Neurosteroid Hydroxylase CYP7B: Vivid Reporter Activity in Dentate Gyrus of Gene-Targeted Mice and Abolition of a Widespread Pathway of Steroid and Oxysterol Hydroxylation*#

Ken Rose*, Adrian Allan†, Stephan Gauldie*, Genevieve Stapleton*,
Lorraine Dobbie*, Karin Dott‡, Cécile Martin‡, Ling Wang§, Eva Hedlund§, Jonathan R. Seckl‡, Jan-Åke Gustafsson§, and Richard Lathe*

From the *Centre for Genome Research and Centre for Neuroscience, University of Edinburgh, King’s Buildings, Edinburgh EH9 3JQ, §Karolinska Institute, Huddinge, Sweden, ‡Transgène SA, 11 Rue de Molsheim, 67000 Strasbourg, France, and the =Molecular Medicine Centre, Western General Hospital, Crewe Road, Edinburgh

Corresponding author: Dr. R. Lathe
CGR, King’s Buildings, West Mains Road
Edinburgh EH9 3JQ
United Kingdom
+44 131 650 5890
Fax +44 131 650 7773
Rlathe@ed.ac.uk
The major adrenal steroid dehydroepiandrosterone (DHEA) enhances memory and immune function but has no known dedicated receptor; local metabolism may govern its activity. We described a cytochrome P450 expressed in brain and other tissues, CYP7B, that catalyzes the 7α-hydroxylation of oxysterols and 3β-hydroxysteroids including DHEA. We report here that CYP7B mRNA and 7α-hydroxylation activity are widespread in rat tissues. However, steroids related to DHEA are reported to be modified at positions other than 7α, exemplified by prominent 6α-hydroxylation of A/andediol (5α-androstane-3β,17β-diol) in some rodent tissues including brain. To determine whether CYP7B is responsible for these and other activities we disrupted the mouse Cyp7b gene by targeted insertion of an IRES-lacZ reporter cassette, placing reporter enzyme activity (β-galactosidase) under Cyp7b promoter control. In heterozygous mouse brain, chromogenic detection of reporter activity was strikingly restricted to the dentate gyrus. Staining did not exactly reproduce the in situ hybridization expression pattern; post-transcriptional control is inferred. Lower level staining was detected in cerebellum, liver and kidney, and which largely paralleled mRNA distribution. Liver and kidney expression was sexually dimorphic. Mice homozygous for the insertion are viable and superficially normal, but ex vivo metabolism of DHEA to 7α-hydroxyDHEA was abolished in brain, spleen, thymus, heart, lung, prostate, uterus and mammary gland; lower abundance metabolites were also eliminated. 7α-hydroxylation of 25-hydroxycholesterol and related substrates was also abolished, as was presumed 6α-hydroxylation of A/andediol. These different enzyme activities therefore derive from the Cyp7b gene. CYP7B is thus a major extra-hepatic steroid and oxysterol hydroxylase and provides the predominant route for local metabolism of DHEA and related molecules in brain and other tissues.
Brain function is subject to hormonal control, notably by steroids synthesized from the adrenal glands and gonads. Accumulating evidence also points to local steroid synthesis and metabolism in brain; a growing field of investigation focuses on the biological role of brain-active steroids, or ‘neurosteroids’ (1-5). Attention has focussed on the major adrenal steroid in primates, dehydroepiandrosterone (DHEA), in view of a possible link with cognitive ageing and immunosenescence. DHEA and related steroids including pregnenolone have memory-enhancing properties in rodents (6-8) as well as immunostimulatory effects (9-11). In primates, levels of DHEA and its sulfate (DHEAS) decline asymptotically with age (12-14). A causal relationship with age-related physiological impairments has been debated (15,16). Because DHEA replacement therapy has brought mixed results (15,17-19) and no dedicated receptor has been described for DHEA, its bioactivity may require local metabolism.

B-ring hydroxylation is a major metabolic route for 3β-hydroxysteroids, including DHEA and pregnenolone, in diverse tissues including brain, heart, liver, mammary and adipose tissue, ovary, pituitary, prostate, spleen and thymus (20-32). In rodent brain, 7α–hydroxylation is the major ex vivo metabolic route for DHEA, pregnenolone, and A/enediol (24,25,31,33-35,29) and, in brain and other tissues, for oxysterols/bile acids (25- and 27-hydroxycholesterol; 3β-hydroxy-5-cholestenoic acid; 3β-hydroxy-5-cholenoic acid, ref. 29), though 7β- and 6α–hydroxylation have also been recorded. Nevertheless, the identities of the enzymes responsible for these diverse activities have not been fully elucidated.

We reported the molecular cloning, from rat and mouse hippocampus, of a new cytochrome P450 with steroid-modifying potential (36). The enzyme is most homologous (39%) to hepatic cholesterol 7α–hydroxylase, CYP7A (37) and more distantly related to CYP8A (prostacyclin synthase; 38) and CYP8B (sterol 12α-hydroxylase; 39). Expressed from recombinant vaccinia virus, the mouse enzyme metabolized DHEA to 7α–hydroxyDHEA (7HD) (ref. 40); pregnenolone, estradiol (E2) and oxysterols including 25-hydroxycholesterol are also converted by the recombinant enzyme (40,41) (Fig. 1A). However, the exact relationship of CYP7B enzyme to the observed ex-vivo steroid hydroxylation activities is not known.

