Expression of 11β-hydroxylase and aldosterone synthase genes in the rat brain

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ABSTRACT

The terminal stages of cortisol and aldosterone production in the human adrenal gland are catalysed by the enzymes 11β-hydroxylase and aldosterone synthase, which are encoded by the *CYP11B1* and *CYP11B2* genes respectively. Recent studies have suggested that aldosterone and cortisol are also made in other tissues such as the brain, heart and vascular system and may play a role in cardiovascular homeostasis. The aim of this study was to confirm the presence of these enzymes and localise them precisely in the rat brain.

Reverse transcription-polymerase chain reaction (RT-PCR)/Southern blotting confirmed transcription of *CYP11B1* and *CYP11B2* in whole brain and hypothalamus minces from Wistar–Kyoto rats. 11β-Hydroxylase and aldosterone synthase were immunolocalised in paraffin-embedded rat adrenal and brain sections using mouse monoclonal antibodies. Negative controls utilised a mouse monoclonal antibody raised against a non-mammalian epitope. In the brain, 11β-hydroxylase and aldosterone synthase were detected in the cerebellum, especially the Purkinje cells, as well as the hippocampus. The specificities of the 11β-hydroxylase and aldosterone synthase antibodies were confirmed by positive immunostaining of the relevant regions of the adrenal cortex.

This is the first direct evidence that steroid hydroxylases involved in the final stages of corticosteroid biosynthesis are present in specific regions of the central nervous system.
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INTRODUCTION

Synthesis and secretion of mineralocorticoids and glucocorticoids from cholesterol occurs in the adrenal cortex, with the initial step, the conversion of cholesterol to pregnenolone, performed by the *CYP11A1* gene product, the side-chain cleavage enzyme. The major glucocorticoid in man is cortisol; in the rat, it is corticosterone. Aldosterone is the major mineralocorticoid in both species. It is synthesised in the zona glomerulosa of the adrenal cortex while cortisol or corticosterone are made mainly in the zona fasciculata (White *et al*. 1992, Vinson *et al*. 1995). The final steps of cortisol and aldosterone biosynthesis are catalysed by 11β-hydroxylase and aldosterone synthase respectively.

11β-Hydroxylase is localised in the zona fasciculata and is regulated by adrenocorticotrophin (ACTH). Aldosterone synthase is unique to the zona glomerulosa and is regulated by the renin–angiotensin system (RAS) (Oertle & Müller 1993). The genes encoding 11β-hydroxylase (*CYP11B1*) and aldosterone synthase (*CYP11B2*) have a high percentage of sequence identity (Imai *et al*. 1990, Nomura *et al*. 1993, Zhang & Miller 1996).

Both aldosterone and cortisol (or corticosterone) have important effects on electrolyte balance and blood pressure by their actions on the kidney and the vasculature (Fraser *et al*. 1989). In addition to these systemic effects, however, it is clear that they exert significant central effects. Three types of evidence exist. First, direct intracerebral

TABLE 1. Oligonucleotide primer sequences used for PCR amplification of cDNA

Primer		Sequence (5′–3′)	Length of product (bp)
CYP11B1/2/3	Upstream	AAC TCC GTG GCC TGA GAC G	342
	Downstream	GCT GTG TGG TGG ACT TGA AC	
Adrenodoxin	Upstream	GAC TCT CTG CTA GAT GTT GTG ATT	289
	Downstream	ATT CTT GCT CAT GTC AAC AGA CTG TCG	
CYP11A1	Upstream	CAA CAT CAC AGA GAT GCT GGC AGG	583
	Downstream	CTC AGG CAT CAG GAT GAG GTT GAA	

