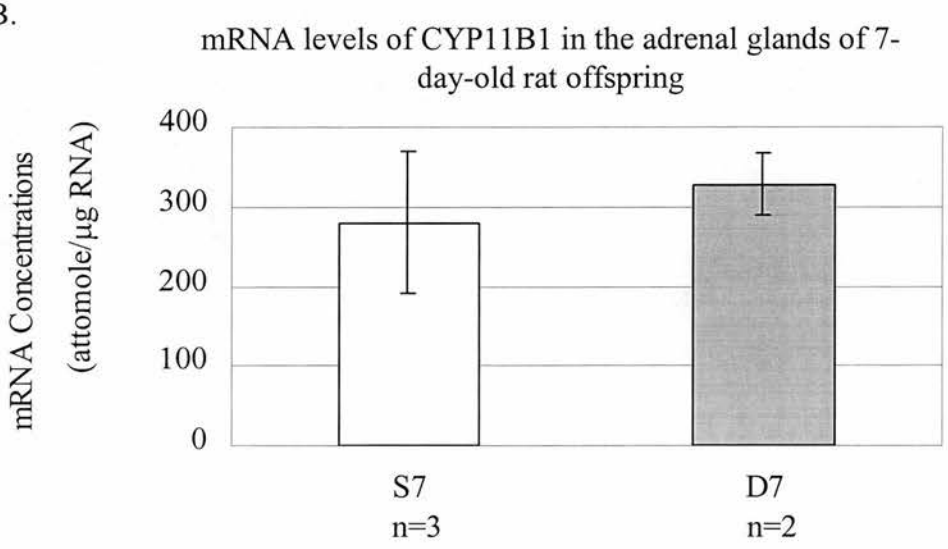


Fig. 5.6 Gene expression of the CYP11B2 (A) and CYP11B1(total) (B) using cRT-PCR in the adrenal glands of 7-day-old rats prenatally treated with saline (S7) or dexamethasone (D7) throughout the gestation. n represents the number of animals tested. Data are expressed as mean±S.E. in the saline-treated groups and the data in dexamethasone-treated groups (n=2) represent the range of the two determinations.

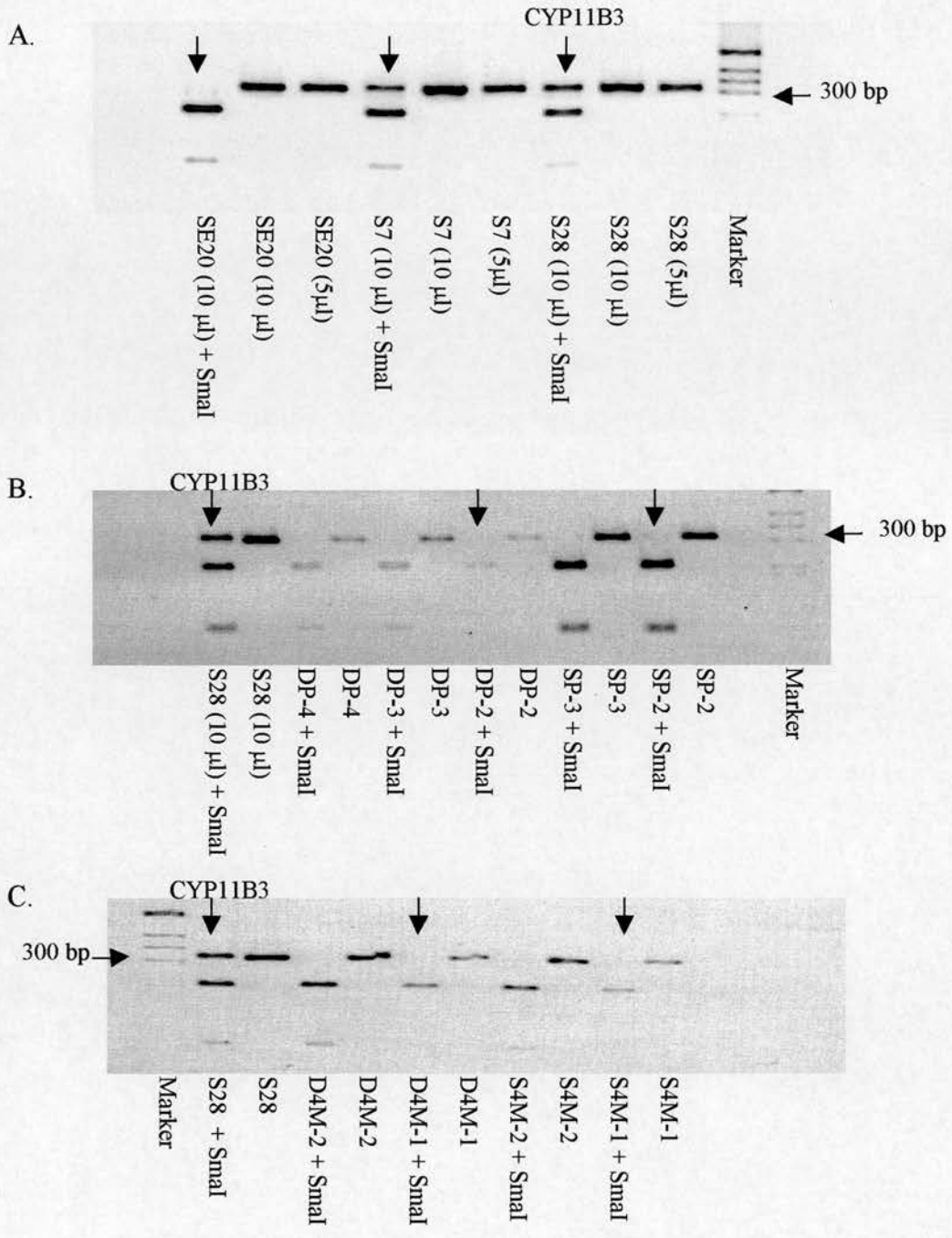
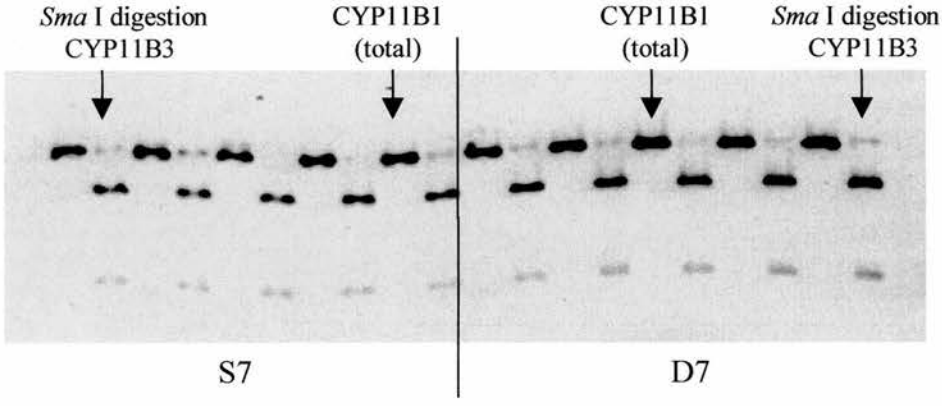


Fig. 5.7 The detection of the gene expression of the CYP11B3 in various developmental stages of adrenal glands of rats prenatally treated with saline- (S) or dexamethasone- (D) throughout the gestation. A; Adrenal RNA (10ng) amplified by CYP11B1 specific primers and digested by restriction enzyme (*Sma* I) from 28 (S28), 7 (S7)-day-old rats and 20-day (E20) embryos. B; Adrenal RNA from with saline or dexamethasone treated pregnant (SP-X *vs* DP-X) rats. C; Adrenal RNA from 4-month-old rats prenatal treated with saline (S4M-X) or dexamethasone (D4M-X) throughout the gestation. *Sma* I digests 312 bp product of CYP11B1 gene into 220 bp and 92 bp fragments. RT-PCR products of CYP11B3 gene are resistant to *Sma* I digestion. X represents an individual animal tested.

A.



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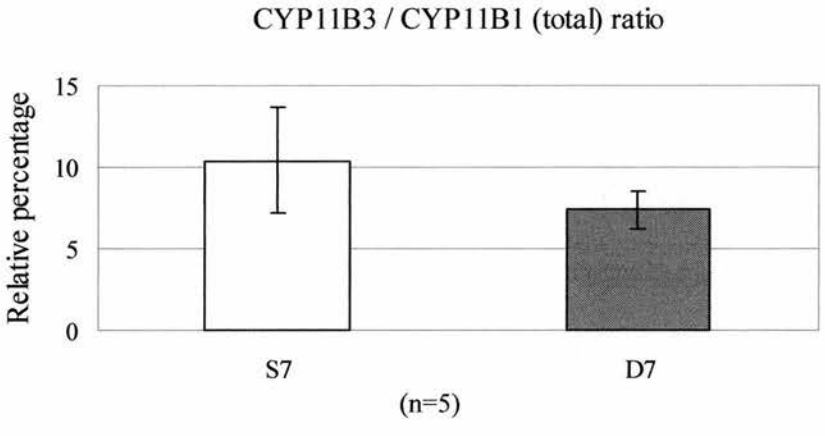


Fig. 5.8 The relative percentage of the gene expression of CYP11B3 and CYP11B1 (total) in the adrenal glands of 7-day-old rats prenatally treated with saline- (S7) or dexamethasone- (D7) throughout the gestation. A; Adrenal RNA (10ng) amplified by CYP11B1 specific primers and digested by restriction enzyme (*Sma* I). *Sma* I digests 312 bp product of CYP11B1 gene into 232 bp and 80 bp fragments. RT-PCR products of CYP11B3 gene are resistant to *Sma* I digestion. B; The relative percentage of CYP11B3 and CYP11B1 (total) gene expression. Values are mean±S.E. n represents the number of animals tested.

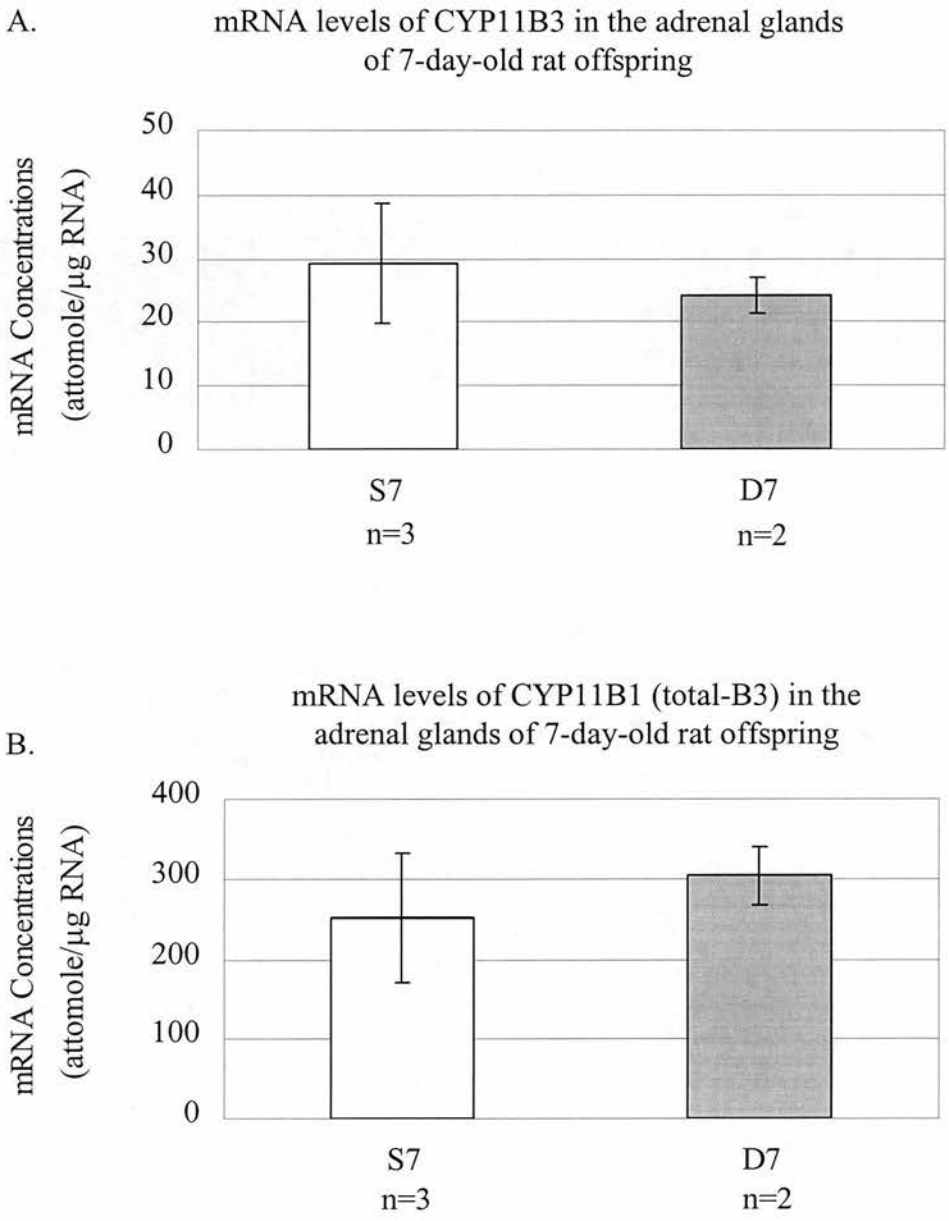


Fig. 5.9 Gene expression of the CYP11B3 (A) and CYP11B1 (total-B3) (B) in the adrenal glands of 7-day-old rat offspring prenatally treated with saline-(S7) or dexamethasone-(D7) throughout the gestation. n represents the number of animals tested. Data are expressed as mean±S.E. in the saline-treated groups and the data in dexamethasone-treated groups (n=2) represent the range of the two determinations.