Multiple enzymes may be present. First, CYP7B emerged from a screen for hippocampus-specific genes (36); steroid hydroxylation in different brain regions may be mediated by other enzyme(s). Second, inhibitor studies argue that separate enzymes catalyse the 7α– and 7β-
hydroxylation of DHEA and pregnenolone in brain (31,33-35). Third, metabolism of A/anediol in rodent brain and also prostate is principally at the 6α position (24-26,28), perhaps indicative of a separate A/anediol hydroxylase enzyme.

To address these questions: the identity of the brain enzyme, its relationship to the observed activities, and expression pattern, we prepared mice harboring a targeted insertion of a reporter gene cassette (IRES-lacZ) into Cyp7b. We describe reporter gene activity in brain and other tissues, and explore alterations in ex-vivo steroid hydroxylation in tissues of Cyp7b -/- mice. We argue that the Cyp7b locus encodes a major pathway of extrahepatic steroid and oxysterol hydroxylation.
EXPERIMENTAL PROCEDURES

Steroid conversions and Northern blotting - Tissue extracts, recombinant vaccinia expressing CYP7B, and assays for hydroxylation activity were as described (40). Substrates [\(^{14}\text{C}\)-] DHEA, [\(^{3}\text{H}\)-] 25-hydroxycholesterol, [\(^{3}\text{H}\)-] A/enediol, [\(^{3}\text{H}\)-] A/anediol, [\(^{3}\text{H}\)-] 5a-androstane-3a,17\(\beta\)-diol; (45-60 mCi/mm for \(^{14}\text{C}\); 20-95 Ci/mm for \(^{3}\text{H}\)) were purchased from New England Nuclear. Tissue homogenates (50 \(\mu\)l) in Waxman’s buffer (0.1 M KPO\(_4\), 1 mM EDTA, 20% w/v glycerol, pH 7.5) were incubated (200 \(\mu\)l volume containing 1 mM NADPH) with radiolabelled substrates for 20 min at 37\(^\circ\)C; steroids were extracted with ethyl acetate, dried, taken up in ethyl acetate, applied to aluminum-backed silica gel TLC plates (Merck), and developed using the buffer system N of Waxman (ethyl acetate/n-hexane/acetic acid, 16:8:1). Chromatograms were visualized by autoradiography. Northern blotting was according to conventional techniques using nylon (Hybond N, Amersham); hybridization used riboprobes at 68\(^\circ\)C under conditions as described (42).

Gene targeting in ES cells – A segment of mouse DNA encompassing exons I to IV of the Cyp7b gene was isolated from a library of 120/Ola genomic DNA in lambda2001 by probing with a sub-genomic clone previously isolated (G. Stapleton, PhD Thesis, University of Edinburgh, 1994). The exon-intron structure is similar to that described for the gene encoding the related enzyme, cholesterol \(\alpha\) hydroxylase (KR, GS and RL, unpublished data; 43,44). An insertion/replacement construct was built in which the IRES-lacZ cassette is stationed within Cyp7b exon II. An 8 kb BamHI-HindIII fragment was subcloned into pBluescriptII and a 5 kb reporter/selection cassette, comprising the LacZ enzyme coding sequence (lacking an artificial nuclear localization signal) prefixed by a viral IRES element, and also containing a neomycin phosphotransferase gene under independent promoter control (MC1neo) and a polyadenylation sequence (45), was introduced at an internal BamHI site within exon II (see Fig. 2). The hybrid construct was suffixed by two copies of a herpes simplex virus thymidine kinase (TK) expression cassette and transfected into E14-TG2a ES cells. Positive-negative selection (46) was used to enrich for targeted clones. Colonies were screened by restriction enzyme digestion (PstI, EcoRI, EcoRI+SalI) and Southern hybridization to separate probes outwith the homology arms (see Fig. 1): external probes were: 150 nt HindIII-NarI fragment from the 5’ end of the mouse cDNA (clone 35 in ref. 36) corresponding to exon I (5’ probe); 480 HindIII-BglII fragment from clone 25 corresponding to exon III (3’ probe).
Generation of transgenic mice - Targeted ES cell clones were injected into the blastocysts of strain C57BL/6 mice; chimeric males were mated to strain C57BL/6 females. Progeny typing was by Southern analysis of tail-tip DNA both using the probes described above. Mice were systematically backcrossed against C57BL/6 animals; the experiments reported use animals of >3 backcross generations. To prepare homozygotes, littermates were intercrossed for each experiment.