administration of doses too small to affect systemic concentrations elicit effects which may differ from their classical systemic effects. For example, small doses of aldosterone raise blood pressure in the rat without the accompanying changes in extracellular sodium or potassium concentrations (Gómez-Sánchez 1997) characteristic of systemic administration. Secondly, the brain is abundantly supplied with corticosteroid receptors. The glucocorticoid receptor is widely distributed but the mineralocorticoid receptor is particularly concentrated in the hippocampus and hypothalamus (De Kloet *et al*. 1998). Thirdly, there is increasing evidence to suggest that corticosteroids can be synthesised in the brain. Both *CYP11B1* and *CYP11B2* appear to be transcribed in brain tissue (Strömstedt & Waterman 1995). There is also some evidence that brain tissue *in vitro* converts precursors to end products (Gómez-Sánchez *et al*. 1997) although *de novo* synthesis from cholesterol has yet to be demonstrated. Rates of synthesis are likely to be small in comparison to those of the adrenal cortex. However, since the brain is highly sensitive to corticosteroids (see above), low level, local synthesis may be physiologically important.

We investigated the transcription of *CYP11B1*, *CYP11B2* and *CYP11A1* in several rat tissues. Evidence of *CYP11A1* expression in extra-adrenal tissues would support the *de novo* formation of corticosteroids. In addition, we also tested for the transcription of adrenodoxin, a cofactor required by mitochondrial cytochrome P450 enzymes such as aldosterone synthase, 11β-hydroxylase and the side-chain cleavage enzyme.

Evidence of corticosteroid synthesis in the rat brain has so far been obtained by reverse transcription-PCR (RT-PCR) or by conversion of steroid precursors to end products in tissue homogenates. To assess its potential role, it is necessary to define more precisely the site(s) of synthesis. With this in mind, evidence of aldosterone synthase and 11β-hydroxylase production, as opposed to gene transcription, and more precise information on their location within the brain have been obtained by immunohistochemistry.

MATERIALS AND METHODS

RNA isolation

Tissues from adult female Wistar–Kyoto rats fed on normal diets were snap frozen in liquid nitrogen for subsequent RNA extraction. Total RNA was extracted from these tissues according to the standard RNAzol B reagent protocol (Biogenesis Ltd, Poole, Dorset, UK). The quality and concentration of the resulting RNA was confirmed by spectrophotometry and electrophoresis on ethidium bromide-stained agarose gels.

RT-PCR

Oligonucleotide primers (Oswel, Southampton, Hants, UK) homologous to parts of the *CYP11B1/B2*, adrenodoxin and *CYP11A1* genes were produced (Table 1). RT-PCR amplification was performed using the GeneAmp RNA PCR kit (Perkin Elmer, Warrington, UK) and oligo-d(T) primers. cDNA was transcribed according to the standard kit protocol. Amplification of *CYP11A1* and adrenodoxin were performed under identical conditions, following the method of Strömstedt & Waterman (1995). There was a final extension step of 7 min at 74 °C, after Nomura *et al*. (1993). Controls had reverse transcriptase enzyme omitted, or water substituted for RNA.

Southern blotting

RT-PCR products were run on 2% agarose gels and Southern blotted to Hybond-N+ membrane (Amersham Life Science, Amersham, Bucks, UK). *CYP11B1* and *CYP11B2* transcript products were

distinguished by hybridising with radiolabelled oligonucleotide probes. The *CYP11B1*-specific oligonucleotide was 5′-TAA ACA TTC AGT CCA ATA-3′; the *CYP11B2*-specific oligonucleotide was 5′-TGG ATG TCC AGC AAA GTC-3′ (position 556–574 bp, exon III). *CYP11A1* (the gene for P450scc) transcripts were identified using the oligonucleotide 5′-GGT GGA GTC TCA GTG TCT CCT TGA TGC TGG CTT TGA G-3′. An adrenodoxin-specific radiolabelled oligonucleotide was not required as any resulting bands were clearly visible on ethidium bromide-stained gels under UV light.

Monoclonal antibody preparation

Synthetic peptides corresponding to hydrophilic areas of aldosterone synthase and 11β-hydroxylase exhibiting minimal homology were prepared by Research Genetics Inc. (Huntsville, AL, USA). The aldosterone synthase monoclonal antibody was generated by immunising CD-1 mice, as described by Tam (1988), with the multiple antigenic peptide MAP-KVRQNARGSLTMDVQQ corresponding to residues 175–190. The 11β-hydroxylase monoclonal antibody was generated in Swiss–Webster mice immunised with the peptide KNVYRE-LAEGRQQS corresponding to residues 272–285, and conjugated to chicken serum albumin. The spleens of the mice with the highest titre were fused to an SP-2 myeloma cell line and the clones selected for their ability to bind immobilised sonicated zona glomerulosa mitochondria from rats on a low sodium diet. Both antibodies were of the IgG1 type.

