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THE ONTOGENY OF 11ß-HYDROXYSTEROID DEHYDROGENASE TYPE 2 AND MINERALOCORTICOID RECEPTOR GENE EXPRESSION REVEAL INTRICATE CONTROL OF GLUCOCORTICOID ACTION IN DEVELOPMENT.

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ABSTRACT

Glucocorticoids play important roles in development and 'fetal programming'. Fetal exposure to excess glucocorticoids reduces birth weight and causes later hypertension. To investigate these processes further we have determined the detailed ontogeny of 11ß-hydroxysteroid dehydrogenase type2 (11ß-HSD2, which potently inactivates glucocorticoids) and the mineralocorticoid receptor (MR) by *in situ* hybridisation from embryonic day 9.5 (E9.5, term = E19) until after birth in the mouse. Widespread abundant 11ß-HSD2 mRNA expression from E9.5-E12.5 changes dramatically at ≈E13 to a limited tissue-specific pattern (kidney, hindgut, testis, bile ducts, lung and a few brain regions (later seen in cerebellum, thalamus, roof of midbrain, neuroepithelial regions in pons and near the subicular hippocampus)). Placenta (labyrinthine zone) and extra-embryonic membranes express abundant 11ß-HSD2 mRNA until E15.5 but this ceases ≈E16.5. It is unclear to what extent rodent term placental 11ß-HSD activity is due to persisting 11ß-HSD2 protein. Convincing MR mRNA expression is seen from E13.5 and includes pituitary, heart, muscle and meninges with expression later in gut, kidney, thymus, discrete areas of lung and several brain regions (including hippocampus, rhinencephalon and hypothalamus). 11ß-HSD2 and MR clearly co-localise ≈E18.5 in kidney and colon and might do so in discrete areas of lung (E14-15) and neuroepithelia near the subicular hippocampus. Probably elsewhere MR are non-selective and 11ß-HSD2 is involved in protecting glucocorticoid receptors in fetal tissues. Comparison with previous enzymology studies suggest the changing pattern of 11ß-HSD2 mRNA is likely to be translated into enzyme activity and have significant parallels in human development.

Glucocorticoids are known to have actions during embryogenesis not only on the structural development of organs but also in the maturation and 'programming' of homeostatic systems(1,2). Excess glucocorticoid exposure of the developing fetus is harmful, reducing birth weight and may predispose to hypertension in adulthood (3). Abundant placental 11ß-hydroxysteroid dehydrogenase type2 (11ß-HSD2) (which inactivates physiological glucocorticoids) protects the developing fetus from high maternal glucocorticoid levels. To investigate the role of glucocorticoids in fetal development and programming it is important to determine the ontogeny of 11ß-HSD2 as this will profoundly affect glucocorticoid access to co-localised glucocorticoid and mineralocorticoid receptors (GR and MR) and the specificity of MR. The fetus develops in a relatively low glucocorticoid environment and so the expression of MR (the receptor with highest affinity for physiological glucocorticoids) may be of particular importance in glucocorticoid action. We now present an *in situ* hybridisation study of the ontogeny of 11ß-HSD2 and MR in the mouse from embryonic day 9.5 (E9.5) until after birth.

MATERIALS AND METHODS

Animals. C57BL/6 mice were time-mated allowing embryos (at known gestations from E9.5) and pups (0.5d postnatal: P0.5) to be obtained and these were frozen immediately (-20°C iso-pentane bath) after death and stored at -80°C.

11ß-HSD2 and MR probes. A mouse 11ß-HSD2 cDNA clone was isolated (Robson. A.C, *et.al.* unpublished) by screening a BALB/c mouse kidney cDNA library in λgt10 with the cDNA of human placental 11ß-HSD2(4). A 666bp mouse 11ß-HSD2 cDNA fragment (corresponding to bases +399-1064 of the human placental 11ß-HSD2 cDNA(4) and with 99% sequence identity to the strain 129/Sv mouse 11ß-HSD2 sequence(5)) with flanking T3/T7 polymerase binding sites was uniquely amplified from the λgt10 clone DNA using polymerase chain reaction (PCR) with primers incorporating T3/T7 sites at their 5' ends. This PCR product (purified on Chromaspin columns (Clontech, Palo Alto CA.)) was used as template for mouse 11ß-HSD2 antisense and sense probes. A 513bp rat MR cDNA clone(6) was linearised separately with HindIII and EcoRI to yield templates for antisense and sense MR probes respectively. ^{35}S-α-UTP labelled RNA antisense and sense probes were synthesised using the appropriate template and phage RNA polymerase, purified using Nensorb columns (NEN/ Du Pont, Stevenage UK) and checked on denaturing acrylamide gels.