Reporter gene expression and in situ hybridization - Frozen tissue sections (10 µM) were transferred to TESPA (2-aminopropyltriethoxysilane)-coated slides, fixed (0.25% w/v glutaraldehyde in 5 mM EGTA, 2 mM MgCl₂, 100 mM NaPO₄ pH 7.3; 5 min.), rinsed (2 mM MgCl₂; 0.01% sodium deoxycholate; 0.01% Nonidet-P40; 100 mM NaPO₄ pH 7.3), and stained (rinse buffer containing 1 mg/ml X-gal, Bethesda Research Labs, 5 mM Kferricyanide, 5 mM Kferrocyanide) at 37°C for 4-16 hours. For in situ hybridization, TESPA slide-mounted tissue sections were fixed (4% w/v paraformaldehyde, 15 min, 4°C), deproteinized (20 µg/ml proteinase K, 1 min; blocking with 0.2% glycine, 5 min), acetylated (0.25% acetic anhydride, 0.1 M triethanolamine pH 8, 0.8% w/v NaCl, 10 min), and dehydrated by passing via successively increasing ethanol solutions (50% to 100% ethanol), immersed in CHCl₃, rinsed in ethanol, and air-dried. For hybridization, sections were incubated overnight at 55°C with [³²P]-riboprobes (prepared by in vitro transcription from pBluescript and pretreated with 10 mM dithiothreitol) in buffer containing 50% v/v deionized formamide, 0.3 M NaCl, 20 mM Tris.Cl pH 8, 5 mM EDTA, 10 mM NaPO₄ pH 8, 10% w/v dextran sulfate, 1 x Denhardt’s solution, 0.5 mg/ml yeast RNA). Higher stringency washing (2 x SSC, 0.1 M dithiothreitol, 65°C, 30 min) was followed by RNase treatment (RNase A, 20 µg/ml, 30 min, 37°C), washing, and dehydration through increasing ethanol concentrations. Slides were exposed for autoradiography (Kodak Biomax).

Immunohistochemistry - Peptides CHEDLEIGAHHLGF and CFEAPEEFRYDRFIEDGKKKTT, designed from the sequences of rat, mouse and human CYP7B (KR and RL, unpublished) and prefixed with N-terminal cysteines, were prepared by chemical synthesis (Albachem Ltd., Edinburgh, UK) and conjugated with keyhole limpet hemocyanin; we gratefully acknowledge Dr. N. Robertson's assistance. Adjuvant-complexed peptides were pooled before inoculation into sheep (Scottish Antibody Production Unit, Carluke, Scotland), generating sera S897 and S898. Immunohistochemistry was performed on perfused and paraformaldehyde-fixed brain sections and developed using a biotinylated second antibody, peroxidase-avidin complex, and diaminobenzidine tetrahydrochloride.
RESULTS

Widespread expression of steroid hydroxylase activity and CYP7B mRNA in rat tissues - We assessed the tissue distribution of steroid hydroxylation activity, measured biochemically, and of CYP7B mRNA, measured by Northern blotting. Fig. 1B confirms CYP7B mRNA in many rat tissues including brain, spleen, heart, prostate, lung and ovary, in addition to kidney and liver as reported (36). Biochemical analysis of different rat tissues revealed that most if not all tissues can convert DHEA to more polar metabolites. Prominent hydroxylation of DHEA to a product comigrating with 7α-hydroxyDHEA (7HD) took place in brain extracts, as previously reported (40), and was also demonstrated in spleen, heart, prostate, seminal vesicles and uterus (Fig. 1C) as well as in lung, uterus and mammary gland (not presented). Some other tissues gave some indication of products comigrating with 7HD (lung, testis, kidney, liver), but many metabolites failed to comigrate with 7HD, suggesting that other pathways also operate. A similar broad distribution of hydroxylation activity was also observed with A/αnediol, A/αenediol, and 25-hydroxycholesterol, while the 3α-hydroxylated substrate 5α-Androstane-3α,17β-diol was not significantly metabolized in most tissues including brain (data not presented). We conclude that CYP7B enzyme and 7α-hydroxylation activities are widely expressed in rat tissues.

Targeting the mouse Cyp7b gene - To address the relationship between CYP7B expression and steroid/sterol hydroxylation, we disrupted the mouse Cyp7b gene. To track CYP7B expression, a bacterial reporter gene (lacZ) was inserted into exon II downstream of the Cyp7b promoter, and prefixed with a viral IRES element permitting efficient translation from the lacZ cistron (Fig. 2A). Homologous recombination in ES cells introduced the IRES-reporter segment into the resident gene. Clones were screened by Southern blotting using internal and external probes; a large proportion gave the expected pattern (Fig. 2B). Male chimeras were generated by blastocyst injection, crossed to C57BL/6 females; heterozygous transgenic progeny of these chimeras appeared at the expected Mendelian ratio (not presented). Further work employed the progeny of one representative targeted line.

Reporter activity in liver and kidney - The targeted locus harbors an insertion of the bacterial β-galactosidase reporter, LacZ, under independent translational control; enzyme activity was anticipated to reiterate the pattern and level of transcription of the endogenous Cyp7b gene. Liver and kidney sections were stained with a chromogenic substrate for LacZ. No staining was seen in non-
transgenic tissues (Fig. 3A, panel a; Fig. 3B, panel a), marked staining was detected in both liver and kidney of transgenic animals and was sexually dimorphic. In male liver, neonatal expression of CYP7B-LacZ was limited to discrete foci (Fig. 3A, panel b); in the adult staining was associated primarily with hepatocytes rather than Kupfer cells or other elements, and was mostly in the perivenous zone of the hepatic lobules (panels c,d). No significant staining was seen in adult female liver (panels e,f). In male kidney, reporter staining was maximal in the outer stripe of the medulla (Fig. 3B, panel b). Within the outer stripe, staining was most intense in cells with more abundant cytoplasm in the S3 segment of the proximal tubule (panels c,d). In female kidney, as in liver, staining was poor to indetectable (panels e,f).