Tissue preparation for immunohistochemistry

Brains and adrenal glands were removed from adult female Wistar–Kyoto rats immediately after lethal injection and fixed for 24 h in 4% (w/v) paraformaldehyde solution before being embedded to form tissue blocks. Sections 5 μm thick were prepared from these, mounted on 3-aminopropyltriethoxysilane-treated slides and air-dried.

Immunohistochemistry

Tissue sections were deparaffinised with xylene and rehydrated through graded alcohols before being rinsed in deionised water and Tris-buffered saline (TBS). The sections were then subjected to microwave antigen-retrieval in 0·01 M citrate buffer, pH 6·0 for 10 min. Subsequent steps were performed using the DAKO Catalysed Signal Amplification (CSA) System Peroxidase Kit (DAKO Ltd, Cambridge, Cambs, UK). Primary antibody was incubated overnight at 4 °C. Immunostaining was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAKO Ltd). Sections were then counterstained with filtered modified Harris haematoxylin solution (Sigma-Aldrich Co. Ltd, Poole, Dorset, UK) for 5 min, dehydrated, and mounted in DPX (BDH Laboratory Supplies, Lutterworth, Leics, UK). Control sections were incubated with mouse IgG1 negative control culture supernatant (DAKO Ltd). In addition, specificity of the monoclonal antibodies was confirmed by incubating them with an excess of their respective antigenic polypeptides (Alta Bioscience, Birmingham, UK), thus quenching any antibody binding.

RESULTS

RT-PCR

CYP11B1 (Fig. 1a) and *B2* (Fig. 1b) were transcribed in the adrenal gland, whole brain and hypothalamus. There was no evidence of expression in the kidney, liver or heart. All the tissues tested were positive for adrenodoxin transcripts (Fig. 1c). There was also evidence of *CYP11A1* transcription in brain tissue (Fig. 1d). All control reactions were negative.

Immunohistochemistry

The specificities of the anti-11β-hydroxylase and anti-aldosterone synthase antibodies were confirmed by immunohistochemistry in rat adrenal gland sections (Fig. 2). The anti-aldosterone synthase antibody produced staining only in the zona glomerulosa whereas the anti-11β-hydroxylase antibody bound to the zona fasciculata and zona reticularis but not to the zona glomerulosa. There were also projections of cells stained for 11β-hydroxylase into the adrenal medulla.