***In situ* Hybridisation.** Sagittal 20 µm cryostat sections were thaw-mounted onto 3-amino propyl triethoxysilane coated slides and stored at -80°C. Tissue sections were fixed, prehybridised, hybridised and washed as described(7), (hybridisation was with ≈3 x 10^6 cpm/section ^{35}S-UTP-labelled RNA probe at 50°C for 12-14 h). Following washing/RNase A treatment slides were dehydrated, dried, and placed against ß-Max. Hyperfilm film (Amersham, Little Chalfont, UK) for 10 days. Sections were then stained with cresyl violet.

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RESULTS.

'Sense' 11ß-HSD2 and MR *in situ* hybridisations showed non-specific background radioactivity only, which was especially low for 11ß-HSD2 (Fig.1 shows examples; sense hybridisation was even lower at other gestations). In contrast, antisense studies showed high levels of specific hybridisation and low background especially for 11ß-HSD2. Several sections at each gestation were analysed and Fig.2 shows results of *in situ* hybridisation from one section at each stage studied. Sections are in the sagittal plane apart from E9.5 and E11.5 which are coronal. To show a greater range of structures the sagittal sections used vary in their distance from the midline, though all pass through kidney except E15.5.

Ontogeny of 11ß-HSD2. High level 11ß-HSD2 mRNA expression was seen throughout the period of study. There is abundant 11ß-HSD2 expression in the labyrinthine zone of the fetal portion of the developing placenta and in the splanchnopleure layer (yolk sac) of the fetal extra-embryonic membranes. The major maternal component of the placenta, decidua, does not express 11ß-HSD2 while 11ß-HSD2 is expressed more peripherally in the uterine wall and mesometrium (see 2, fig.2). At E14.5 11ß-HSD2 mRNA is abundant in labyrinthine zone and fetal extra-embryonic membranes, whilst by E16.5 it is virtually absent.

11ß-HSD2 mRNA is highly expressed in the developing fetus and there is a remarkable change in its distribution between E12.5 and E13.5, being widespread before and much more restricted afterwards. Kidney and its antecedents (metanephros, mesonephros and nephrogenic cords in the primitive urogenital ridge) express 11ß-HSD2 highly and renal distribution in later gestation resembles the adult (see E17.5). Expression is also seen in gonadal ridge, and in the developing male reproductive system (testis, mesonephric duct/ductus deferens and in the bulbar urethral region) and in the paramesonephric duct in the female (see P0.5, the only female stage examined; 11ß-HSD2 and MR mRNA expression are otherwise similar in male and female at this stage). 11ß-HSD2 mRNA is expressed at very high level in the GI tract between E9.5 and E12.5 being present at E12.5 in hindgut and midgut (including stomach) and also at lower level in oropharynx, tongue and hepatobilary primordia. By E14.5-E15.5 strong expression is limited to hindgut, gallbladder/major bile ducts and a remarkable

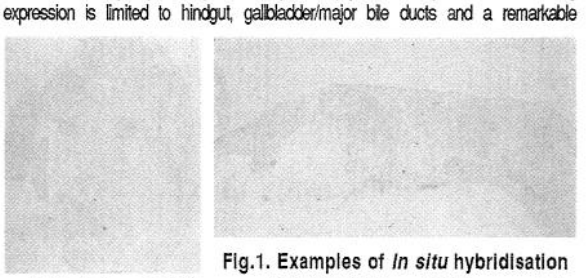


Fig.1. Examples of *In situ* hybridisation controls. *Left panel,* E18.5 + sense 11ß-HSD2. *Right panel,* P0.5 + sense MR.