To determine whether reporter activity paralleled expression of the hybrid gene, in situ hybridization was performed using probes specific for lacZ or for CYP7B. In liver and kidney, CYP7B expression was robust but lacZ mRNA was difficult to detect (Fig. 4AB; for brain expression see sections following). mRNA patterns (Fig. 4) broadly reflected the distribution of LacZ staining (Fig. 3). CYP7B mRNA was detected in both male and female tissues. In male kidney strong expression was seen in the S3 segment of the outer stripe of the outer medulla with some expression extending as medullary rays into cortex, confirmed using a LacZ probe (Fig. 4A). It was not possible from our data to define unambiguously the cell type within each nephron segment. Nevertheless, the distribution was only compatible with expression in the S3 segment of the nephron, with minor extension to the medullary portion of the collecting duct. The cortical distal convoluted and connecting tubules were apparently negative for expression. In female CYP7B mRNA levels were much lower, but again highest in the S3 segment of the outer stripe with some faint linear low-intensity signal in the inner stripe/inner medulla in the collecting ducts (Fig. 4A). In liver, CYP7B mRNA was dispersed widely through the tissue of both male and female animals (Fig. 4B) predominantly in the perivenous zones; overall expression levels were substantially lower in female than in male. In female kidney and liver the LacZ probe failed to detect hybrid mRNA.

**Brain expression: reporter activity is robustly and selectively expressed in the dentate gyrus of hippocampus with lesser expression in cerebellum** – Brain sections from heterozygous mice were stained for reporter enzyme activity. Vivid staining was observed, almost exclusively restricted to the dentate gyrus of the hippocampal formation (Fig. 5A). On prolonged incubation lesser but significant reporter activity was also detected in cerebellum (Fig. 5B). Higher magnification revealed reporter
stain close to dentate granule neurons and a subset of neurons in the dentate hilus; regions CA3 and CA1 of hippocampus were essentially negative. Dentate gyrus staining was not precisely colocalized with the cell bodies of the dentate granule neurons; instead punctate staining was displaced asymmetrically into the axo-dendritic fields surrounding the dentate neurons (Fig. 5A).

In situ hybridization was used to evaluate the extent to which reporter enzyme activity mirrors CYP7B gene expression. The patterns of lacZ mRNA and CYP7B mRNA were similar but not entirely identical: e.g. lacZ mRNA appeared more strongly in cerebellum than in cortex; the reverse was true using CYP7B probe (Fig. 4C). This result may reflect sequence-specific mRNA turnover. A striking difference emerged between the staining pattern for LacZ reporter activity and the distribution of either CYP7B or lacZ mRNA (compare Fig. 5A with Fig. 4C). High levels of CYP7B message were associated with the dentate gyrus, and predominantly with the neuronal cell layer: this mRNA is probably responsible for the intense reporter staining seen adjacent to the dentate gyrus. However, comparable, if marginally lower, levels of CYP7B mRNA were present in other regions that failed to display any reporter gene activity, notably in CA3, possibly indicative of post-transcriptional control.

To address the brain distribution of endogenous CYP7B enzyme, polyclonal antibody was raised in sheep against synthetic CYP7B peptides and used to probe for CYP7B antigen in brain of non-transgenic animals. Pre-incubation with the primary immunogen confirmed the specificity of the antibody. However, staining was reduced but not abolished by the targeted gene disruption (not shown), the peptide epitopes selected may be present in other proteins.

Generation of null mice: Mice homozygous for the insertion are viable but fail to accumulate CYP7B mRNA - To assess the contribution of CYP7B enzyme to steroid hydroxylation in brain and other tissues, mice heterozygous for the IRES-LacZ insertion were intercrossed to produce animals in which CYP7B function is absent. The distribution of genotypes obtained (46 +/-, 86 +/-, 31 -/-) among intercross progeny was not significantly different from a purely Mendelian ratio. Nevertheless, the sex ratio among the homozygotes (M, 10; F, 21 from 163 intercross progeny) suggested, but did not prove, selective loss of males. To confirm disruption of the Cyp7b gene, Northern analysis was performed; the expected transcripts at ~2 kb (see Fig. 1) were absent, confirming that the reporter cassette has inserted into the Cyp7b gene (not presented). Mice harboring the insertion appeared superficially normal, at least to 12 months of age, although no detailed studies on homozygote fertility,
liver, kidney, brain or immune function have yet been performed in these mice. We conclude that CYP7B enzyme, in mouse, is not essential for viability.