In the brain, immunostaining of both 11β-hydroxylase and aldosterone synthase occurred in the cerebellar cortex (Fig. 3), particularly within the Purkinje cells. 11β-Hydroxylase and aldosterone synthase were also detected in the hippocampus, with greatest intensity in the dentate gyrus and CA3 cells (Fig. 4). No evidence of translation could be found in the hypothalamus. Immunostaining could not be seen in control sections. Positive immunostaining was quenched by prior incubation with excess antigenic peptide.
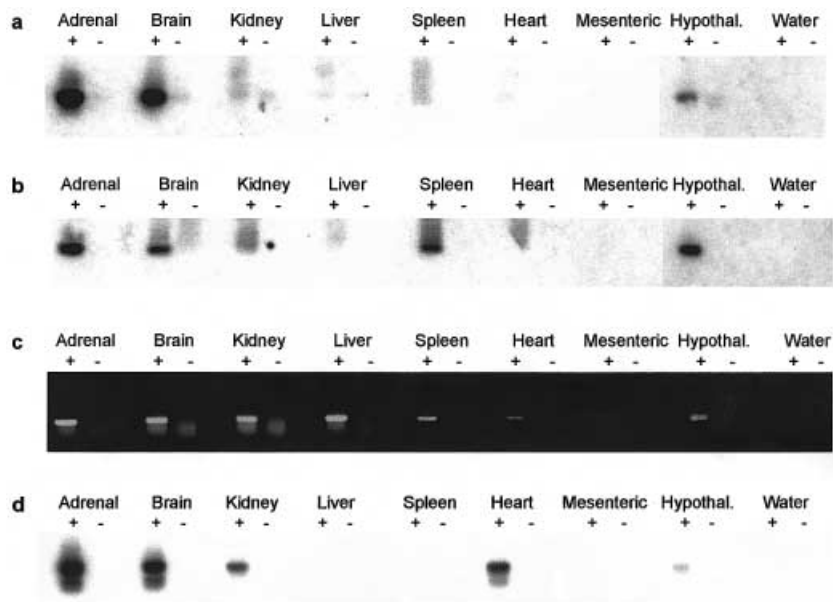
FIGURE 1.   RT-PCR of RNAs from various tissues in the presence (+) and absence (−) of reverse transcriptase. (a) *CYP11B1*, (b) *CYP11B2*, (c) adrenodoxin, (d) *CYP11A1*. Products were detected by Southern blotting using specific, end-labelled radioactive oligonucleotides, except for adrenodoxin transcripts which were detected by ethidium bromide staining under UV light.
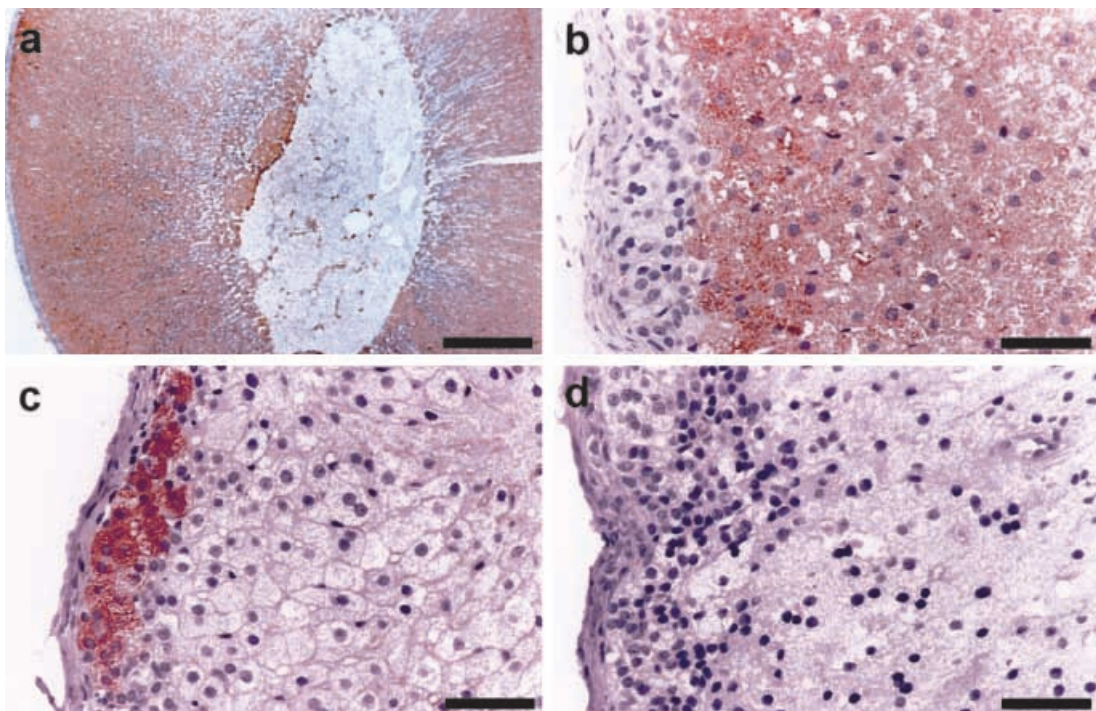