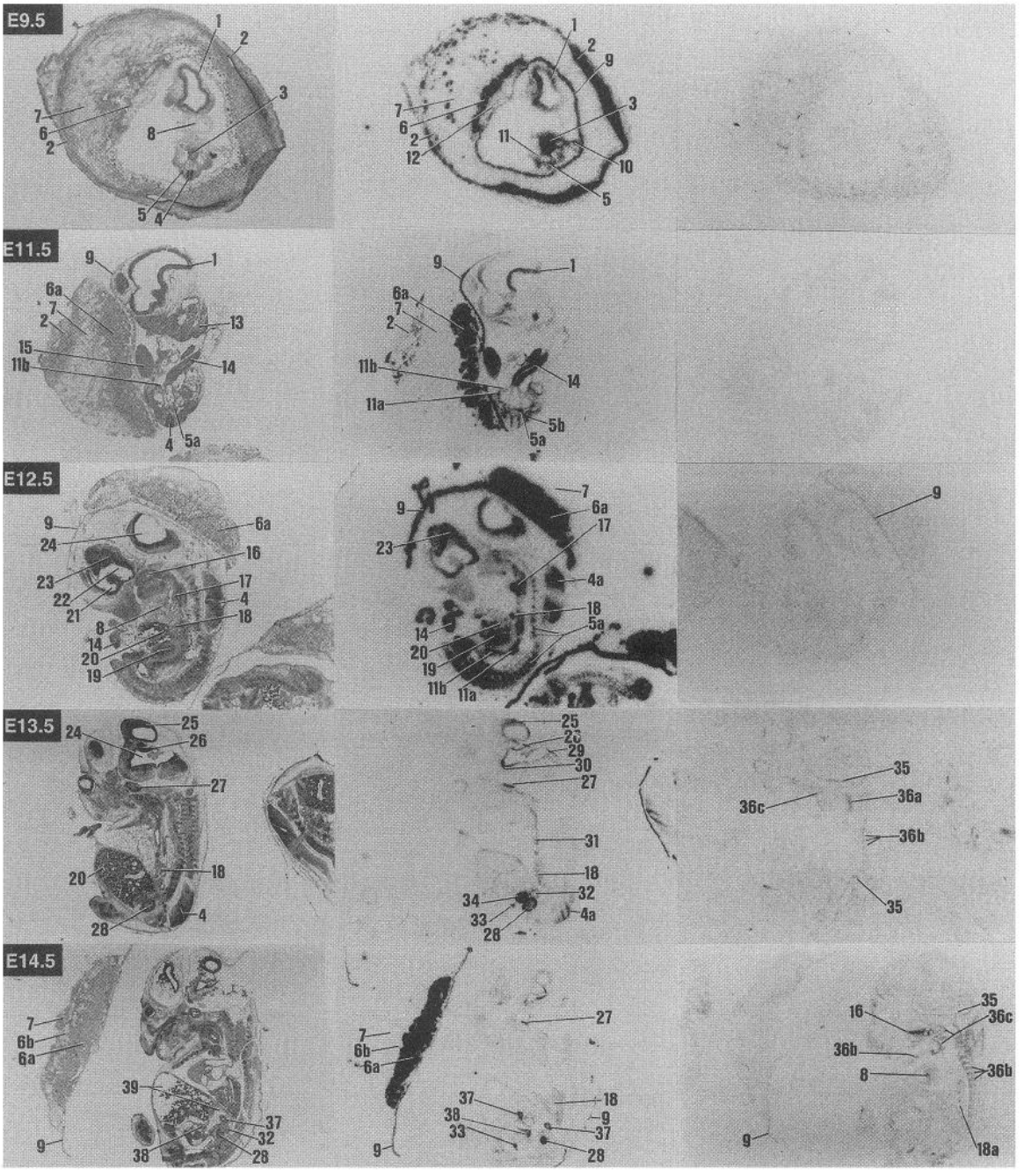
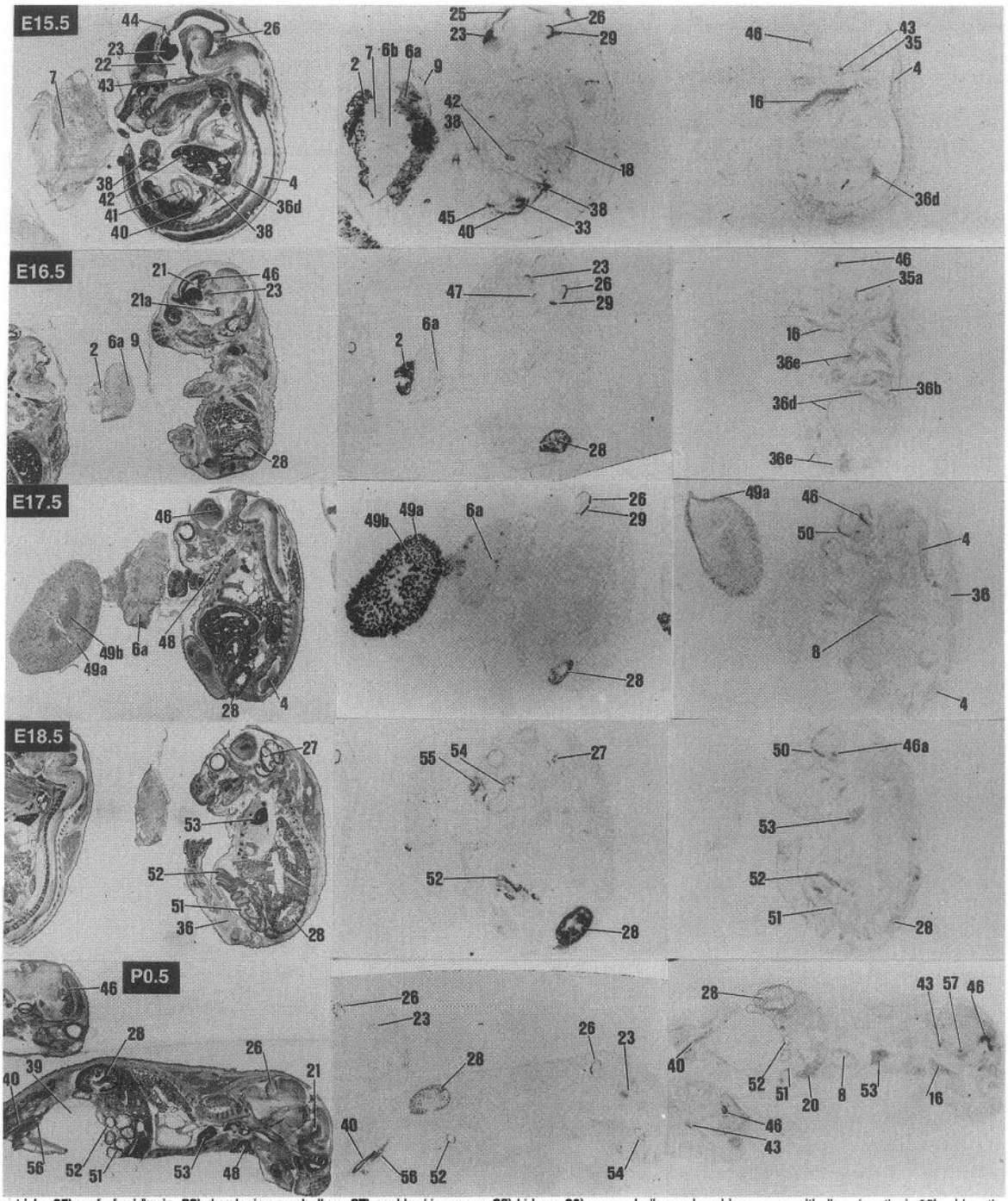