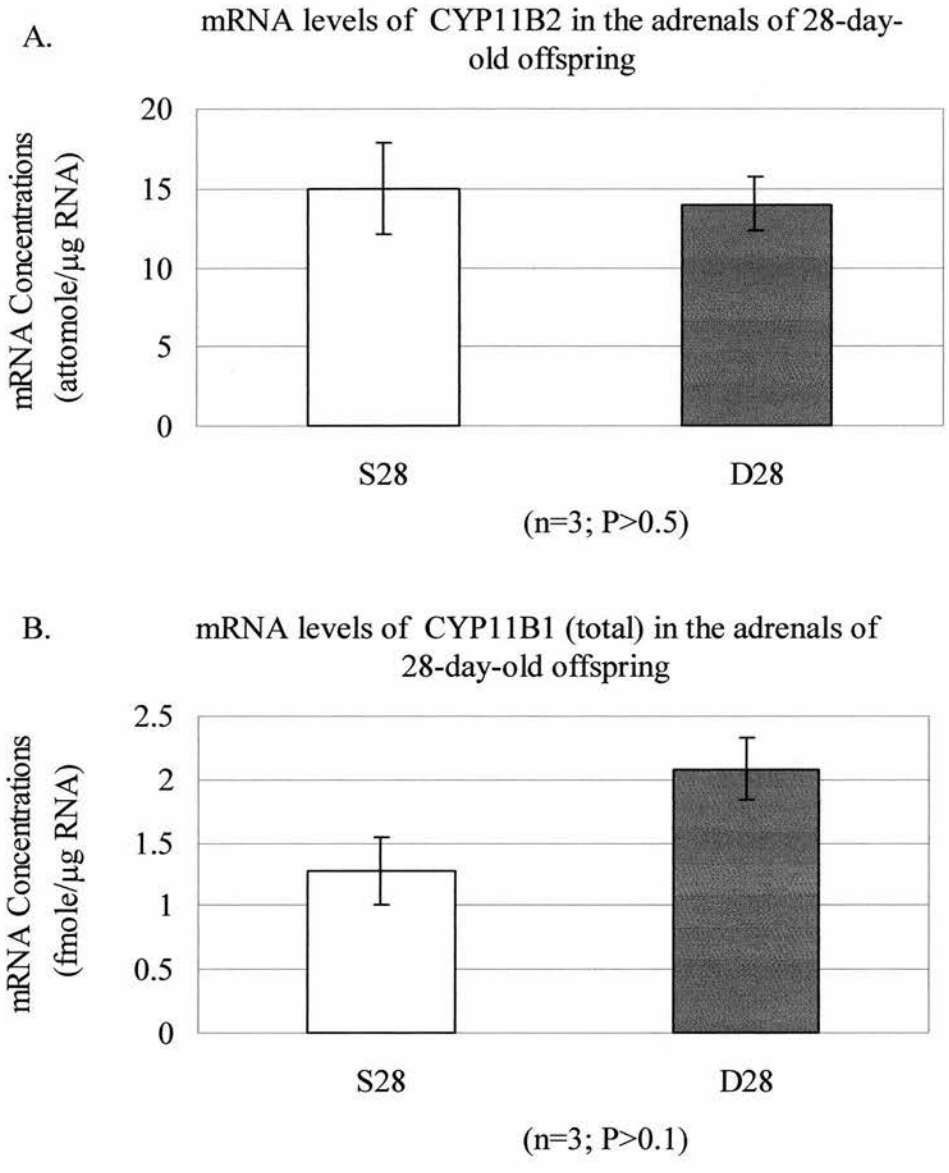
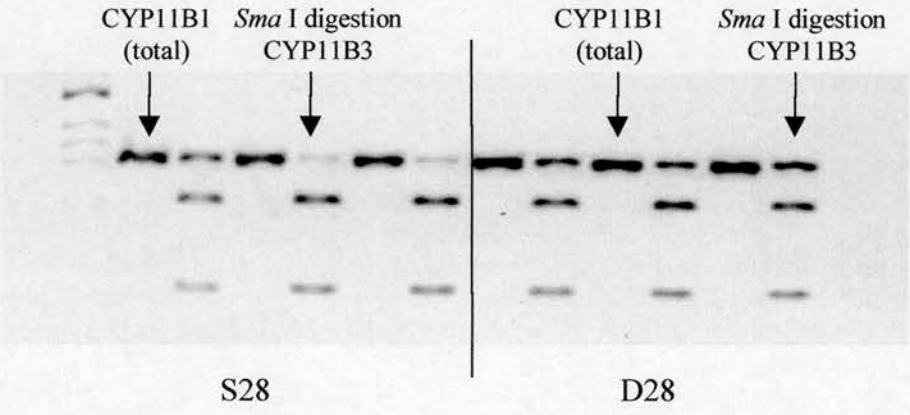


Fig. 5.10 Gene expression of CYP11B2 (A), and CYP11B1/B3 (B) using cRT-PCR in the adrenal glands of 28-day-old rats prenatally treated with saline- (S28) or dexamethasone- (D28) throughout the gestation. Values are mean ±S.E. n represents the number of animals tested. Data were analysed by Student's t-test.

A.



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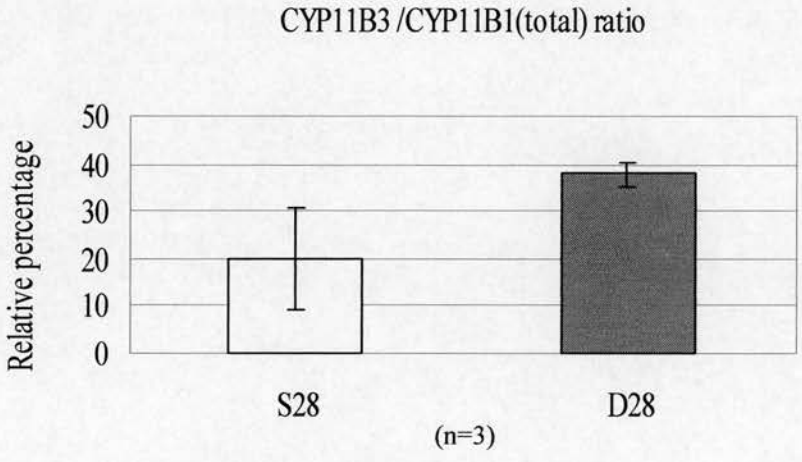

Fig. 5.11 The relative percentage of the gene expression of CYP11B3 and CYP11B1 (total) in the adrenal glands of 28-day-old rats prenatally treated with saline- (S28) or dexamethasone- (D28) throughout the gestation. A; Adrenal RNA (10ng) amplified by CYP11B1 specific primers and digested by restriction enzyme (*Sma* I). *Sma* I digests 312 bp product of CYP11B1 gene into 232 bp and 80 bp fragments. RT-PCR products of CYP11B3 gene are resistant to *Sma* I digestion. B; The relative percentage of CYP11B3 and CYP11B1 (total) gene expression. Values are mean±S.E. n represents the number of animals tested.

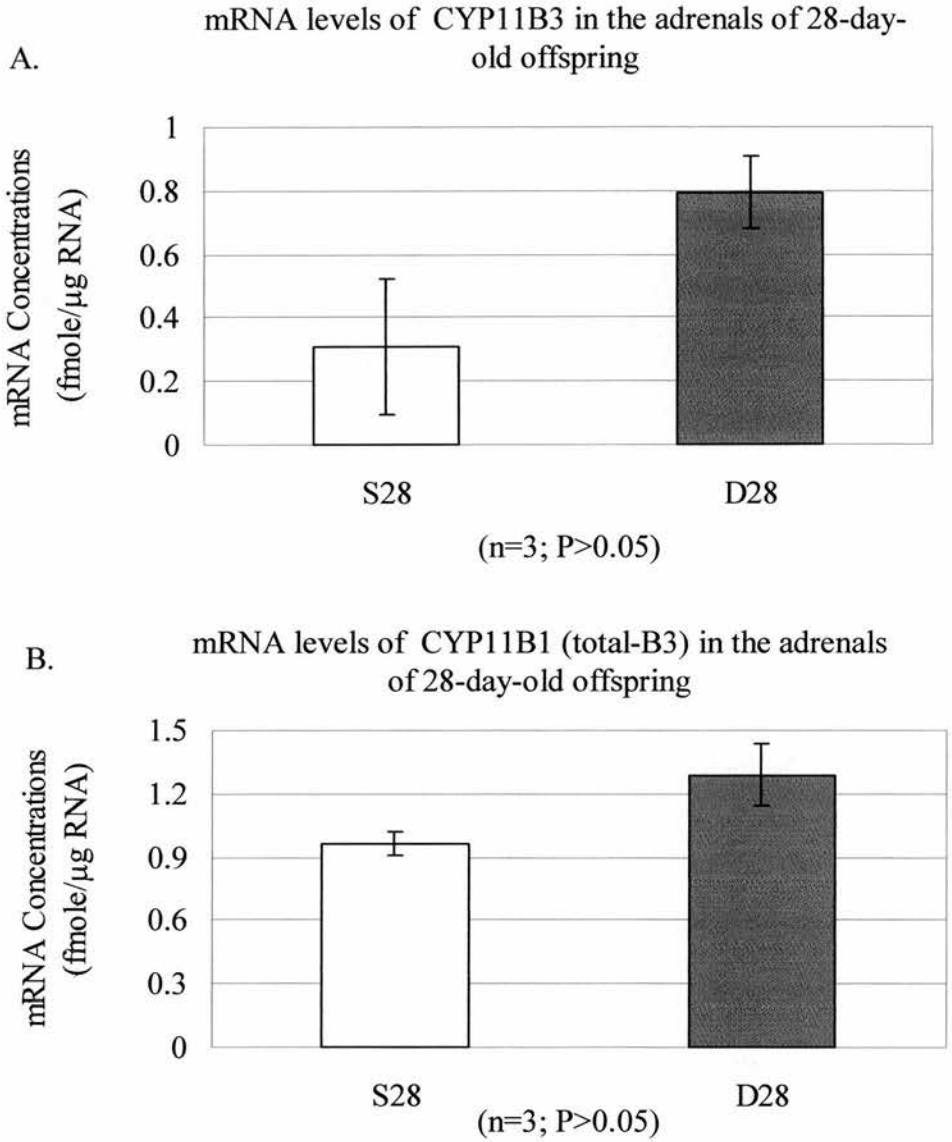

Fig. 5.12 Gene expression of the CYP11B3 (A) and CYP11B1 (total-B3) (B) in the adrenal glands of 28-day-old rats prenatally treated with saline (S28) or dexamethasone (D28) throughout the gestation. Values are mean±S.E. n represents the number of animals tested. Data were analysed by the Student's t-test.