FIGURE 2.   Immunohistochemical localisation of enzymes in rat adrenal tissue. (a) Staining for 11β-hydroxylase in the zona fasciculata, zona reticularis and adrenal medulla (bar=500 μm), (b) staining for 11β-hydroxylase in the zona fasciculata (bar=50 μm), (c) staining for aldosterone synthase in the zona glomerulosa (bar=50 μm), (d) absence of staining in control section incubated with antibody raised against non-mammalian epitope (bar=50 μm).
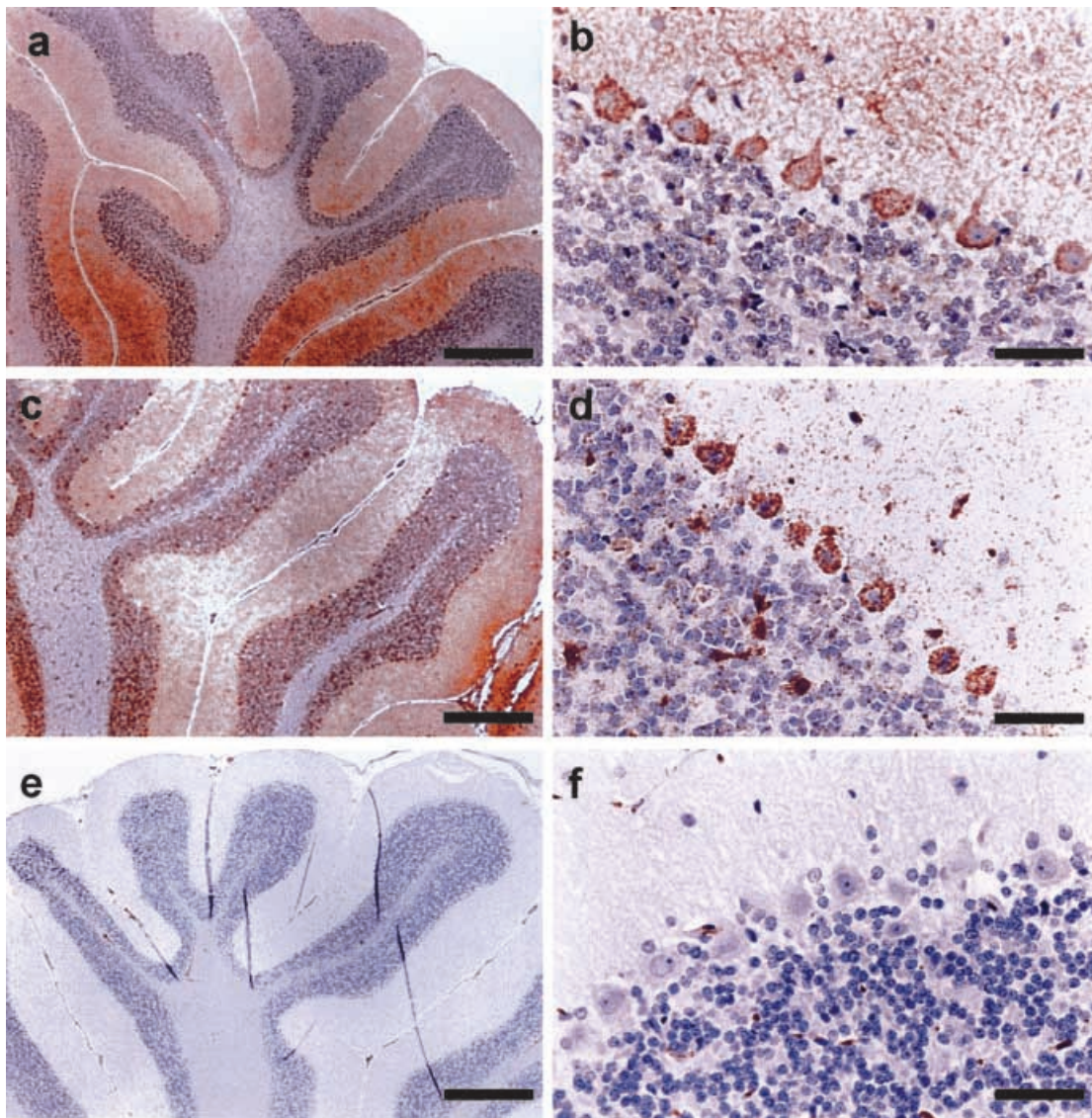
FIGURE 3.   Immunohistochemical localisation of enzymes in rat cerebellum. (a) Staining for 11β-hydroxylase (bar=500 µm), (b) detail showing 11β-hydroxylase staining in Purkinje cells (bar=50 µm), (c) staining for aldosterone synthase (bar=500 µm), (d) detail showing aldosterone synthase staining in Purkinje cells (bar=50 µm), (e) absence of staining in control section incubated with antibody raised against non-mammalian epitope (bar=500 µm), (f) detail showing Purkinje cells in control section (bar=50 µm).