Cyp7b gene disruption abolishes DHEA 7α−hydroxylation - To evaluate the contribution of CYP7B to steroid/sterol hydroxylation in different tissues, ex vivo minces from Cyp7b −/− and control mice were incubated with radiolabelled steroids; reaction products were examined by thin-layer chromatography (TLC) and autoradiography. Total brain extracts from wild-type mice (including cortex, cerebellum, hippocampus and other brain regions) predominantly converted DHEA to two products (Fig. 6A). The more abundant polar derivative (21% input material, 15 min reaction time) comigrated on TLC with recombinant CYP7B enzyme product (Fig. 6A); this we previously showed on the basis of TLC mobility, gas chromatography with mass-spectroscopy, and tritium-release experiments to be identical to 7α−hydroxyDHEA (7HD, ref. 40). The second species, termed Molecule 1, migrated closely behind the DHEA substrate, and is inferred to be the product of 17β-HSD activity on DHEA, androstenediol (A/enediol); the extent of brain conversion to this product was usually low (~3% input material, 15 min reaction).

Brain extracts from heterozygous animals displayed reduced substrate conversion to 7HD (53% of wild-type level); in homozygous −/− brain no conversion of DHEA to 7HD was recorded (Fig. 6A); scanning radiography revealed that 7HD was not detectable over background (less than 0.1% of wild-type conversion level). Reduced 7HD production was accompanied by a small increase in molecule 1 (~2x in −/− extracts). Conversion of A/enediol (Fig. 6A), A/anediol and 25-hydroxycholesterol (Fig. 6B) were also abolished.

Other mouse tissues were examined. Spleen, thymus, heart, lung (male), prostate, uterus and mammary gland (Fig. 6C) from Cyp7b −/− mice failed to convert DHEA to more polar derivatives including 7α−hydroxy DHEA or were severely impaired in the conversion (female lung, testis). Liver and kidney metabolism of DHEA and other steroids is complex; there were nevertheless significant differences in the profiles obtained, particularly in males (Fig. 6C), consistent with the sexual dimorphism of expression.

CYP7B and steroid 6α−hydroxylation. Incubation of extracts of wild-type mouse brain with radiolabelled A/anediol 6α−androstane-3β,17β-diol) yielded 1 major and 2 minor products on TLC; the major product comigrated with the in vitro conversion product of obtained with recombinant CYP7B enzyme (see Fig. 6B; also data not shown) and inferred to be 6α−hydroxyA/anediol; minor
products are inferred to be 7α- and 7β-hydroxylated derivatives. Incubation of total brain extracts from mutant mice with radiolabelled A/enediol failed to produce more polar derivatives; scanning quantitation of TLC plate revealed that the extent of conversion was less than 0.1% of control values while extracts from animals heterozygous for the gene disruption showed intermediate levels of conversion. We conclude that A/enediol is not hydroxylated in extracts of Cyp7b -/- mutant brain. The predominant pathway of A/enediol hydroxylation in brain and other tissues including prostate is reported by several groups to be at the 6α position, rather than at 7α. Because B-ring stereochemistry differs between 5α-reduced steroids (such as A/enediol) and 5-ene steroids (including DHEA), we infer but have not proven here that CYP7B catalyses 6α-hydroxylation of A/enediol. Because this conversion is abolished by the targeted mutation, we conclude that CYP7B is responsible for metabolism of A/enediol, and most likely to 6α-hydroxyA/enediol (5α-androstane-3β,6α,17β-triol).

Because tissues of Cyp7b -/- mice fail to hydroxylate DHEA, A/enediol, A/enediol or 25-hydroxycholesterol, and hydroxylation at both the 7α (DHEA, A/enediol, 25-hydroxycholesterol) and presumably 6α (A/enediol) positions is abolished by the gene disruption, Cyp7b is likely to encode a major extra-hepatic pathway of steroid/sterol B-ring hydroxylation.
DISCUSSION

We have used gene-targeting to address the relationship of CYP7B enzyme to the steroid and sterol hydroxylation activities reported in rodent tissues. Li-Hawkins et al. (47) recently and independently reported mice in which the Cyp7b gene had been disrupted, their studies centred on cholesterol metabolism in liver. We now describe transgenic mice in which a reporter gene (lacZ) is inserted into the Cyp7b gene; we report expression studies and steroid/sterol metabolism in extra-hepatic tissues of the mutant mouse.

Our principal observations are as follows. First, steroid hydroxylation activity and CY7B mRNA are widely distributed in brain and other tissues. Second, targeting the mouse Cyp7b gene with an IRES-lacZ construct generates reporter enzyme activity in multiple tissues including brain, kidney and liver; brain reporter expression was dramatically if superficially restricted to the dentate gyrus. Third, the pattern of reporter activity in brain failed to match exactly the distribution of CYP7B mRNA or lacZ mRNA, though the reporter activity/mRNA patterns in liver and kidney were largely coincident. Fourth, mice lacking CYP7B activity are viable and superficially normal. Fifth, ex vivo extracts of homozygous Cyp7b -/- animals fail to catalyse hydroxylation of the steroid (and sterol) substrates tested, including DHEA and 25-hydroxycholesterol. Sixth, the targeted mutation also abolished hydroxylation (presumed to be at 6α) of the 5α-reduced steroid, A/anediol. These aspects are discussed separately below.