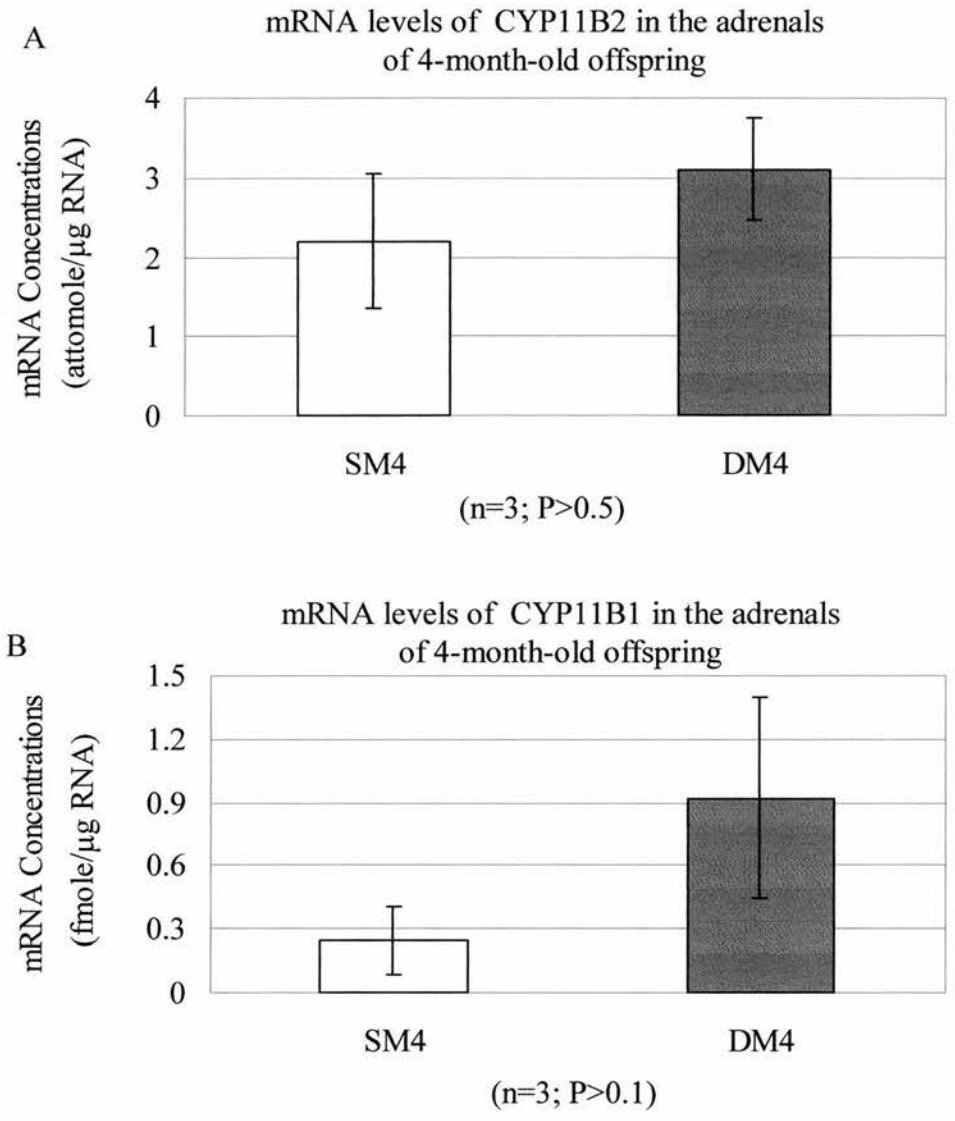

Fig. 5.13 Gene expression of the CYP11B2 (A) and CYP11B1 (B) using cRT-PCR in the adrenal glands of 4-month-old rat offspring prenatally treated with saline (SM4) or dexamethasone (DM4) throughout the gestation. Values are mean±S.E. n represents the number of animals tested. Data were analysed by the Student's t-test.

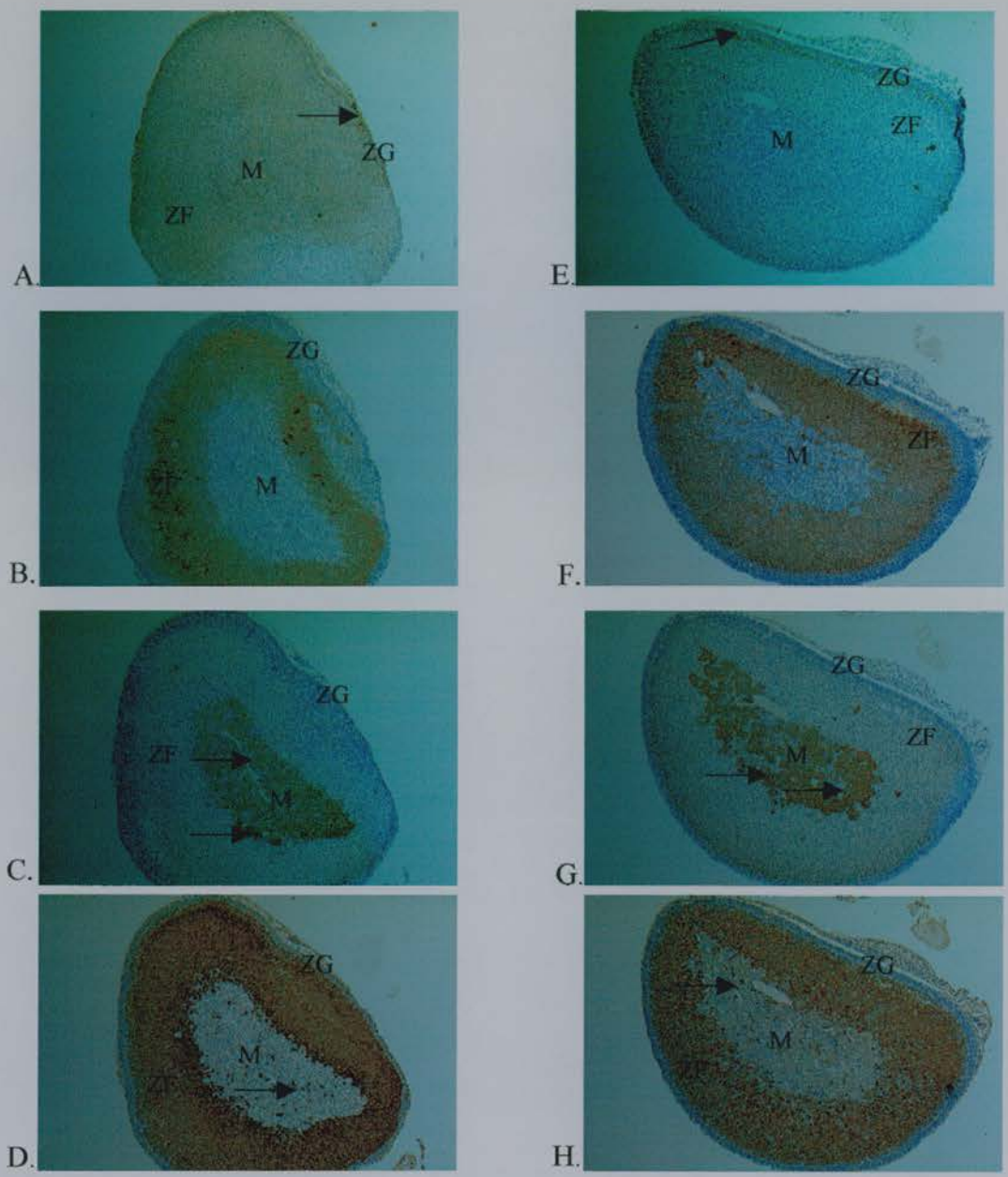

Fig. 5.14 Serial staining with various antibodies in the adrenal glands of prenatally saline- (A-D) or dexamethasone- (E-H) treated 7-day-old rats. A and E: CYP11B2, arrows showed positive staining for CYP11B2, B and F: CYP11B1, C and G: Chromogranin A, arrows showed two different staining intensities for chromaffin cells, D and H: 3 β-Hydroxysteroid dehydrogenase, arrows showed cortical cells in medulla. ZG, ZF and M denote zona glomerulosa, zona fasciculata and medulla, respectively. Magnification: 50X.

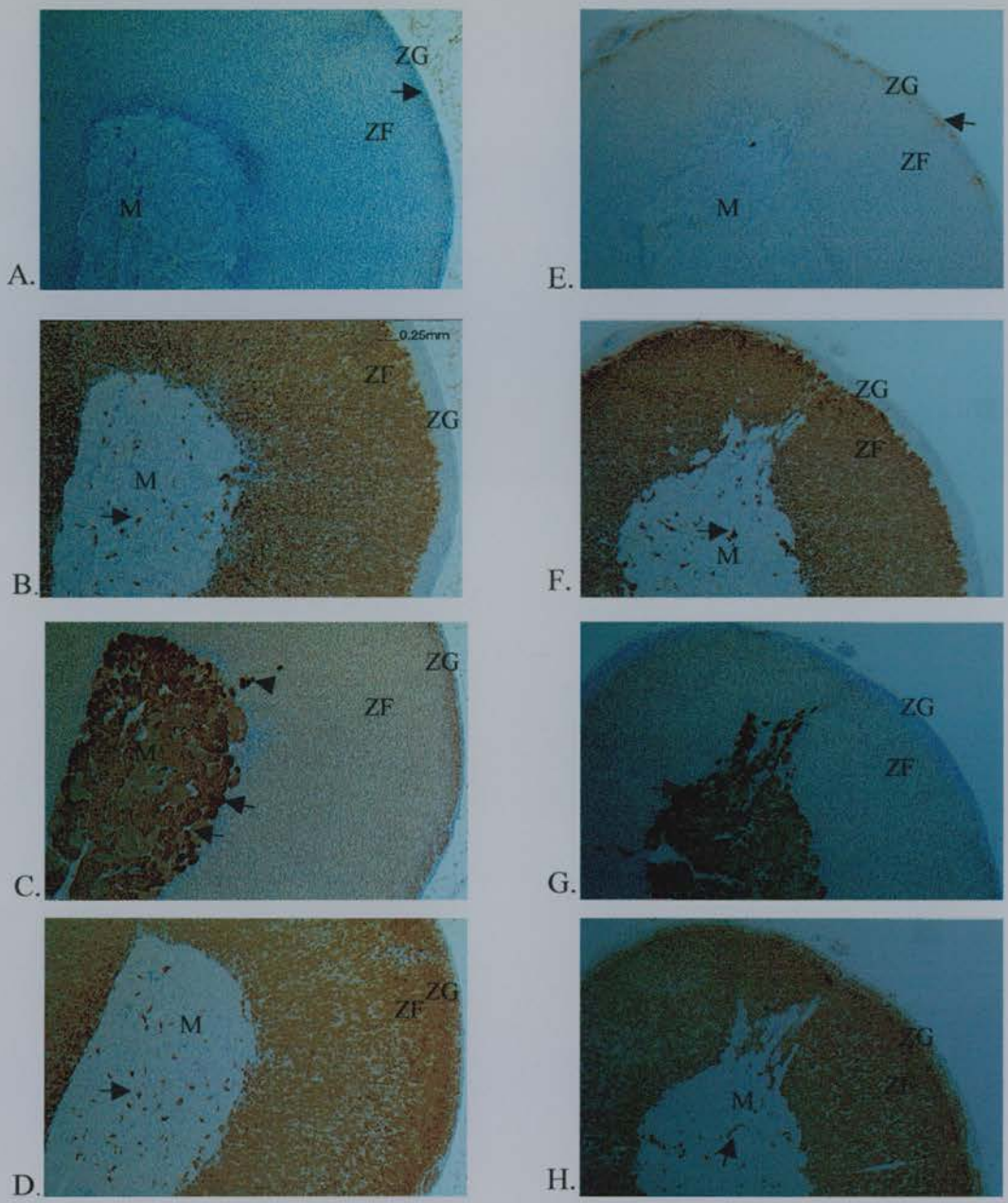

Fig. 5.15 Serial staining of various antibodies in the adrenal glands of prenatally saline- (A-D) or dexamethasone- (E-H) treated 28-day-old rat offspring. A and E; CYP11B2, arrows showed positive staining for CYP11B2, B and F; CYP11B1, arrows showed cortical cells in medulla, C and G; Chromogranin A, arrows showed two different staining intensities for chromaffin cells, arrowhead showed chromaffin cells in cortex, D and H; 3β-Hydroxysteroid dehydrogenase, arrows showed cortical cells in medulla. ZG, ZF and M denote zona glomerulosa, zona fasciculata and medulla, respectively. Magnification: 50X.