DISCUSSION

Until recently, it was believed that steroid hormone biosynthesis takes place in a limited range of tissues including the adrenal cortex, gonads and placenta. However, it is well known that neurosteroids such as pregnenolone, progesterone and dehydroepiandrosterone (DHEA) are synthesised in the central nervous system (CNS) (Baulieu

& Robel 1990). There is now increasing evidence to suggest that corticosteroid production, once regarded as confined to the adrenal cortex, can also occur in the brain and in vascular tissue (Baulieu & Robel 1990, Strömstedt & Waterman 1995, Takeda *et al*. 1996, Gómez-Sánchez *et al*. 1997). In this study, we have investigated the possibility of local corticosteroid production in a number of tissues, but with particular emphasis on the CNS. RT-PCR
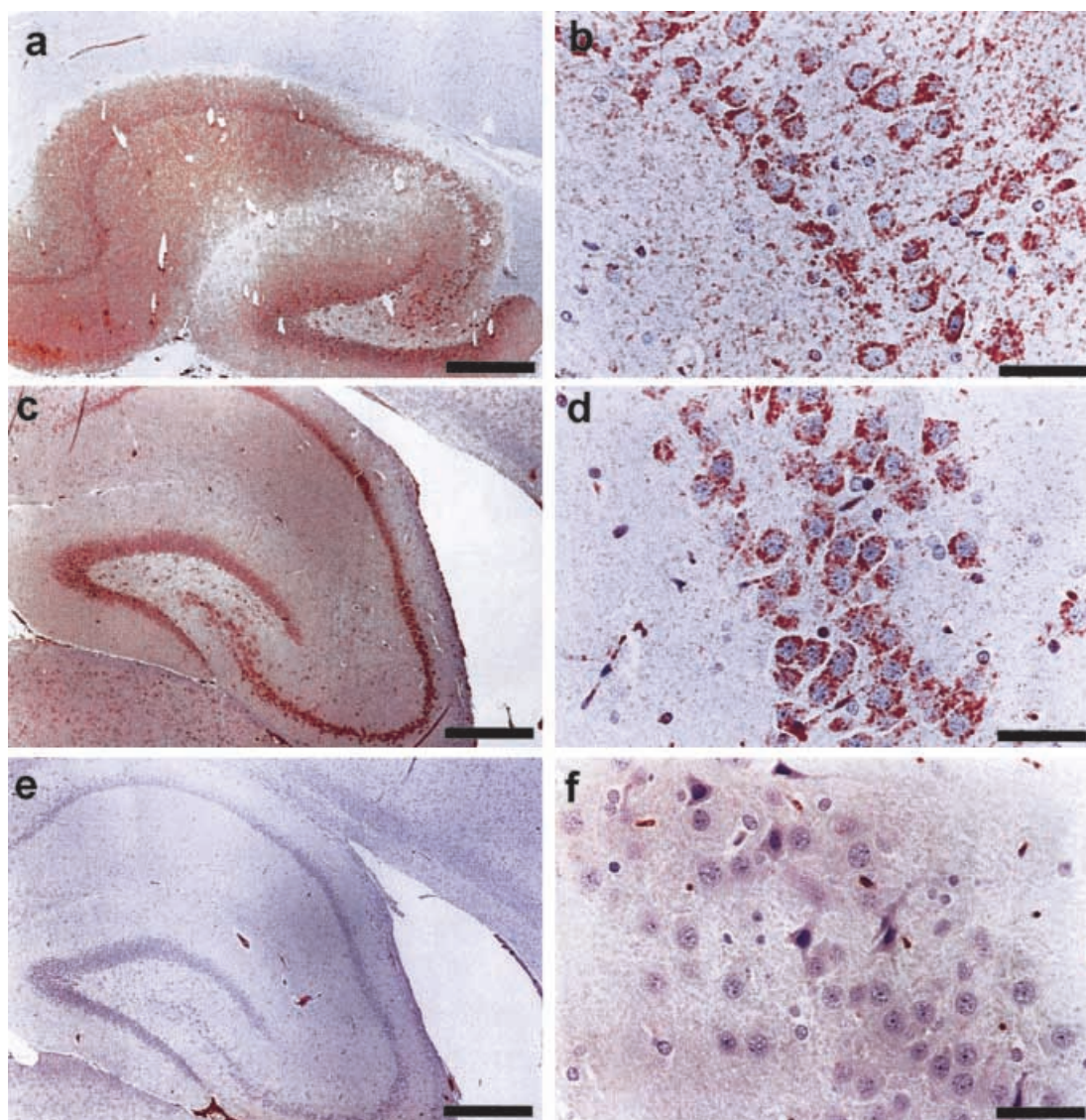
Figure 4.   Immunohistochemical localisation of enzymes in rat hippocampus. (a) Staining for 11β-hydroxylase (bar=500 µm), (b) detail showing 11β-hydroxylase staining in CA3 cells (bar=50 µm), (c) staining for aldosterone synthase (bar=500 µm), (d) detail showing aldosterone synthase staining in CA3 cells (bar=50 µm), (e) absence of staining in control section incubated with antibody raised against non-mammalian epitope (bar=500 µm), (f) detail showing CA3 cells in control section (bar=50 µm).