CYP7B expression, originally suspected to be most robustly expressed in hippocampus, is found more widely. Transcripts were readily detected in rat brain, but also at significant levels in spleen, heart, prostate, lung and ovary, in addition to kidney and liver expression in both mouse and rat (this work and ref. 36). Relative levels of expression are difficult to assess precisely, but appeared highest in brain, and particularly in the dentate gyrus of the hippocampus; high levels are present in prostate, with significant levels in liver, kidney, heart and spleen. The level of mRNA expression in ovary was lower. Three mRNAs are present in brain and some other tissues: a pair of transcripts at 2kb (1.8 and 2.1) and a much larger RNA (~5kb) that is prominent in rat but not mouse brain; while the two smaller transcripts are thought to arise by alternative polyadenylation site utilization (36) the origin of the larger transcript is not known. 6α-hydroxylation activity is also widespread, and we report conversion of DHEA to a molecule comigrating with 6α-hydroxyDHEA in several rat and mouse tissues including brain, spleen, thymus, heart, lung, testis, prostate, uterus, ovary and mammary gland,
though the efficiency of conversion varied between tissues. Liver and kidney metabolism was complex, precluding specific analysis of B-ring hydroxylation. Most of these tissues hydroxylate A/α-androstanediol and 25-hydroxycholesterol (this work and data not presented) in addition to DHEA (this work). In contrast, the potent anesthetic steroid 5α-androstane-3α,17β-diol was very poorly metabolized by the majority of these tissues, including brain (not presented). In addition to 7α-hydroxylation of DHEA, we also observe a slightly more slowly migrating species, Molecule 1, that is inferred, on the basis of other experiments (E. DeGryse, P. Vico and RL, unpublished data), to be the 17β-HSD product of DHEA, androstenediol, also a substrate for CYP7B (40). This emphasizes previous reports that 17β-HSD activity is also widespread in brain and other tissues (48).

We generated mice harboring a targeted insertion of an IRES-lacZ reporter cassette. Chromogenic staining was observed in both liver and kidney, organs in which CYP7B mRNA is present (36, this work); the staining pattern was similar to the patterns of CYP7B mRNA or LacZ sequences detected by in situ hybridization. Staining in kidney was associated with the S3 segment of the outer stripe of the medulla, a region of acid-base and electrolyte exchange, fuel resorption and metabolic activity. Liver staining appeared largely in the perivenous zone of the lobules, where glycolysis predominates. The function of CYP7B in these regions is not known. No staining was observed in other tissues analyzed (not presented), probably because of the insensitivity of this technique, with the exception of peri-follicular staining in ovary and intense staining in seminiferous tubules (to be published).

In the brain, strikingly vivid reporter staining was seen in the dentate gyrus of the hippocampus, with only lower levels in cerebellum. Overt coloration was absent from other brain regions including cortex and olfactory bulb. Punctate staining in the dentate gyrus is reminiscent of the pattern seen previously with the *kin* gene-trap insertion (49), and suggestive of association of the reporter enzyme with discrete membrane components; these could be a subclass of synapses or other unidentified structures. Here, in contrast to liver and kidney, reporter expression failed to parallel either CYP7B or LacZ mRNA. Staining was restricted to dentate gyrus and, at lower levels, cerebellum; hippocampal regions CA1-3 were essentially negative. In contrast, CYP7B mRNA and transgene-encoded LacZ mRNA were through much of cortex, hippocampus, olfactory bulb and cerebellum. Nevertheless, localized LacZ activity reiterates the restricted expression exploited for CYP7B cDNA isolation (36). Because DHEA 7-hydroxylation activity is present in microdissected
brain regions including olfactory bulb, cortex, cerebellum, brainstem, and is abolished by the mutation (unpublished), our results would seem to exclude a second unidentified enzyme with significant ex vivo activity. Possible explanations for the reporter/mRNA discrepancy include: [1] tissue specificity of IRES elements (ref. 50), [2] structure and/or processing of the hybrid RNA may differ between dentate and the CA regions, [3] β-galactosidase enzyme is multimeric: LacZ staining may show a threshold effect, [4] possible post-transcriptional control of CYP7B expression: 7-hydroxylation, presumably mediated by CYP7B, is modulated by cell density (51,52) though the mechanism has not been determined.

Intercrossing heterozygous Cyp7b +/- animals generated homozygous +/- animals at or near the expected frequency. Although our data do not rule out some selective perinatal loss of male homozygotes, we conclude that Cyp7b gene function is not essential for viability in adult mice; a similar conclusion was reached by another group (47). This contrasts with the situation in human, where CY7B deficiency was associated with abnormalities of hepatic cholesterol metabolism and was incompatible with survival (43). Species differences in hepatic cholesterol metabolism were previously suggested by studies on CYP27 (cholesterol 27-hydroxylase): human mutations produce disordered lipid metabolism, atherosclerosis and mental retardation (cerebrotendinous xanthomatosis [CTX]; 53), mice lacking CYP27 display no CTX-related pathological abnormalities (54).