Fig2. Expression of 11ß-HSD2 and MR in mouse development. Results of histological staining (cresyl violet) and *in situ hybridisation* for 11ß-HSD2 and MR are shown in the *left, middle and right panels* respectively for each gestation(E9.5-P0.5). Apart from extra-embryonic structures, labelling in the *middle and right* panels is restricted to areas positive in the *in situ* hybridisation. The following are labelled:- 1) brain neuroepithelium, 2) uterine wall, 3) hindgut, 4) spinal cord ((4a) mantle layer), 5) somite including sclerotome (5a) and myotome (5b), 6) developing placenta (fetal) with labyrinthine (6a) and spongiotrophoblast (6b) zones, 7) decidua (maternal), 8) heart, 9) fetal extra-embryonic membranes, 10) allantois, 11) urogenital ridge ((11a) mesonephros, (11b) developing gonad) 12) allantoic cavity/ chorionic plate, 13) first branchial arch, 14) midgut, 15) limb bud, 16) tongue, 17) 3rd/4th branchial arches, 18) developing lung, (18a) discrete intra pulmonary structures possibly airways or vessels, 19) stomach, 20) developing liver, 21) lateral ventricle and (21a) tip of its posterior horn, 22) 3rd ventricle, 23) developing thalamus (dorsal diencephalon), 24) 4th