was used to show transcription of *CYP11B1* (11β-hydroxylase) and *CYP11B2* (aldosterone synthase). Immunohistochemical techniques in brain tissue, utilising specific monoclonal antibodies raised against the respective enzymes, provided firm evidence of translation. This approach was validated by the specificity of the antibodies in the adrenal cortex. Furthermore, following SDS-PAGE and immunoblotting, the anti-11β-hydroxylase antibody identified a protein band in adrenal tissue with a molecular weight of ∼52 000 (data not shown) which approximates to the theoretical molecular weight of the enzyme. Aldosterone synthase concentration is too low to be detected by this method.

RT-PCR showed clearly that *CYP11B1* and *CYP11B2* are transcribed in the brain. Some transcription could be attributed more precisely to the hypothalamus, which was analysed separately. This confirms previous work by other groups (see

above). In addition, we and others (Mellon & Deschepper 1993, Strömstedt & Waterman 1995, Gómez-Sánchez *et al*. 1997) have shown that the genes encoding side-chain cleavage enzyme, 21-hydroxylase and adrenodoxin are also present in brain tissue. Indeed, adrenodoxin appears to be transcribed ubiquitously. These findings suggest that the full complement of enzymes and co-factors necessary for *de novo* production of corticosteroids from cholesterol is present in the brain.

The presence of gene transcripts does not provide unequivocal evidence that translation to a functional protein occurs. However, other groups have shown that brain minces from intact and adrenalectomised rats can synthesise corticosterone and aldosterone from endogenous precursors and also from radio-labelled 11-deoxycorticosterone (DOC) (Gómez-Sánchez *et al*. 1997). Production can be inhibited by metyrapone, a relatively specific inhibitor of 11β-hydroxylase (Gower 1974). It is also possible that adrenal-derived precursors such as DOC will act as substrates for 11β-hydroxylase and aldosterone synthase in other tissues. However, in man this seems unlikely given the low circulating levels of DOC (low nM range) and the reported K_m values (μM range) of the enzymes in question (Fardella *et al*. 1996).