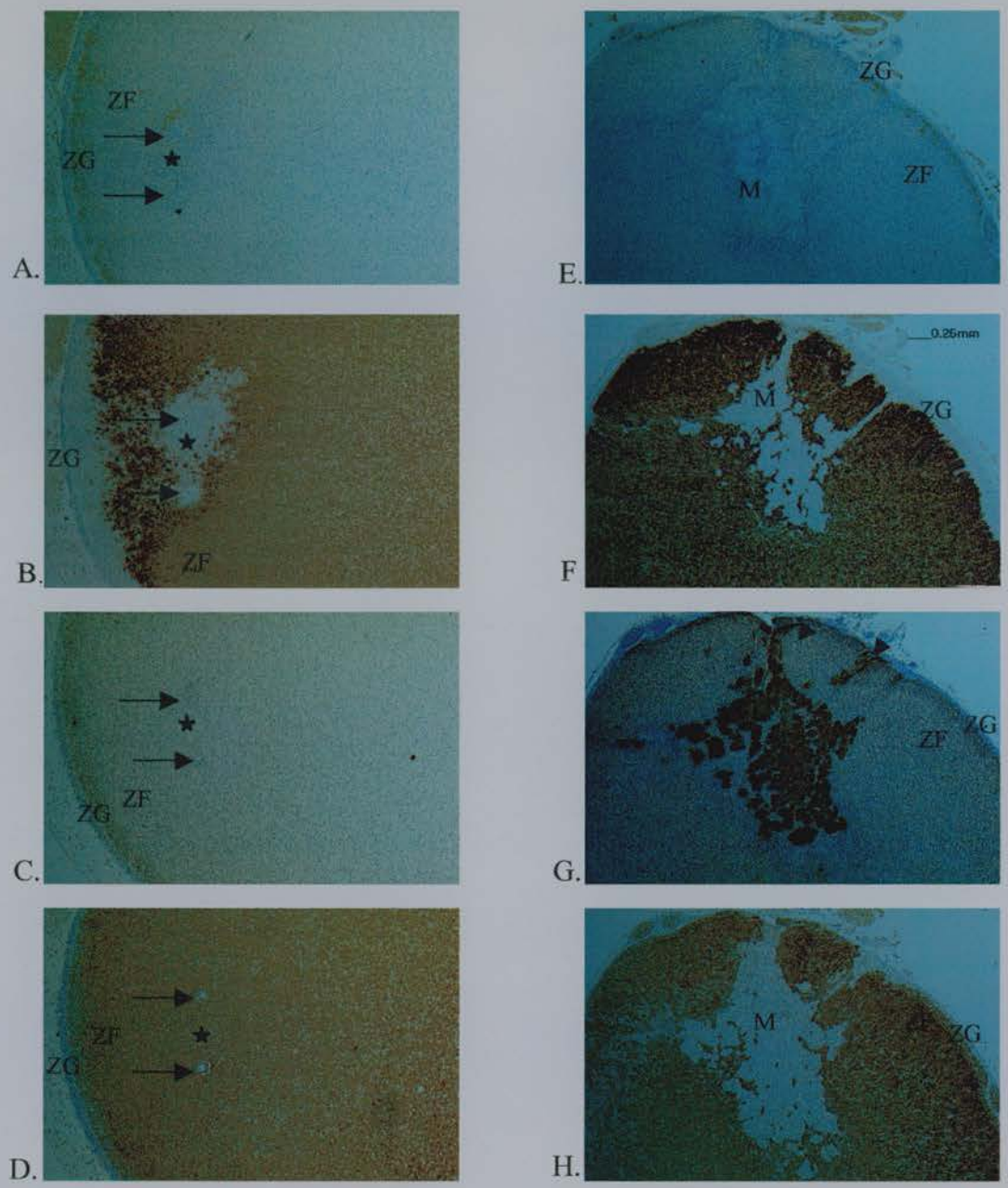

Fig. 5.16 Serial staining of various antibodies in the adrenal glands of prenatally dexamethasone-treated 28-day-old rat offspring. A and E; CYP11B2, B and F; CYP11B1, C and G; Chromogranin A, D and H; 3β-Hydroxysteroid dehydrogenase. ZG, ZF and M denote zona glomerulosa, zona fasciculata and medulla, respectively. Arrows showed the blood vessels, stars showed cortical cells without CYP11B2 and CYP11B1 staining, arrowheads showed the chromaffin cells protruded into the ZG. Magnification: 50X.

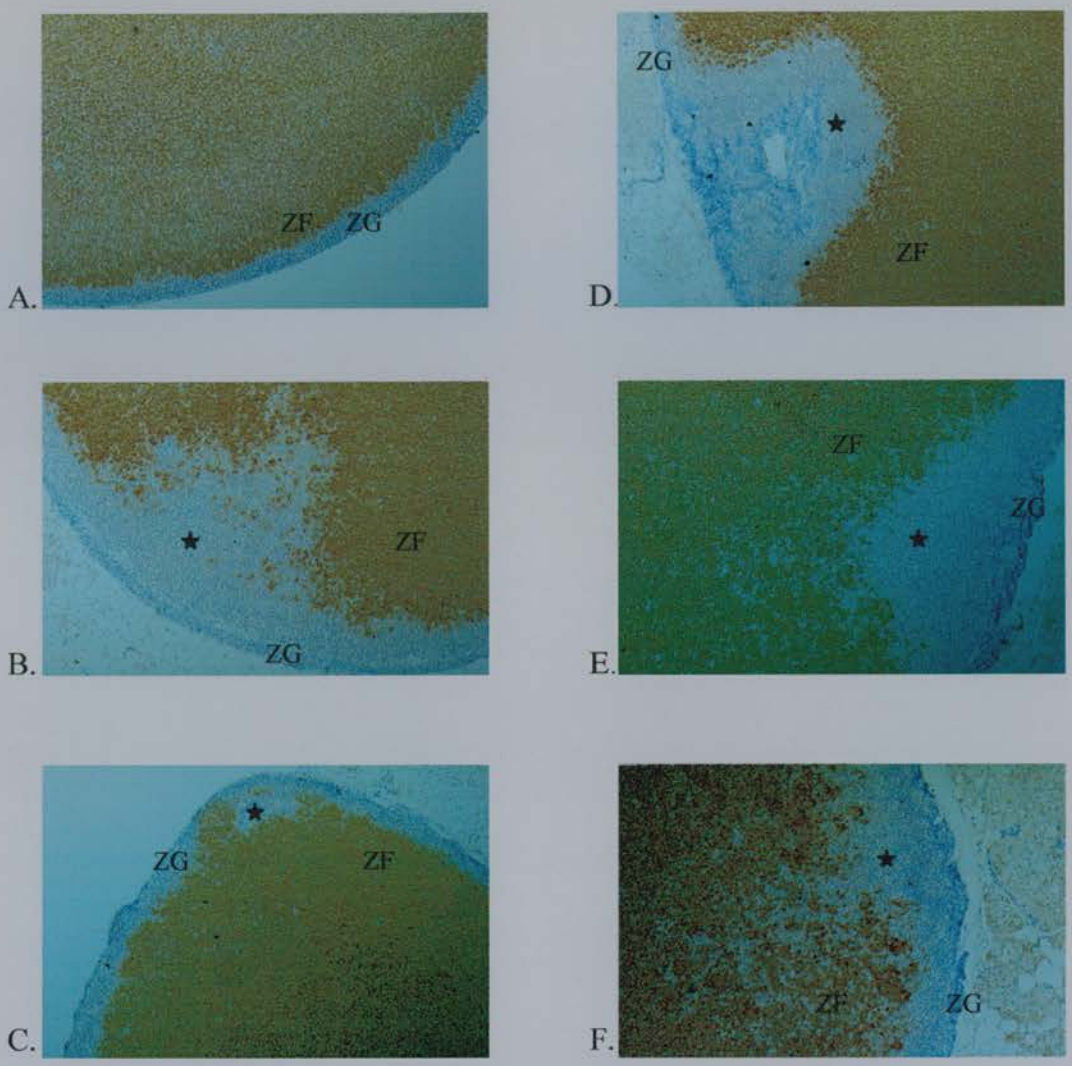

Fig. 5.17 Immunostaining for CYP11B1 in the adrenal glands of prenatally saline- (A) or dexamethasone- (B-F) treated 28-day-old rat offspring. Stars showed cortical cells without CYP11B2 and CYP11B1 staining. ZG and ZF denote zona glomerulosa and zona fasciculata, respectively. Magnification: 50X.

Chapter 6 Characterisation of a Steroidogenic Acute Regulatory (StAR) protein antibody in the rat adrenal glands

6.1 Introduction

The conversion of cholesterol to pregnenolone is the initial step of all the steroid biosynthesis. This reaction is catalysed by the cytochrome P450 cholesterol side-chain cleavage enzyme complex (CYP11A), which is localised on the inner mitochondrial membrane. It was hypothesised for many years that a labile, rapidly synthesised protein was necessary for the transport of cholesterol through the aqueous intermembrane space in response to acute hormonal stimulation (Jefcoate et al., 1986). Steroidogenic acute regulatory (StAR) protein (Clark et al., 1994) was first purified, cloned and sequenced from mouse MA-10 Leydig tumor cells and then expressed in COS-1 cells to verify functionality. The expression of StAR in these cells resulted in significant increases in steroid hormone synthesis in the absence of trophic hormone stimulation. In addition, the protein was synthesised in a timely- and dose-responsive manner to hormone stimulation, its synthesis was sensitive to cycloheximide treatment and the protein was found to be localised in the mitochondria. Cherradi et al. (1997) also demonstrated that StAR protein was localised to a greater extent in the intermembrane spaces than in the outer membrane or mitochondria matrix. These features suggested that StAR might be a key protein, which could potentially be responsible for cholesterol transport. It was further confirmed clinically that StAR protein mutation in humans was the cause of congenital lipoid adrenal hyperplasia, an autosomal recessive condition with severe deficiencies in steroid hormones (Lin et al., 1995).

Extensive *in vitro* and *in vivo* studies on the regulation of StAR protein have been conducted. Changes in StAR protein and mRNA have been linked to physiological changes in steroid secretion. In sheep, hypophysectomy resulted in decreased StAR

mRNA levels in the corpus luteum and serum progesterone levels (Juengel et al., 1995). Under acute stimulation by ACTH, StAR mRNA increased followed, after a 3-5 hour delay, by an increase in the level of StAR protein in the rat. On the other hand, when rat adrenals were hypertrophied by chronic ACTH stimulation, the expression of StAR protein was increased in the ZG and ZFR (Lehoux et al., 1998). Sodium restriction also affected StAR protein expression in the ZG, but not in the ZFR. This may imply that StAR protein is involved in the action of sodium restriction in promoting aldosterone secretion (Lehoux et al., 1999; Peters et al., 1998). Cherradi et al. (1997) also demonstrated that physiological increases in cytosolic calcium, produced either with a Ca^{2+} inonophore or with AII were effective in stimulating specific StAR protein accumulation, especially in the inner mitochondrial membrane.

On the basis of these previous studies, it was of interest to investigate whether factors regulating mineralocorticoid and glucocorticoid synthesis in adults, might have influences on adrenal StAR protein expression during development. Though time constraints did not allow analysis of changes in StAR protein expression with changes in mineralocorticoid and glucocorticoid levels in the experiments described in previous chapters, this chapter describes the characterisation of a novel StAR protein antibody for the specific analysis of StAR protein by immunohistochemistry and immunoblotting, which could potentially be employed in future physiological studies in the rat.

6.2 Results

6.2.1 Localisation by immunohistochemistry

6.2.1.1 StAR protein immunohistochemistry in rat adrenal gland

To loosen or break the crosslinkages caused by formalin fixation, various antigen retrieval techniques are often used for immunohistochemistry. In the rat adrenal gland, microwave-based antigen retrieval was essential for StAR protein antibody immunohistochemical optimisation. The optimal concentration of StAR protein primary antibody was found to be 1:100 in PBS buffer when various dilution ranges

were tried from 1:50 to 1:1000. The HRP-conjugated secondary antibody concentration was found to be optimal at 1:200. When the primary antibody was omitted from the immunostaining protocol, no staining was observed (Fig. 6.1A). The brown positive staining for StAR protein was noted in the three zones of the adrenal cortex and was differentially distributed. The most intense staining was found in the ZG and the ZF (Fig. 6.1B). The ZR only presented a weak staining. Unexpectedly there was even more intense in the central area of the rat adrenal (Fig. 6.1C and D).