ventricle, 25) roof of midbrain, 26) developing cerebellum, 27) cochlea/ inner ear, 28) kidney, 29) precerebellar and cochlear neuroepithelium (pontine), 30) midpontine floor of 4th ventricle, 31) bronchus, 32) adrenal, 33) mesonephric duct/ ductus deferens, 34) testis, 35) meninges and (35a) tentorium, 36) muscle groups (and specifically 36a) cervical prevertebral, 36b) segmental intervertebral and intercostal, 36c) pharyngeal-laryngeal, 36d) diaphragm (including crus) and abdominal wall, 36e) fore- and hindlimb/ limb girdle muscles), 37) developing major bile duct, 38) dorsal mesentery of midgut (not pancreas), 39) artifactual spaces, 40) distal colon/ rectum, 41) bladder, 42) gall bladder, 43) pituitary, 44) pineal, 45) bulbar urethra, 46) hippocampus, (46a) vicinity of subicular hippocampus, 47) neuroepithelium around tip of posterior horn of lateral ventricle, 48) submandibular (salivary) gland, 49) maternal adult kidney ((49a) cortex and (49b) medulla), 50) rhinencephalon, 51) small intestine, 52) colon, 53) thymus, 54) developing tooth, 55) whisker follicles, 56) paramesonephric duct, and 57) hypothalamus. Actual embryo crown rump length: E9.5=3mm, E18.5=22mm.

expression in the mesentery of the midgut. Expression declines thereafter, but persists postnatally in colon. Limb buds and somites express abundant 11ß-HSD2 involving myotomes and sclerotomes (developing muscular and bony tissues respectively) at E11.5, while expression ceases by E13.5. Lung and the adrenal cortex continue moderately high 11ß-HSD2 mRNA expression on E13.5 though this ceases by E14.5 for adrenal and declines by E16.5 in lung. 11ß-HSD2 expression in CNS is abundant, non-uniform and increases to E12.5 declining sharply by E13.5. Expression in spinal cord by E13.5 is diminished and restricted to the dorsal aspect and is absent by E14.5. Beyond E12.5 brain neuroepithelial 11ß-HSD2 expression declines and by E15.5 has become restricted to several discrete areas which include thalamus, cerebellar primordia, roof of midbrain, regions of pontine neuroepithelia (precerebellar, cochlear and possibly midpontine adjacent to the floor of the fourth ventricle), a region next the tip of the posterior horn of the lateral ventricle (near subicular hippocampus/amygdala see 47 E16.5) and possibly at lower level in infundibulum/neurohypophysis (faint at E15.5). Expression in thalamus and cerebellum persist postnatally. Finally expression is also seen in developing teeth (E15.5-postnatal) and the inner ear (at least E13.5-E18.5), including the cochlea in the vicinity of the basilar membrane (between scala tympani and scala media) which is at the perilymph/ endolymph interface.