There are discrepancies between the data gained from RT-PCR and from immunohistochemistry. For example, there was no evidence of the enzymes' expression in the hypothalamus using immuno-histochemistry, despite suggestions from RT-PCR to the contrary. However, RT-PCR is a highly sensitive technique and the levels of translated enzyme may be beyond immunohistochemistry's present detection capabilities. Another possibility is that *CYP11B1* and *CYP11B2* are regulated at the translational rather than the transcriptional level (e.g. transcripts in certain tissues may be unstable and degrade quickly). Moreover, the dissection of the brain into specific regions before isolating RNA is technically demanding and the risk of cross-contamination between brain regions is high. For this reason, RT-PCR is not as reliable for the localisation of expression as immunohistochemistry. These conflicting findings emphasise the importance of following up RT-PCR studies with confirmation of enzyme synthesis.

The second part of this study was designed to localise aldosterone synthase and 11β-hydroxylase precisely within the tissue. Demonstration of the expected distribution of aldosterone synthase in the zona glomerulosa and of 11β-hydroxylase in the zonae fasciculata and reticularis using immuno-histochemistry, previously shown by Ogishima *et al*. (1992), confirmed the performance of this method.

Our experiments show unequivocally that there is 11β-hydroxylase-like and aldosterone synthase-like immunoreactivity in discrete areas of the rat CNS. These include the hippocampus, in particular the dentate gyrus, and the cerebellum, especially the Purkinje cells. Furukawa *et al*. (1998) discovered a similar distribution for the transcripts of other enzymes involved in this pathway (StAR, P-450scc and 3β-HSD) using *in situ* hybridisation, and this further supports the hypothesis that *de novo* production of corticosteroids can occur in certain regions of the brain.

Although the presence of the enzymes in the CNS is clear, their function and regulation are not. Glucocorticoid and mineralocorticoid receptors are abundant in the CNS, the latter especially so in the hippocampus, an area associated with memory and learning (Agarwal *et al*. 1993, De Kloet *et al*. 1998). Activation of the central mineralocorticoid receptor raises blood pressure and alters thirst and salt appetite (Gómez-Sánchez 1997). The effects on blood pressure of mineralocorticoid administration direct to the CNS have already been remarked upon.

Although the techniques employed here are, at best, semi-quantitative, it is beyond dispute that the corticosteroidogenic enzymes in the brain are found at much lower levels than in the adrenal cortex. However, the resulting amounts of corti-costeroid, even if low, could be capable of significant physiological effects. Gómez-Sánchez (1997) showed that intracerebroventricular admin-istration of very small doses of aldosterone to the rat increased systemic blood pressure (see earlier). This effect could be specifically inhibited by mineralocorticoid receptor antagonists. Thus, even small amounts produced locally could exert signifi-cant physiological effects via paracrine or autocrine actions at this site. Aldosterone produced locally may be implicated in vascular tone control in the rat (Takeda *et al*. 1995, Takeda *et al*. 1997) and, in man, aldosterone administration has recently been shown to modulate baroreceptor function (Yee & Struthers 1998).

Glucocorticoids have widespread actions on the brain, and assist in the regulation of hypothalamic/pituitary/adrenal function by interacting with glucocorticoid receptors in the hypothalamus and anterior pituitary. (The hippocampus is important here.) They could also have major effects on long-term cardiovascular homeostasis (Seckl 1997). Increased CNS exposure to glucocorticoids during fetal development has lifelong effects on cardiovas-cular and metabolic function. Local CNS synthesis of corticosteroids may provide another mechanism to determine tissue glucocorticoid exposure at key

developmental times. Deviations above or below normal glucocorticoid levels in the hippocampus are also known to change the function of hippocampal cells and even result in their apoptosis (Reagan & McEwen 1997). Local production in the rat hippocampus may be involved in these processes.

Having confirmed that the corticosteroidogenic apparatus does occur locally, the control mechanisms regulating its expression in the CNS, which may differ from those in the adrenal cortex, should be investigated. The clinical implications of extra-adrenal corticosteroidogenesis remain to be explored.

ACKNOWLEDGEMENTS

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