6.2.1.2 Rat adrenal serial sections staining by various antibodies

To identify the cell types of the central area of the adrenal gland which showed StAR protein positive staining (Fig. 6.2A), serial sections of adrenal glands were stained with antibodies, which recognise specific cells of the adrenal cortex or medulla.

3β-HSD was localised in all three zones of the adrenal cortex (Fig.6.2B). The most intense staining area was between the ZG and the ZF in rat adrenal tissues. Most of the area of the medulla showed no staining, except where some cortical cells were clustered within the medulla (Fig.6.2B).

A chromogranin A antibody was used as a marker for medullary cells. The staining for chromogranin A appeared mainly in the adrenal medulla. Some chromaffin cells presented directly under the adrenal capsule or in the cortex (Fig.6.2C), where there was no staining for 3β-HSD in the consecutive sections (Fig.6.2B).

To compare the staining pattern of the peptide StAR protein antibody with another StAR protein antibody that was raised against the full length recombinant mouse StAR protein (mouse StAR protein antibody, generously provided by Dr. Buck Hales), the consecutive sections, incubated with the mouse StAR protein antibody, showed positive staining only in the adrenal cortex, especially in ZG and the outer ZF

(Fig.6.2D).

6.2.1.3 Comparisons of two separate StAR protein antibodies in mouse adrenal sections

The staining patterns of the two StAR protein antibodies in mouse adrenal sections are shown in Fig.6.3. Both of the negative control sections showed the absence of positive staining for peptide StAR protein and mouse StAR protein antibodies (Fig. 6.3 A and D). Immunostaining for both the antibodies was found intensely and homogeneously in all cortical zones (Fig. 6.3 B and E). However, the peptide StAR protein antibody also showed positive staining in the adrenal medulla (Fig 6.3 B and C) whereas the mouse StAR protein antibody did not exhibit any immunoreactivity in the medulla (Fig 6.3 E and F). In addition, both the antibodies showed weak staining for the StAR protein in the area between the cortex and the medulla (Fig 6.3 C and F).

6.2.1.4 StAR protein immunohistochemistry in the rat testis

The negative control section showed the absence of StAR protein staining (Fig. 6.4A) in the rat testis. StAR protein localised in most of the seminiferous tubules (Fig. 6.4B) and interstitial connective tissues (Fig. 6.4C) of the rat testis. Some Leydig cells displayed the positive staining (Fig. 6.4D). However, spermatocytes, Sertoli cells and spermatids also showed intense staining.

6.2.2 Immunoblotting

Subcellular samples of the adrenal cortex and total medulla homogenate were analysed by immunoblotting in the rat (Fig. 6.5 A and B). Immunoblotting experiments showed intense bands for StAR protein (30 kDa) in the mitochondria of rat ZG and ZF/R. A weak 30 kDa band was also detected in the microsomes in the rat ZF/R. No StAR protein was found in the rat medulla (Fig. 6.5A). A 42 kDa protein,

corresponding to 3β-HSD, was detected in both the mitochondrial and microsomal subcellular fractions of the ZG and the ZF/R. (Fig. 6.5B).

Immunoblot analyses of total protein homogenate from various rat tissues indicated that StAR protein was present only in the adrenal and ovary. There was no specific signal detected in brain, heart, liver, kidney and testis (Fig. 6.5C).

6.2.3 Expression of StAR mRNA transcripts

RT-PCR with specific StAR primers amplified a 494 bp fragment of the StAR mRNA (Fig 6.6). To define the profiles of StAR mRNA expression in rats during development, adrenal total RNA was extracted from pregnant rats and at various stages of development in offspring of rats treated with saline throughout gestation. StAR transcripts were expressed in all adrenal samples (Fig 6.6) although at different levels. Adrenals from 28-day-old rats had the highest expression levels of StAR mRNA among all the samples. The abundance of StAR mRNA in adrenals of 20 day embryos was similar to that observed in 7-day-old rat adrenals. In contrast, relatively lower levels of the StAR mRNA were observed in the 4-month-old and pregnant rat adrenals.

6.3 Discussion

The localisation of StAR protein has been confined to the adrenal cortex, testicular and ovarian tissues of adult mice (Clark et al., 1995), and humans (Pollack et al., 1997). The distribution of StAR protein and mRNA in the rat adrenal gland has also been established (Peters et al., 1998; Lehoux et al., 1999). In these studies, the StAR gene and protein were expressed in the three zones of the adrenal cortex with high intensity in the ZF. A few cells also appeared positive for StAR protein in the adrenal medulla (Lehoux et al., 1999). Peters et al. (1998) detected single cells with StAR protein gene expression in the adrenal medulla, whereas CYP11B2 mRNA was always negative in this area using *in situ* hybridization techniques. In the present study, the staining for StAR protein was shown, in agreement with others, mainly in

the adrenal ZG and ZF with less intensity in the ZR. However, the adrenal medulla also exhibited intense immunoreactivity for the peptide StAR antibody. Moreover, these immunopositive areas were further confirmed to be medullary cells rather than cortical cells, except for a few clusters of cortical cells contained within the medulla.

The mouse adrenal cortex has a region called the X-zone that resides between the ZF/R and the medulla. The expression of the X-zone in mice varies with strains, genetic condition, age and reproductive status (Deacon et al, 1986; Parker and Schimmer, 2001). To compare the staining patterns in rat adrenal tissues, mouse adrenal sections were stained by both peptide StAR protein antisera and mouse StAR protein antisera in the present studies. Similar results were also shown in mouse adrenal sections, whereas localisation using mouse StAR protein antibody was demonstrated mainly in the mouse adrenal cortex. Whether the X-zone is responsible for the steroid synthesis and expressing StAR protein needs further investigation.

Adrenocortical cells have been found in the medulla in contact with chromaffin cells forming islets or retaining some contact with the rest of the cortex (Bornstein et al., 1991; Berka et al., 1996). Because of the close arrangement of cortical and medullary cells, it has been considered that functionally relevant intra-adrenal paracrine interactions between these two different endocrine tissues occur (Gallo-Payet et al., 1987; Bornstein et al., 1991; Bornstein and Ehrhart-Bornstein, 1992; Bornstein et al., 1994).

The specificity of the peptide StAR protein antibody was examined by immunoblotting. Consistent with other studies, a 30 kDa band, corresponding to StAR protein, was detected mainly in the mitochondria of the rat ZG, ZF and the total protein homogenate of the adrenal and ovary. Immunoreactive protein was not found in the rat medulla by immunoblotting, but this may relate to the limited amount of pure rat medullary tissue available. However, one should also consider that immunohistochemical findings do not always correlate with immunoblotting results for some antibodies. This may be due to the fact that the antigenic determinants

presented to the antibodies are different when comparing fixed tissue sections with tissue homogenates in which the proteins have become denatured. A similar conclusion may be relevant to the results seen in the rat testis which showed no indication of the existence of StAR protein by immunoblotting. Clark et al. (1995) suggested that the restricted StAR protein in the Leydig cells that compose only 5-8% of the testicular cell content resulted in a low detection level by immunoblotting (Clark et al., 1995). However, the immunostaining results indicated that not only the Leydig cells, but also spermatocytes and Sertoli cells showed StAR protein positive staining in rat testis. Pollack et al. (1997) reported the presence of StAR protein in human Sertoli cells, renal tubules and fetal oocytes, which do not express CYP11A and are not able to convert cholesterol into pregnenolone. Gregory et al. (1998) also detected StAR protein in mitochondria of cultured rat Sertoli cells incubated with follicle-stimulating hormone. These authors suggested that StAR protein might have roles in metabolic process in addition to facilitating pregnenolone synthesis (Pollack et al., 1997; Gregory et al., 1998). At this preliminary stage one can not rule out the possibilities that the studies reported by other groups and by myself in this chapter are detecting a protein which is not StAR protein, but a closely related protein with common antigenic determinants.

It has been demonstrated that StAR protein is differentially regulated at different developmental stages in the rat adrenal. StAR transcripts were also expressed in the adrenal primordium at days 11 of the embryo (E11) and in the adrenal cortex in the adult. During the gonadogenesis, StAR protein expression was restricted to testicular Leydig cells from E12.5 throughout the embryogenesis and in the adult testis (Hasegawa et al., 2000). On the other hand, the expression of StAR transcripts was detected in the corpus luteum of adult ovary (Clark et al., 1995). Recently, Furukawa et al. (1998) found that StAR transcripts were constitutively expressed in the adult rat brain using more sensitive molecular biological methods. In StAR protein knockout mice, the adrenal gland was affected most severely at birth and exhibited progressive increases in lipid deposits with aging, especially marked changes in the ZG and the ZF (Hasegawa et al., 2000).

In this preliminary study, the expression of StAR mRNA was relatively high in the adrenals of the 20 days of embryos and in early postnatal rats (7-day- and 28-day-old rats) compared to the expression levels in the adult and pregnant rat adrenals. It is known that basal plasma corticosterone levels are very low during postnatal stress-hyporesponsive period in rats (Schapiro, 1962). In contrast, maternal and fetal corticosterone levels are high during late pregnancy in rats (Dupouy et al., 1975). It is not clear why the expression of StAR mRNA was not correlated to the changes in plasma corticosterone levels during these developmental stages. Accordingly, StAR protein may have some other as yet unidentified functions in adrenal gland, especially in the adrenal medulla. Alternatively, there may have other protein in the adrenal medulla, which exhibits similar epitope to the structure or function to StAR protein. Notably, however, no immunoreactive species other than the 30 kDa one, was demonstrated either in total rat adrenal or testis homogenates. Clearly, future studies are required to increase our knowledge in this area in order to determine the significance of the findings already reported in the literature by other workers and the current findings described in this chapter.

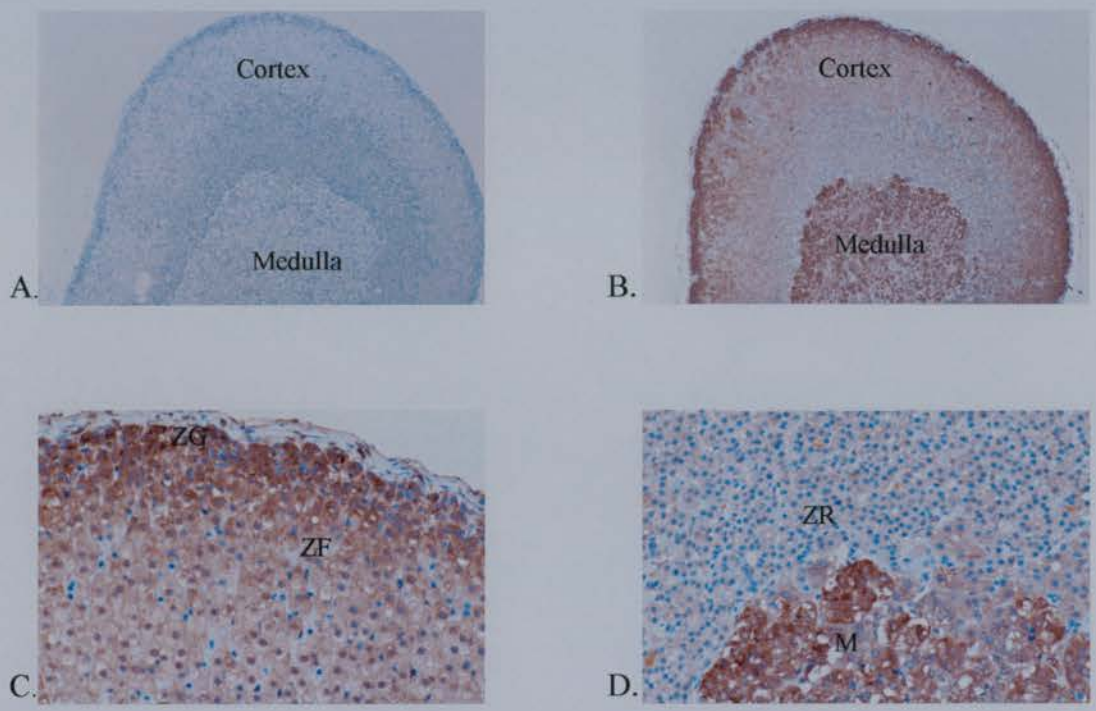

Fig. 6.1 Immunohistochemical localisation of StAR protein in the rat adrenal gland. A, negative control section shows the absence of StAR protein positive staining (48x magnification). B, localisation of StAR protein shows brown positive staining in the adrenal cortex and medulla (48x magnification). C, high power image of the ZG and ZF (120x magnification). D, high power image of the ZR and medulla (300x magnification). ZG, ZF, ZR and M denote the zona glomerulosa, zona fasciculata, zona reticularis and medulla, respectively.