Ontogeny of MR. Convincing MR mRNA expression is first seen at E13.5 in muscle, meninges and on other sections (not shown) is clearly abundant in pituitary, heart and root of tongue. From E14.5-E16.5 a punctate pattern of expression is seen in lung (possibly related to airways or pulmonary vessels) and there is a dramatic spread of expression in muscle throughout the body (see E16.5). However by E18.5 this is greatly reduced or absent, though MR persists postnatally in heart and tongue. MR mRNA is clearly expressed in intestine (colon > small bowel), renal cortex and thymus by E18.5 and postnatally. Lower level expression is seen in bowel as early as E15.5 but increases selectively in colon at E18.5. Similarly in kidney, lower level MR expression is present a least 1 day prior to the increase in renal cortex at E18.5-P0.5. MR mRNA expression in brain is clearly seen from E15.5 (hippocampus) and is seen in several other discrete areas including rhinencephalon (see E17.5), hypothalamus (see P0.5) and in the vicinity of the subicular hippocampus (see E18.5). MR mRNA appears to be present in spinal cord (E15.5-E17.5) but diminishes at the end of gestation. Finally, at P0.5 there is MR mRNA expression in liver. Thus moderate-high expression of MR was seen, as described, in the mouse fetus from E13.5 onwards. Defining regions with no MR mRNA is very difficult as background makes it impossible to exclude expression at very low level.

DISCUSSION.

There is evidence for abundant 11ß-HSD (inactivating glucocorticoids) enzyme activity in placenta and fetus in mouse and human. Burton *etal*(8) showed both mouse placenta and fetus had levels of 11ß-dehydrogenase activity comparable to adult kidney. Human studies suggest high placental 11ß-dehydrogenase activity spans 10-20wk to full term(9), whilst high fetal tissue 11ß-dehydrogenase activity early (10-20wk(9,10)) reduces dramatically and in most tissues (kidney and gonad being exceptions) levels fall to ≈zero by full term(9). Moreover this reduction is probably already advanced by 23-26wks(4)(based on the absence of 11ß-HSD2 mRNA expression in some tissues previously expressing 11ß-HSD2 activity). The similarity of these changes in 11ß-HSD enzyme activity to the 11ß-HSD2 mRNA ontogeny described here suggests the changing pattern of 11ß-HSD2 mRNA is likely to be translated into enzyme activity and have significant parallels in human development.

The remarkable changes in 11ß-HSD2 expression are likely to profoundly influence glucocorticoid action on the fetus. Between E9.5-E12.5 11ß-HSD2 mRNA is widely expressed in the fetus at high level (heart being an exception) and combined with 11ß-HSD2 in the developing placenta will severely restrict glucocorticoid access to most fetal tissues (GR mRNA is reported present from E8(11)). Dramatic change occurs by E13.5 with widespread silencing of 11ß-HSD2 expression and appearance of MR mRNA. Thus glucocorticoids reaching the fetus between ≈E13-15 will be restricted (abundant placental 11ß-HSD2) but may have access to unprotected MR (pituitary, muscle heart and brain regions especially hippocampus) and GR (mRNA reported abundant in liver, heart, palate, and pituitary/hypothalamus), while access to receptors in kidney, hindgut, testis, biliary tree, brain regions (e.g. thalamus) and lung may be more restricted. Surprisingly between E15.5 and E16.5 placental 11ß-HSD2 mRNA switches off; whether 11ß-dehydrogenase activity present in term rodent placenta(8,12) is due to 11ß-HSD2 protein persisting and/or another isoform is unknown. Between E17.5 and birth MR mRNA expression increases greatly in colon and kidney where high 11ß-HSD2 expression will make such receptors aldosterone-selective, as in adults.

The ontogeny of MR has not previously been studied except in the gut(13) and late gestation/postnatal brain in the rat(14) where the findings are similar to those in mouse. Our findings raise the real possibility of MR directed gene effects occurring in developing muscle, heart, discrete areas of lung and thymus as well as more familiar sites e.g. kidney. Functional glucocorticoid receptors are certainly present in mouse tissues at an even earlier stage (in palate(15) and chromaffin cell precursors(1)). MR mRNA expression in muscle is particularly unexpected and occurs at an important stage; by E13 myoblasts are proliferating rapidly, by E15 primary muscle fibres are formed and contractile with secondary muscle fibres appearing by E17-18 when MR mRNA ceases. However at present one can only speculate as to the significance of MR mRNA in these tissues. Finally in patients with defects in 11ß-HSD2(16,17) phenotype varies in severity but is dominated by the effects of failure to protect MR from glucocorticoid in kidney; whether there are other abnormalities explicable by loss of 11ß-HSD2 at other sites or during development remains to be elucidated, though the very high incidence of low birth weight(16) is suggestive of just such overexposure of tissues to glucocorticoid.