We inspected tissues from Cyp7b +/- mice for their ability to catalyze steroid and oxysterol hydroxylation ex vivo. 7α-hydroxylation of DHEA was abolished in brain, spleen, thymus, lung, heart, uterus and mammary gland; gene disruption not only abolished 7α-hydroxylation of DHEA, but also of pregnenolone, A/enediol, and 25-hydroxycholesterol. The complexity of steroid and sterol conversions in transgenic and control liver precluded analysis of specific B-ring modification. In brain and other tissues, homozygous disruption of the Cyp7b gene also abolishes the production of two minor products, probably 7β-hydroxyDHEA (ascertained by TLC comigration) and a second product with a TLC migration slightly faster than 7α-hydroxyDHEA: this is inferred (but not proven), on the basis of this and other work (40,31,34,35), to correspond to the 6α-hydroxy derivative of DHEA. Both products are generated in vitro by recombinant CYP7B enzyme (40). We conclude that minor modification of DHEA at the 7β and probably 6α positions is an inherent property of CYP7B enzyme. This contrasts with the conclusion, on the basis of inhibitor studies, that different enzymes in brain and prostate are responsible for 7α- and 7β- hydroxylation of DHEA and pregnenolone (34,35,31).
Incubation of A/anediol with extracts of mouse brain (this work) or recombinant CYP7B enzyme (this work and ref. 40) yielded, like DHEA, one major and two minor polar metabolites. The major metabolite of A/anediol produced by brain ex-vivo is 6α-hydroxyA/anediol (5α-androstane-3β,6α,17β-triol) as reported (24-26,28); this comigrated with the major CYP7B product. We infer, but have not formally proven, that CYP7B catalyzes 6α-hydroxylation of A/anediol, and that the less abundant A/anediol metabolites correspond to 7α- and 7β-hydroxlated derivatives. Ex vivo production of all these molecules was abolished by Cyp7b gene disruption (<0.1% of the wild-type conversion level in Cyp7b -/- extracts).

Disruption of the Cyp7b gene therefore abolishes hydroxylation of DHEA, pregnenolone, A/anediol, and 25-hydroxycholesterol, at both major (7α) and minor (7β, 6α?) positions. It also abolishes hydroxylation of A/anediol, both at the major (6α) position and at minor positions. We suggest that one gene product, CYP7B enzyme, is responsible for all these activities.

This study does not rule out formally the possibility that different transcripts from the CYP7B gene might encode physically distinct enzymes with separate substrate specificities and hydroxylation stereochemistry. We think this unlikely. First, substrate specificity and stereochemistry for these conversions can vary according to reaction conditions (26,31). Second, the hydroxylation profiles of DHEA and A/anediol by brain and recombinant CYP7B enzyme are indistinguishable (unpublished).

CYP7B is thereby likely to furnish a major extra-hepatic and broad-spectrum steroid/sterol B-ring hydroxylase, with predominant hydroxylation at the 7α position (exemplified by DHEA and oxysterols) complemented by 6α-hydroxylation of some atypical substrates (exemplified by A/anediol). CYP7B is not the only extra-hepatic B-ring hydroxylase: a testosterone 7α-hydroxylase has been described in testis (CYP2A9/15: refs. 55,56) while, in human (but not rodent) prostate, 6α-hydroxylation is reported to be performed by a non-P450 enzyme that, unlike CYP7B (this work), modifies steroids with the 5α-3α configuration (57,32).

What might be the biological role of steroid/sterol B-ring hydroxylation? In liver, hydroxylation promotes metabolic elimination: oxysterol conversion to bile acids is promoted by CYP7A and CYP7B operating in parallel with a dedicated liver-specific 24(S)-hydroxycholesterol 7α-hydroxylase, CYP39A1 (58). Hydroxylation may also promote metabolic elimination of testosterone and progesterone derivatives following 5α-reduction and 3β-HSD action (see ref. 28). In
other tissues a specific regulatory role for B-ring modified steroids has been suggested. In addition to
feedback control of cholesterol synthesis by 6- and 7-hydroxylated cholesterols (59,60), B-ring
modified sterols may regulate cell death processes and cognitive and immune function (ref. 5 for
review).

More generally, CYP7B activity may gate steroid access to receptor targets, either by
preventing or potentiating receptor interactions. The major adrenal steroid DHEA is a case in point: 7-
hydroxylation may generate, or be on the metabolic pathway towards, the bioactive derivatives that
enhance cognitive, immune and other physiological processes, notably those that decline (like DHEA
levels) with age. While the specific receptors targeted by 7-modified steroids of this class remain to be
identified, a report that 7-oxoDHEA is more effective than DHEA in promoting long-term memory
retention in old (22 mo) mice (61) is suggestive of this interpretation, and further emphasizes that
CYP7B-mediated hydroxylation may not be the end of the metabolic pathway (62). Notably, a
mammalian 7α-hydroxysteroid dehydrogenase activity has been described (63).

Receptor gating may also be indirect. Hydroxylation of DHEA and related steroids may
divert these precursors from local synthesis of more active steroids including corticosteroids and sex
steroids. This might be important in liver and kidney where the striking sexual dimorphism reflects a
similar male preponderance of androgen-sensitive gene expression. Further, the most potent anesthetic
steroids, with the 3α-5α configuration, are not CYP7B substrates, but may compete with the relatively
inert A/anediol for access to cell-surface receptor channels: hydroxylation of A/anediol could gate this
process (28). A similar process may take place at nuclear receptors: inert 7-oxo steroids might compete
with active hormone (64). Studies on mutant mice will be required to test these possibilities.
Investigations of cognitive, neuroendocrine and electrophysiological parameters in the mutant mice are
planned, particularly as a function of age.