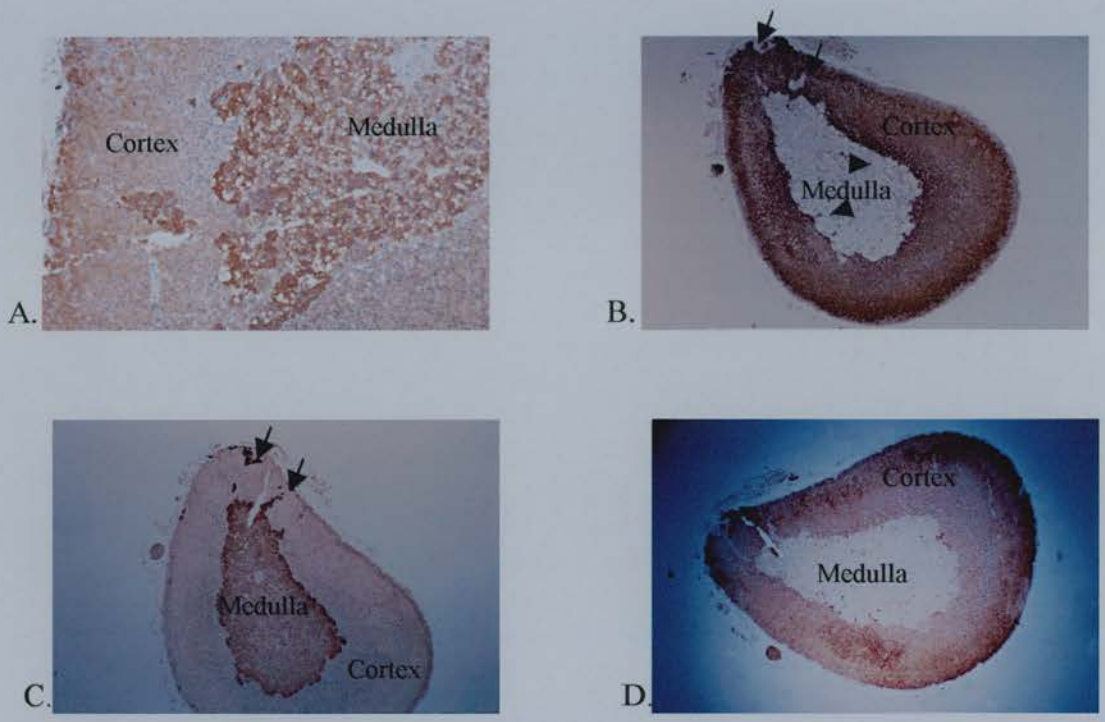

Fig. 6.2 Consecutive sections of the rat adrenal gland were incubated with different antibodies. A, section incubated with the StAR peptide antibody shows positive staining in the adrenal cortex and medulla (48x magnification). B, localisation of 3β-HSD shows positive staining in adrenal cortex (Arrowheads show cortical cells within the medulla. Arrows show chromaffin cells under the adrenal capsule and in the cortex.) (30x magnification). C, localisation of chromogranin A shows positive staining in adrenal medulla. Some medullary cells present in cortex and under adrenal capsule (arrows). (30x magnification). D, section incubated with the mouse StAR protein antibody shows positive staining in adrenal cortex only (30x magnification).

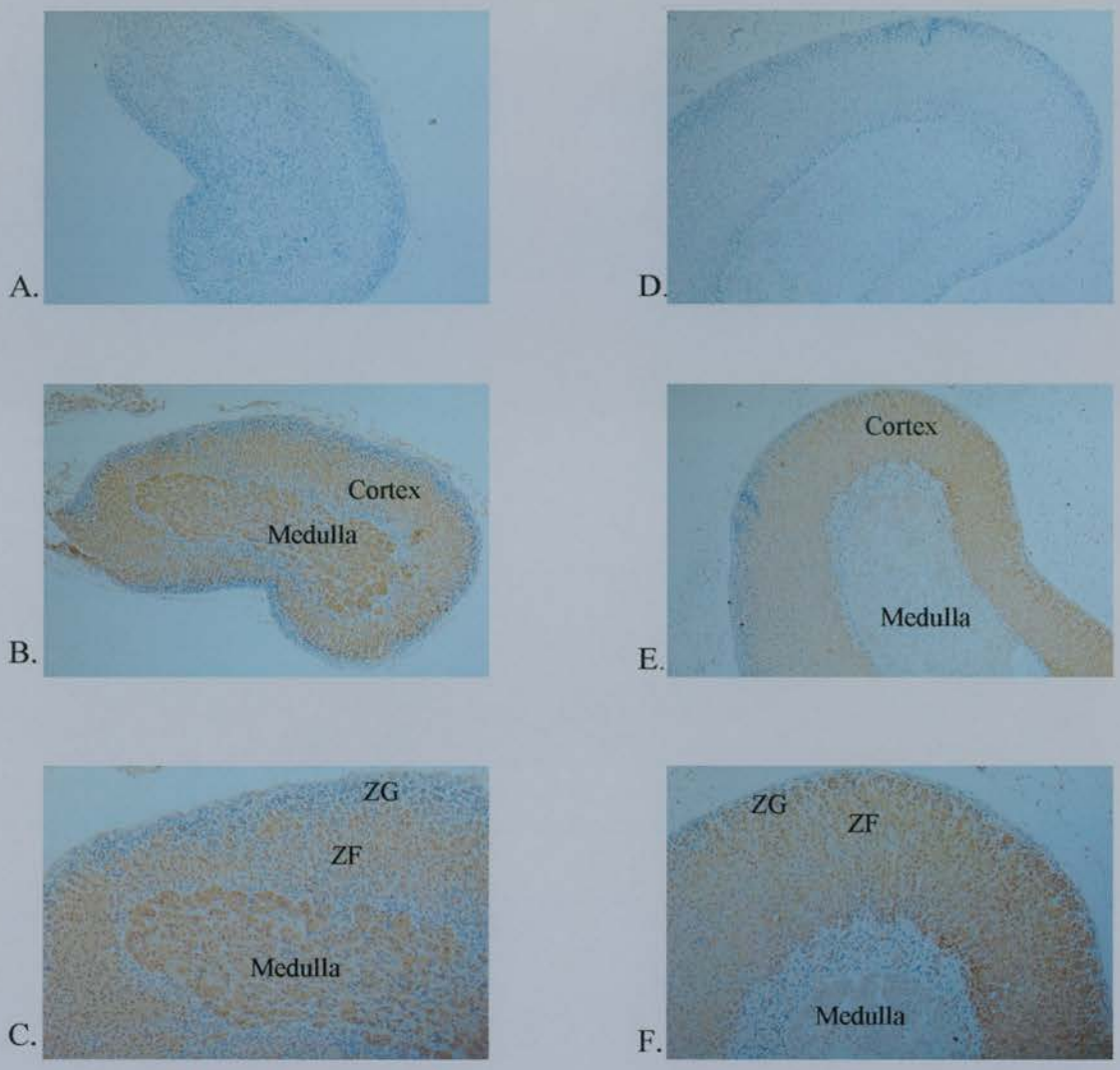

Fig. 6.3 Immunohistochemical localisation of StAR protein in the mouse adrenal gland. A-C, sections were incubated with or without peptide StAR protein antisera. D-F, sections were incubated with or without mouse StAR antisera. A and D, negative control sections show the absence of StAR protein positive staining (50x magnification). B and E, localisation of StAR protein shows positive staining in the adrenal cortex and the medulla (50x magnification). C and F, high power images of the ZG, ZF, ZR and medulla (100x magnification). ZG and ZF denote the zona glomerulosa and zona fasciculata, respectively.

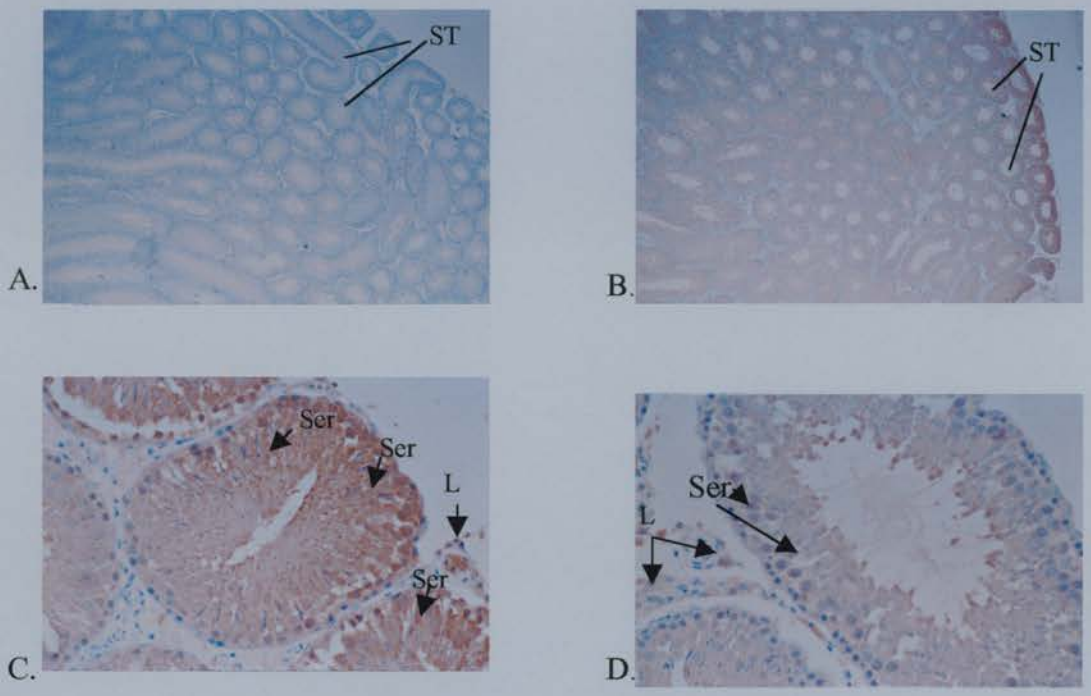

Fig 6.4 Immunohistochemical localisation of StAR peptide antibody in the rat testis. A, negative control section shows the absence of StAR protein positive staining (30x magnification). B, localisation of StAR protein shows positive staining in seminiferous tubules (ST) (30x magnification). C and D, high power images of the seminiferous tubules and inetstitial connective tissues of the rat testis (300x magnification). Positive staining is showing in spermatocytes (ST), Sertoli cells (Ser), and Leydig cells (L).