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REFERENCES.

1. **Cole TJ, Blendy JA, Monaghan AP, Kreiglstein K, Schmid W, Aguzzi G, Fantuzzi G, Hummler E, Unsicker K,Schutz G** 1995 Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes and Development 9:1608-1621

2. **Seckl JR, Brown RW** 1994 11-Beta-hydroxysteroid dehydrogenase: On several roads to hypertension. J Hypertens 12:105-112

3. **Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR** 1993 Dysfunction of placental glucocorticoid barrier: Link between fetal environment and adult hypertension? Lancet 341:355-357

4. **Brown RW, Chapman KE, Kotelevtsev Y, Yau J, Lindsay RM, Brett L, Leckie C, Murad P, Lyons V, Mullins JJ, Edwards CRW, Seckl JR** 1995 Cloning and Production of Antisera to Human Placental 11ß-Hydroxysteroid Dehydrogenase Type 2. Biochem J (In Press)

5. **Cole TJ** 1995 Cloning of the mouse 11ß-hydroxysteroid dehydrogenase type 2 gene: tissue specific expression and localisation in distal convoluted tubules and collecting ducts of the kidney. Endocrinology 136: 4693-4696

6. **Arriza JL, Simerly RB, Swanson LW, Evans RM** 1988 The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. Neuron 1:887-900

7. **Wilkinson DG** 1992 In situ hybridisation. A practical approach. Oxford University Press.

8. **Burton AF, Turnell RW** 1968 11-dehydrocorticosteroids in tissues of mice. Canadian Journal of Biochemistry 46:497-502

9. **Murphy BE** 1981 Ontogeny of cortisol-cortisone interconversion in human tissues: a role for cortisone in human fetal development. J Steroid Biochem 14:811-817

10. **Stewart PM, Murry BA, Mason JI** 1994 Type 2 11beta -hydroxysteroid dehydrogenase in human fetal tissues. J Clin Endocrinol Metab 78:1529-1532

11. **Rajan V, Bard JBL, Ross A, Seckl JR** 1994 Glucocorticoid receptor gene expression during mouse development. J.Endocrinol 140(Suppl):p96

12. **Brown RW, Chapman KE, Edwards CRW, Seckl JR** 1993 Human placental 11beta -hydroxysteroid dehydrogenase: Evidence for and partial purification of a distinct NAD-dependent isoform. Endocrinology 132:2614-2621

13. **Fuller PJ, Verity K** 1990 Mineralocorticoid receptor gene expression in the gastrointestinal tract: Distribution and ontogeny. J Steroid Biochem 36:263-267

14. **Van Eekelen JAM, Bohn MC, de Kloet ER** 1991 Postnatal ontogeny of mineralocorticoid and glucocorticoid receptor gene expression in regions of the rat tel- and diencephalon. Developmental Brain Research 61:33-43

15. **Abbott BD, McNabb FMA, Lau C** 1994 Glucocorticoid receptor expression during the development of the embryonic mouse palate. J Craniofac Genet Devel Biol 14:87-96

16. **Mune T, Rogerson FM, Nikkilä H, Agarwal AK., White PC** 1995 Human hypertension caused by mutations in the kidney isozyme of 11ß-hydroxysteroid dehydrogenase. Nature Genetics 10:394-399

17. **Wilson RC, Krozowski ZS, Li K., Obeyesekere VR, Razzaghy-Azar M, Harbison MD, Wei JQ, Shackleton CHL, Funder JW, New MI** 1995 A mutation in the HSD11B2 gene in a family with apparent mineralocorticoid excess. J. Clin. Endocrinol Metab. 80:2263-2265