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LEGENDS TO FIGURES.

FIG. 1. **Steroid hydroxylation and CYP7B mRNA in rat tissues.** A. Reaction catalyzed by CYP7B enzyme. B. Northern analysis of rat tissues probed with CYP7B or ribosomal protein S26 (internal control). C: Ex vivo DHEA hydroxylation in rat tissues analyzed by ascending TLC. Tissues (panels B and C) were: Br, brain; Sp, spleen; He, heart; Pr, prostate; Se, seminal vesicle; Ut, uterus; Te, testis; Lu, lung; Ov, ovary; Ki, kidney; Li, liver (extracts of male, m, and female, f, animals); DHEA, substrate only. m1, molecule 1 (see text); 7HD, 7α-hydroxyDHEA. Filled arrow, sample application; open arrow, DHEA substrate.

FIG. 2. **Gene-targeting of the mouse Cyp7b locus.** A: Exons I-V of the Cyp7b gene, showing insertion of the IRES-lacX-pMC1-neo cassette into exon II; restriction sites are P, PstI; B, BamHI, H, HindIII; E, EcoRI; S, Sall; X, XhoI; Bg/B, BglII/BamHI fusion site. HSV TK is the thymidine kinase gene of herpes simplex virus. B: Southern analysis of targeted clones; restriction sites and digests as in panel A.

FIG. 3. **Expression of chromogenic reporter activity in gene-targeted mice.** Tissues of wild-type (WT) and Cyp7b +/- mice stained with a substrate for LacZ enzyme activity (blue staining). Panel A: Liver; panels are a, WT, female; b, +/-, male, neonate; cd, +/-, male, adult; e,f, +/-, female, adult. Scale bars, filled, 100 µm; open, 1 mm. Panel B: Kidney, a, WT, male, adult; b-d, +/-, male, adult; e, WT, female, adult; f, +/-, female, adult, scale bars as in panel A.

FIG. 4. **Expression of CYP7B and LacZ mRNA in gene-targeted mice.** In situ hybridization of kidney (panel A), liver (panel B) and brain (panel C) used probes as indicated; in A the first two panels are stained sections; all other panels are in situ hybridization (contact autoradiography); Ma, male, Fe, female; +/-, genotype at Cyp7b locus; wt, wild-type. Subregions CA1, CA3 and dentate gyrus (DG) of the hippocampal formation are indicated in Panel C.

FIG. 5. **Reporter staining in brain of Cyp7b +/- mice.** A, brain and hippocampal staining: top panel, complete sagittal section of brain (hematoxylin/eosin staining, red coloration; LacZ enzyme activity,
blue coloration), enlargements below; B, cerebellar staining. Scales are given as original magnifications (10x or 40x).

**FIG. 6** Ex vivo steroid metabolism (TLC analysis) in tissues of Cyp7b -/- mice. Panel A: Brain metabolism of DHEA and A/enediol is abolished by Cyp7b gene disruption; lanes are V7b, recombinant CYP7B enzyme expressed from vaccinia virus; Sp, spleen, Br, brain, +/-, +/-. -/-; genotype at the Cyp7b locus; S, substrate (no extract); filled arrows, position of substrate application; open arrows, substrate. Panel B: Metabolism of DHEA, A/anediol and 25-hydroxycholesterol (25-OHChol) is abolished in both spleen (Sp) and brain (Br). Panel C: Metabolism of DHEA to 7α-hydroxyDHEA (horizontal line) is abolished or diminished in several tissues of male and female mutant mice; paired tracks are extracts from wild-type (left) and mutant (right) mice; tissues were Br, brain; Sp, spleen; He, heart; Te, testis; Pr, prostate; Lu, lung; Th, thymus; Li, liver; Ki, kidney; Ut, uterus; Ma, mammary gland. m1, molecule 1 (see text); 7HD, 7α-hydroxyDHEA.
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#To whom correspondence should be addressed: Centre for Genome Research, Edinburgh University, West Mains Road, Edinburgh EH9 3JQ, UK

1. The abbreviations used in this work are: A/anediol, 5α-androstane-3β,17β-diol (androstanediol); A/enediol, 5-androstene-3β,17β-diol (androstenediol); DHEA, dehydroepiandrosterone; 7HD, 7α-hydroxyDHEA; IRES, internal ribosomal entry signal; HSD, hydroxysteroid dehydrogenase; TLC, thin layer chromatography.
Fig. 2

Diagram A shows a genetic map with restriction sites and gene sequences. Diagram B illustrates the distances between probes and their hybridization patterns for different genotypes (WT, +/-).
Fig. 4

A: kidney

B: liver

C: brain

Cyp7b

LacZ

CA1

CA3

DG
Fig. 6
Neurosteroid Hydroxylase CYP7B: Vivid Reporter Activity in Dentate Gyrus of Gene-Targeted Mice and Abolition of a Widespread Pathway of Steroid and Oxysterol Hydroxylation

Ken Rose, Adrian Allan, Stephan Gauldie, Genevieve Stapleton, Lorraine Dobbie, Karin Dott, Cécile Martin, Ling Wang, Eva Hedlund, Jonathan R Seckl, Jan-Åke Gustafsson and Richard Lathe

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