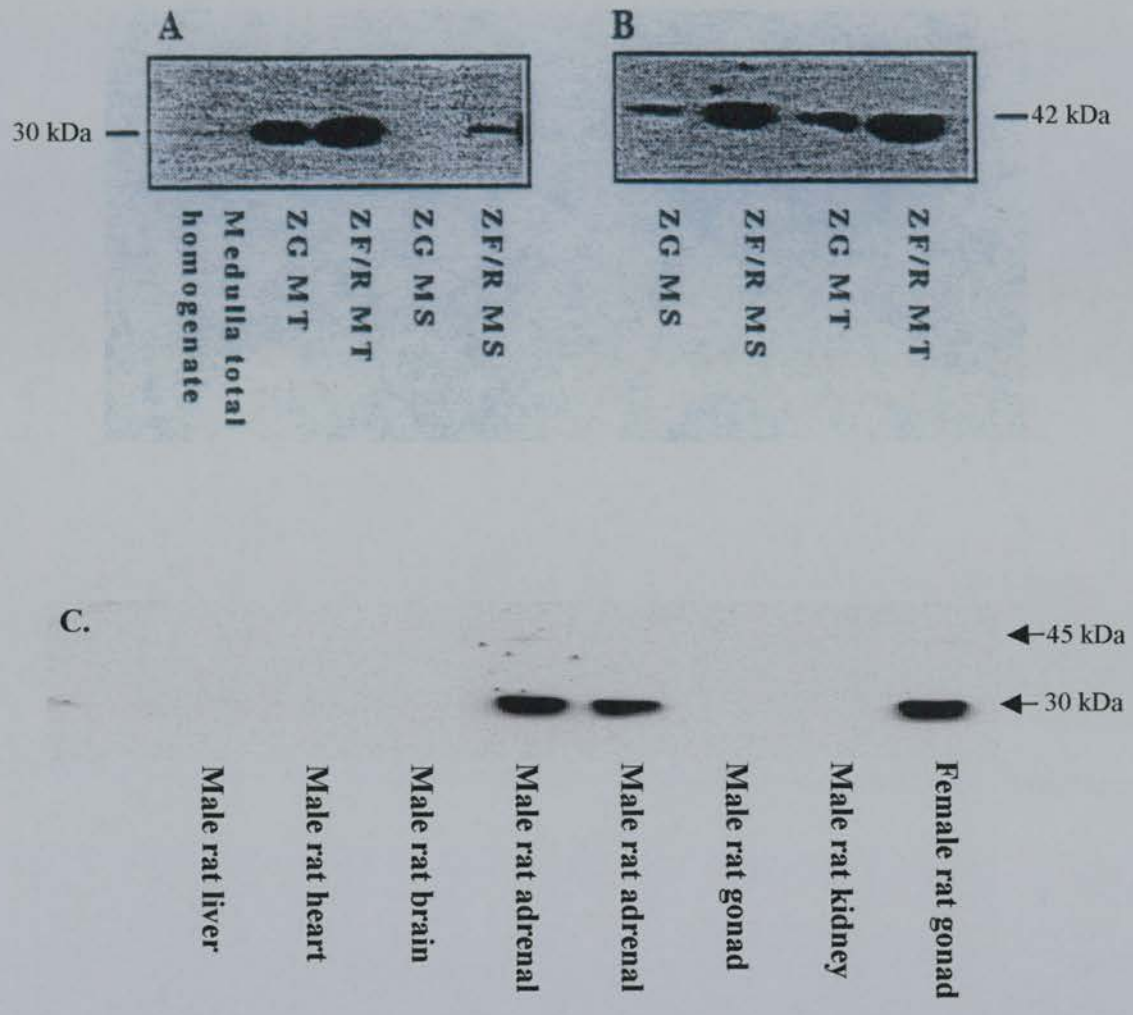

Fig 6.5 Immunoblotting of peptide StAR protein (A and C) and 3β-HSD (B) antibodies. A, detection of StAR protein in mitochondria (MT) and microsomes (MS) from the ZG, ZF/R and medulla of rat adrenal glands. B, detection of 3β-HSD in mitochondria and microsomes from the ZG and ZF/R of rat adrenal glands. C, immunoblotting of peptide StAR protein. Total protein homogenates were extracted from different rat tissues. Protein loading was 25 μg/ per lane.

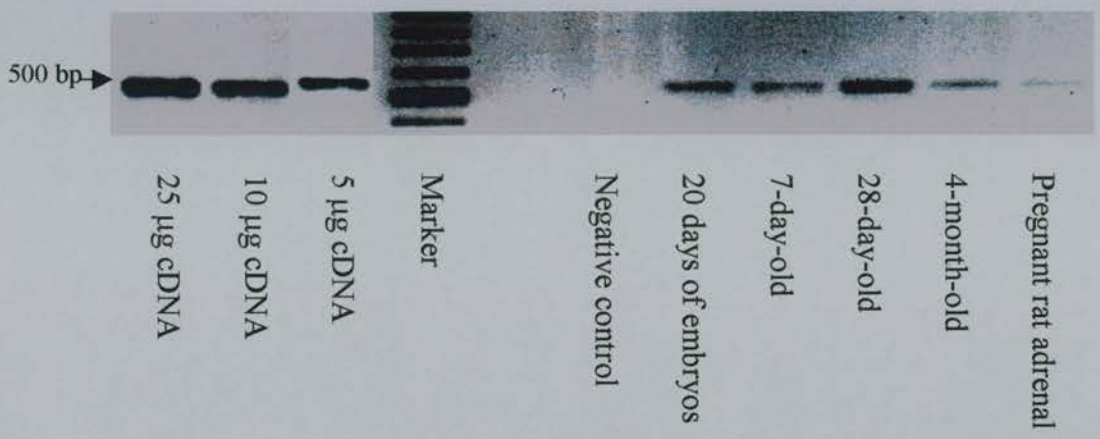

Fig. 6.6 StAR cDNA optimisation with various amounts of plasmids and mRNA expression of StAR using RT-PCR in rat adrenal glands obtained from pregnant rats and at various developmental stages in rat offspring treated with saline throughout pregnancy (see Chapter 4 and 5). Analyses were performed on 10 ng of total RNA templates.

Chapter 7 General discussion

7.1 Summary of results

Functional zonation of the rat adrenal cortex may be defined by the expression of the mitochondrial enzymes CYP11B2 and CYP11B1 that regulate the secretion of the steroids aldosterone in the ZG and corticosterone in the ZF, respectively. Maternal manipulation of (i) the RAS system by changing dietary sodium, and (ii) the HPA axis by chronic administration of dexamethasone may affect the distribution and expression of CYP11B2 and CYP11B1 in the adrenal cortex of fetuses and offspring. On the other hand, synthesis of these steroids is also dependent on StAR protein that facilitates the transport of cholesterol into the mitochondria. In order to provide a tool for the study of StAR protein expression in rat adrenal paradigms, an antibody against a bovine StAR peptide sequence was characterised. The results of this thesis are summarised below:

1. Low-salt-diet caused significant increases of ZG width in non-pregnant and pregnant rats whereas high-salt-diet markedly reduced ZG width in both non-pregnant and pregnant rats compared with tissues of rats fed a normal-salt-diet. Cell hypertrophy was seen in the ZG of the adrenals from both the non-pregnant and pregnant low-salt-treated rats. Adult and fetal adrenal CYP11B2 and CYP11B1 expression results are summarised in Table 7.1. These results suggest that dietary sodium restriction may activate the RAS to induce the expression of CYP11B2 in fetal adrenals but that responses of protein and mRNA are muted compared with changes in maternal adrenal expression.

Table 7. 1 Summary of salt diet experiments

	Methods	[Aldo]		CYP11B2		[B]		CYP11B1	
		High-salt	Low-salt	High-salt	Low-salt	High-salt	Low-salt	High-salt	Low-salt
NP	IHC			↓↓↓	↑			NS	NS
Preg	IHC			↓↓↓	↑			NS	NS
	WB			↓	↑↑↑↑			NS	NS
	cRT-PCR			↓↓↓↓	↑↑↑↑			NS	NS
E20	IHC			↓	↑↑↑↑			NS	NS
	WB			NS	NS			NS	NS
	cRT-PCR			NS	NS			NS	NS
	RIA	NS	↑↑↑ ↑			NS	NS		

IHC: Immunohistochemistry; WB: Western blotting; cRT-PCR: competitive RT-PCR; RIA: radioimmunoassay; [Aldo]: plasma aldosterone; [B]: Plasma corticosterone; NP: Non-pregnant rats; Preg: Pregnant rats. E20: 20-day rat embryos.
↑ or ↓: $P<0.5$; ↑↑ or ↓↓: $P<0.1$; ↑↑↑ or ↓↓↓: $P<0.05$; ↑↑↑↑ or ↓↓↓↓: $P<0.01$ compared to control salt diet group by Tukey's test.
NS: Not significant compared to control salt diet group by one-way ANOVA analysis.

2. Chronic daily injections of DEX throughout gestation significantly reduced the protein and mRNA expression of the CYP11B1 and caused modest increases in CYP11B2 mRNA and protein in adrenals of DEX-treated pregnant rats. Intrauterine DEX-treated rats exhibited fetal growth retardation with differential effects on heart, kidney and adrenal weights. Some of these effects persisted after birth when DEX-treatment had been stopped. Postnatal body weights continued to be lower at age 7-day, 28-day and 4-month-old whereas heart weight was proportionately greater after DEX-treatment in utero, particularly at days 7 and 28. Protein and mRNA levels of CYP11B2, CYP11B1 and CYP11B3 in the adrenals of offspring are summarised in Table 7.2. These results suggest that DEX readily crosses the placenta and down-regulates fetal HPA axis activity. Furthermore, maternal DEX-treatment may programme the physiological state of the adult offspring via changes in the activity of the HPA axis or possibly the RAS system.

3. Immunohistochemical studies in adult rat adrenal glands, using a peptide StAR antibody, showed the distribution of StAR protein in the ZG and ZF with less intense staining in the ZR. In addition, intense immunoreactivity was seen in the adrenal medulla. The specificity of the peptide StAR antibody was examined by immunoblotting. A 30 kDa band, corresponding to StAR, was detected mainly in the total protein homogenate of the adrenal and ovary and the mitochondria of the ZG and ZF. These results suggest that StAR protein may have some other as yet unidentified functions in the adrenal gland, especially in the medulla. Alternatively,

Table 7. 2 Summary of dexamethasone experiments

	Methods	CYP11B2	CYP11B1	CYP11B3
Preg	**IHC**	NS	↓↓↓↓	?
	WB	↑↑↑	↓↓↓	?
	cRT-PCR	↑	↓↓↓	ND
E20	**IHC**	↑	↓↓	?
	WB	NS	NS	?
	cRT-PCR	↑	↓↓↓↓	ND
7-Day	**IHC**	↑↑↑	NS	?
	WB	↓	↓	?
	cRT-PCR	↑	NS	NS
28-Day	**IHC**	↑	NS	?
	WB	NS	NS	?
	cRT-PCR	NS	↑↑	↑↑
4-Month	**IHC**	–	–	?
	WB	↑↑	↑	?
	cRT-PCR	NS	↑	ND

IHC: Immunohistochemistry; WB: Western blotting; cRT-PCR: competitive RT-PCR; Preg: Pregnant rats. E20: 20-day rat embryos. 7-Day: 7-day-old rat offspring; 28-Day: 28-day-old rat offspring; 4-Month: 4-Month-old rat offspring.

↑ or ↓: $P<0.5$; ↑↑ or ↓↓: $P<0.1$; ↑↑↑ or ↓↓↓: $P<0.05$; ↑↑↑↑ or ↓↓↓↓: $P<0.01$ compared to control group by the Student's t test.

NS: Not significant compared to control group by Student's-t test. ND: Not detected.

?: Unknown;

–: not measured

the adrenal medulla may contain a protein closely related to StAR, sharing common antigenicity.

7.2 Possible mechanisms of fetal adrenal CYP11B enzyme regulations by maternal mineralocorticoid or glucocorticoid manipulations

It has been widely reported that maternal environments may programme fetal and postnatal physiological and metabolic processes when a stimulus or insult is encountered during a critical period of development. These stimuli include maternal dietary interventions, stress or exposure of the fetus to glucocorticoid hormones. Rats exposed to a low sodium diet *in utero* and on into adult life show growth retardation and higher blood pressure than rats fed a control diet (unpublished data). The preliminary data suggest that the intrauterine programming effect caused by the sodium-restricted diet during pregnancy is similar to other studies in rats exposed to glucocorticoids and low protein diets *in utero*. A number of studies have demonstrated that the intrauterine programming has led to disproportionate growth retardation of peripheral organs, high blood pressure or other metabolic diseases in adult life (Benediktsson et al., 1993; Lindsay et al., 1996; Langley-Evans, 1997; Langley-Evans et al., 1998; Seckl et al., 1998; Langley-Evans et al., 1999)(Fig. 7.1). The mechanisms responsible for the programming effect remain incompletely defined. One of the mechanisms may be related to the regulation of the HPA axis through the MR or GR receptors in the brain, which is a major target for glucocorticoids and has a role in the control of adrenal corticosterone secretion through the HPA axis. However, it has been established that maternal mineralocorticoids and glucocorticoids can cross the placenta and reach the fetal

adrenal thereby affecting the fetal steroidogenesis. In addition, at the period around birth the fetal adrenal is exceptionally active both in terms of cell proliferation and differentiation (Josimovich et al., 1954; Roos, 1967). Therefore, it is reasonable to hypothesis that the adrenal gland plays a crucial role in the intrauterine programming, especially in relation to the enzymes responsible for the biosynthesis of mineralocorticoids and glucocorticoids which are involved in blood pressure regulation. In this thesis, it has been clearly demonstrated that the supply of a low sodium diet and DEX treatment throughout pregnancy cause a significant increase of CYP11B2 and a decrease of CYP11B1, respectively in pregnant rats. In both animal models, the attenuation of the expression of CYP11B2 and CYP11B1 in fetal (E20) adrenals is similar to those in maternal adrenals though the effects are less obvious in fetal adrenals than in the maternal ones. Furthermore, in the DEX-treatment experiments, adrenal CYP11B2 shows a slightly increased response to intrauterine DEX-treatment compared to that of CYP11B1 in the offspring. It is known that glucocorticoid excess in the fetus suppresses the development of fetal adrenal function because of the feedback inhibition of ACTH secretion by maternal glucocorticoid. However, experiments in rats and sheep have shown that fetal expression of genes for renin, angiotensinogen and AII receptors are all regulated by glucocorticoid treatment (Reul et al., 1989; Everett et al., 1991; Sato et al., 1994; Segar et al., 1995; Whorwood et al., 2001). Therefore, it is likely that programming of blood pressure or cardiovascular disease by maternal mineralocorticoid and glucocorticoid manipulations might be mediated by sensitisation of the RAS system.

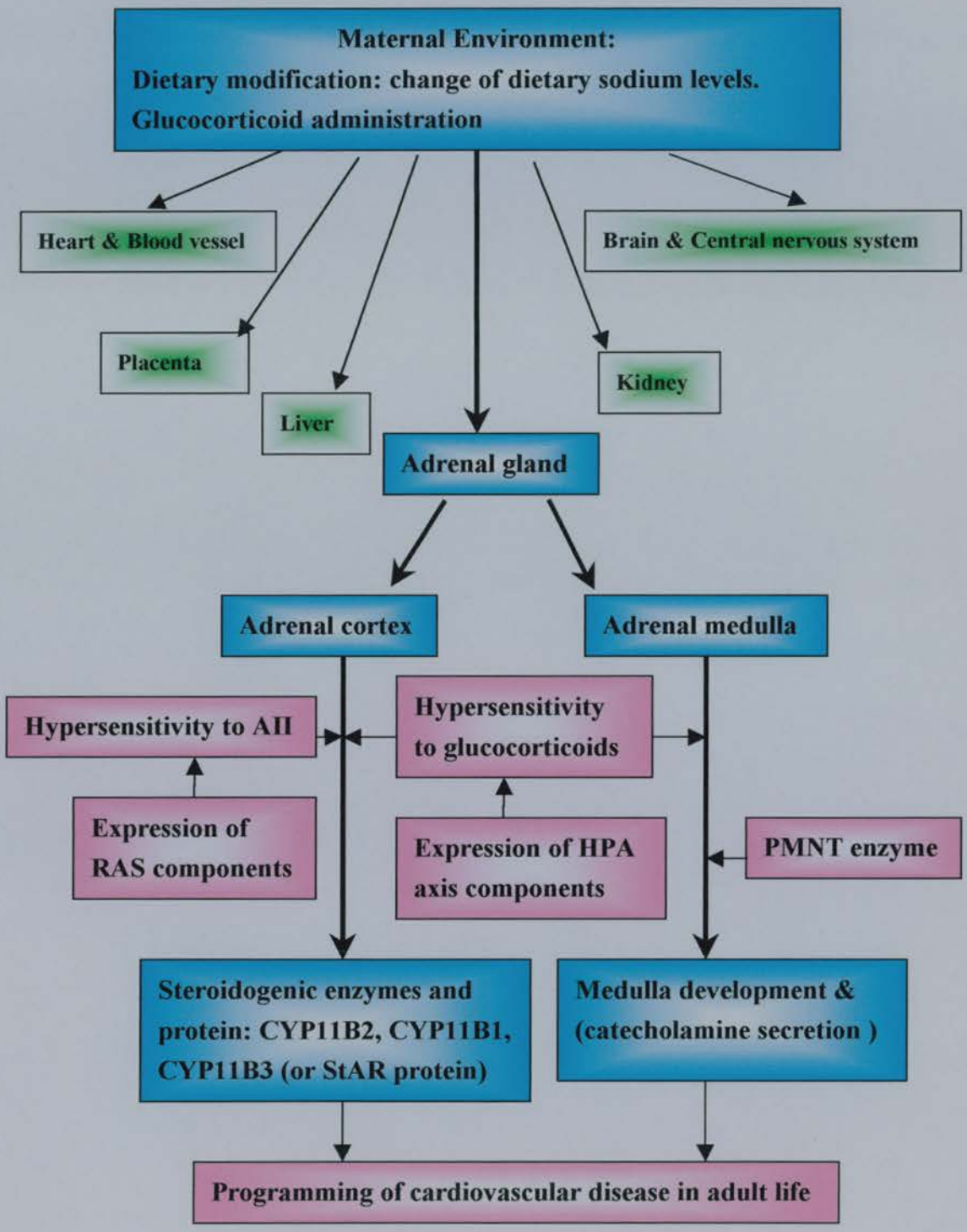

Fig. 7. 1 Possible roles and mechanisms of adrenal steroidogenic enzymes in prenatal programming of cardiovascular disease. Broad lines indicate the main

On the other hand, lifelong exposure of rats to low sodium diet causes a higher blood pressure in adult life that is associated with the increased vascular responsiveness to noradrenaline and vasopressin (unpublished data communicated by Dr. C.J. Kenyon). Glucocorticoids affect the maturation of fetal organs, including the growth and innervation of the fetal heart. Infused glucocorticoids raise blood pressure and increase reactivity to noradrenaline in fetal sheep (reviewed by Clark, 1998). The administration of DEX also results in transient ventricular hypertrophy in premature neonates. In this thesis, the morphological changes of the adrenal medulla were also noticed. Whether the prenatal changes would persist into later life is still unknown. However, these changes in the medulla may indicate another possible mechanism underlying the programming of adult disease by maternal mineralocorticoid and glucocorticoid treatments.

7.3 Possible roles of CYP11B3 in the adrenal development

The physiological function of the CYP11B3 enzyme and why the rat adrenal expresses CYP11B3 gene only for a limited time interval presently are unclear. In this thesis, the expression levels of CYP11B3 mRNA were measurable in newborn rat adrenals, but not in fetal, adult and pregnant rat adrenals, which were in agreement with other studies (Mellon et al., 1995; Zhou et al, 1995). It has also been demonstrated that the function of CYP11B3 enzyme is more similar to CYP11B1 (Mellon et al., 1995; Zhou et al., 1995). Also, Mellon et al. (1995) reported that the CYP11B3 gene was negatively regulated by ACTH in the male adrenal gland (Mellon et al., 1995). It is known that basal and stress-stimulated plasma

corticosterone levels are very low during the hyporesponsive period. This is associated with a decreased ACTH secretion (Schapiro, 1962; Sapolsky and Meany, 1986; Walker et al., 1986). Speculation about the function of CYP11B3 is whether the expression of the CYP11B3 has been made on related to the hyporesponsiveness during the neonatal period.

7.4 Possible roles of StAR protein in adrenal development

The regulation of StAR protein expression has been established somewhat extensively *in vitro* and *in vivo*. Changes in StAR protein and mRNA have been linked to physiological changes in steroid secretion. In humans, StAR protein mutation causes congenital lipoid adrenal hyperplasia with severe deficiencies steroid hormones (Lin et al., 1995). Recently, StAR protein was found to be involved in both the action of sodium restriction and ACTH stimulation in promoting aldosterone and corticosterone formation in adult rat adrenals (Lehoux et al., 1998; Lehoux et al., 1999; Peters et al., 1998). It would be of interest to investigate whether maternal mineralocorticoid and glucocorticoid manipulations affect the change of StAR protein or mRNA expression during various developmental stages and whether these changes are concomitant with those of CYP11B2 and CYP11B1 in fetal or postnatal rat adrenals.

7.5 Conclusion and future plans

The concept of fetal programming proposed by Barker and colleagues is now widely accepted. The mechanisms linking maternal environments and later raised blood pressure or other metabolic diseases could include programming of the HPA axis or the RAS system. The hypothesis arising from this thesis is that over–exposure

of the fetus to maternal glucocorticoids and to particular levels of sodium during pregnancy leads to the alteration of steroidogenic enzyme expression, especially CYP11B2 and CYP11B1 in fetal and postnatal rat adrenals. However, whether such changes would persist into later life needs further investigations on several aspects. According to the current results, some of the experiments can be expanded. This thesis also promotes some future works on examining the RAS system in kidneys and adrenal glands, especially renin expression levels in these two animal models. Plasma aldosterone and corticosterone levels and blood pressure need to be monitored in offspring. Langley-Evans et al. (1999) indicated that fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the adult rat offspring (Langley-Evans et al., 1999). Therefore, it is of interest to know whether the renal function is impaired in these two animal models, especially the salt diet experiments. Another interesting observation in this thesis is the relationship between medulla development and the intrauterine treatment with glucocorticoids. Investigation of the catecholamine secretion would be of great interest in addition to PMNT enzyme expression in relation to the histology in the adrenal medulla. Finally, the StAR protein expression may be another key factor to study in more detail in the rat paradigms described in this thesis.

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Appendix I

SPECIAL DIET SERVICES Ltd

Formula [1653] - Low Na Rat Diet

Predicted Analysis					
Moisture	10.5	%	Glycine	1.51	%
C Fat	7	%	Aspartic	0.85	%
C Protein	14.9	%	Glutamic	1.86	%
C Fibre	11.6	%	Proline	0.68	%
Ash	3.03	%	Serine	0.67	%
NFE	39.6	%	Hydroxypro	0.028	%
D C Fat	6.3	%	Calcium	0.66	%
D C Protein	13.4	%	Total P	0.53	%
T D Fibre	8.8	%	Phy P	0.115	%
Pectin	1.0	%	Av P	0.413	%
Hemicellulose	5.3	%	Na	0.014	%
Cellulose	1.96	%	Cl	0.054	%
Lignin	0.57	%	K	0.466	%
Starch	37.55	%	Mg	0.13	mg/kg
Sugar	7.7	%	Fe	55.0	mg/kg
G. Energy	16.1	MJ/Kg	Cu	10	mg/kg
D Energy	14.1	MJ/Kg	Mn	38.2	mg/kg
M Energy	12.9	MJ/Kg	Zn	29.	mg/kg
Myristol	0.05	%	Co	26.5	mg/kg
Palmitol	0.26	%	I	39.4	µg/kg
Oleic	1.7	%	F	12.5	mg/kg
Linoleic	2.7	%	Se	59	µg/kg
Linolenic	0.09	%	Retinol	1320	µg/kg
Arachidonic	0.013	%	Vit A	4356	mg/kg
Lauric	0.024	%	α Tocoph	11	mg/kg
Myristic	0.025	%	Vit E	62.1	mg/kg
Palmitic	0.059	%	Vit B1	6.55	mg/kg
Stearic	0.114	%	Vit B2	5.45	mg/kg

Arginine	0.95	%	Vit B6	6	mg/kg
Lysine	0.74	%	Vit B12	1.1	µg/kg
Methionine	0.3	%	Vit C	2.75	mg/kg
Cystine	0.23	%	Vit K	2.57	mg/kg
Tryptophan	0.15	%	Folic	0.17	mg/kg
Histidine	0.36	%	Nico	30.6	mg/kg
Threonine	0.56	%	Panto	12.0	mg/kg
Isoleucine	0.66	%	Choline	651	mg/kg
Leucine	1.33	%	Inositol	1.65	µg/kg
Phenylalanine	0.74	%	Biotin	55	µg/kg
Valine	0.72	%	Carotene	2.65	mg/kg
Tyrosine	0.59	%	Xanthine	8.25	mg/kg

Actual Analysis

0.03% Sodium **0.29% Chloride** **0.6% Potassium**

Appendix II

Publications and conference abstracts arising from this research

Y-C. Lo, S. Mackenzie, A.F. Howie, D.K. Apps, J.I. Mason, B.C. Williams S.D. Morley "Properties of an adrenal medullary protein immuno-related to steroidogenic acute regulatory (StAR) protein." Endocrine Research 26(4):737-748(2000)

Y-C. Lo, C.E. Gomez-Sanchez, C.J. Kenyon, J.I. Mason and B.C. Williams "Effects of dietary sodium on aldosterone synthase, 11β-hydroxylase and 3β-hydroxysteroid dehydrogenase expression in pregnant and foetal wistar rats. Molecular Steroidogenesis Frontiers Sciences Series No. 29:349-350(1999)

Y-C. Lo, L. Brett, C.J. Kenyon, S.D. Morley, J.I. Mason and B.C. Williams "StAR protein is expressed in both medulla and cortex of the bovine and rat adrenal gland." Endocrine Research 24(3&4): 559-563 (1998)