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Citation for published version:

Digital Object Identifier (DOI):
10.1677/JOE-07-0439

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published in:
Journal of Endocrinology

Publisher Rights Statement:
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Effects of ACTH, dexamethasone, and adrenalectomy on 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) gene expression in the rat central nervous system

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Abstract

Using a highly sensitive quantitative RT-PCR method for the measurement of CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase) mRNAs, we previously demonstrated that CYP11B2 expression in the central nervous system (CNS) is subject to regulation by dietary sodium. We have now quantified the expression of these genes in the CNS of male Wistar Kyoto (WKY) rats in response to systemic ACTH infusion, dexamethasone infusion, and adrenalectomy. CYP11B1 and CYP11B2 mRNA levels were measured in total RNA isolated from the adrenal gland and discrete brain regions using real-time quantitative RT-PCR. ACTH infusion (40 ng/day for 7 days, n=8) significantly increased CYP11B1 mRNA in the adrenal gland, hypothalamus, and cerebral cortex compared with animals infused with vehicle only. ACTH infusion decreased adrenal CYP11B2 expression but increased expression in all of the CNS regions except the cortex. Dexamethasone (10 μg/day for 7 days, n=8) reduced adrenal CYP11B1 mRNA compared with control animals but had no significant effect on either gene's expression in the CNS. Adrenalectomy (n=6 per group) significantly increased CYP11B1 expression in the hippocampus and hypothalamus and raised CYP11B2 expression in the cerebellum relative to sham-operated animals. This study confirms the transcription of CYP11B1 and CYP11B2 throughout the CNS and demonstrates that gene transcription is subject to differential regulation by ACTH and circulating corticosteroid levels.

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Introduction

Corticosteroid hormones such as aldosterone and corticosterone are synthesized by specific enzymes in the adrenal cortex but now there is strong evidence that these enzymes are also present in other tissues (Davies & MacKenzie 2003). Although these extra-adrenal sites are not capable of corticosteroid production on the same scale as the adrenal cortex, due to a much lower level of relevant gene expression, their proximity to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) is consistent with a paracrine/autocrine mode of steroid hormone action. We and others have shown that the brain is a major extra-adrenal site of corticosteroid production (Gomez-Sanchez et al. 1997, MacKenzie et al. 2000a). The genes for all of the enzymes required to form aldosterone and corticosterone de novo from cholesterol are transcribed, including those of such early pathway enzymes as the steroidogenic acute regulatory protein, the side-chain cleavage enzyme (P450sc), and 3β-hydroxysteroid dehydrogenase (3β-HSD; Furukawa et al. 1998). Furthermore, we have shown that the enzymes that perform the terminal stages of corticosteroid biosynthesis, aldosterone synthase, and 11β-hydroxylase are themselves present in rat brain (MacKenzie et al. 2000a). These studies showed expression of all these genes to occur in areas throughout the brain, with expression strongest within the hippocampus and dentate gyrus as well as the cerebellar granule layer and Purkinje cells.

In the rat, 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) convert 11-deoxycorticosterone (DOC) to corticosterone and aldosterone respectively. Their genes, CYP11B1 and CYP11B2, possess highly homologous coding regions but differ markedly in their regulation. In the adrenal cortex, CYP11B1 expression is controlled by adrenocorticotropic hormone (ACTH) while CYP11B2 is regulated mainly by the renin-angiotensin system. In the adrenal cortex, the expression of these genes is subject to strict zonation: CYP11B2 is confined to the zona glomerulosa and CYP11B1 to the zona fasciculata/reticularis. ACTH increases CYP11B2 expression acutely but long-term administration of high doses results in regression of the zona glomerulosa and the consequent suppression of aldosterone synthase expression.
(Mitani et al. 1996). In the rat brain, however, the distribution of CYP11B1 and CYP11B2 expression appears to be identical, so that the two enzymes are present within the same cells, such as the cerebellar granule layer and Purkinje cells (MacKenzie et al. 2000a).

We previously developed a quantitative RT-PCR method for accurate, sensitive measurement of CYP11B1 and CYP11B2 mRNA in brain tissue. The detection limit of 100 copies/μg total RNA permitted the detection of both CYP11B1 and CYP11B2 transcription throughout the rat brain and revealed that local CYP11B2 transcription increases in response to dietary sodium restriction (Ye et al. 2003). Furthermore, although the presence of CYP11B2 transcripts was clearly demonstrable in brain stem, cerebral cortex, hippocampus, hypothalamus, and cerebellum, CYP11B2 expression only responded to dietary sodium restriction in the hippocampus and the cerebellum, suggesting that regulation may vary throughout the brain.

The aim of this study was to extend our previous observations by comparing and contrasting the effects of systemic infusion of ACTH and dexamethasone on CYP11B1 and CYP11B2 expression in the adrenal gland and central nervous system (CNS). In addition, the effect of adrenalectomy on central CYP11B1 and CYP11B2 expression was also studied.

Materials and Methods

Experimental animals

The authors confirm that all animal experimentation described in this paper was conducted in accord with accepted standards of humane animal care and UK legal requirements.

ACTH and dexamethasone infusion

Three groups of adult male Wistar rats (250–300 g, N=8), fed on normal chow (Special Diet Services Ltd, Witham, Essex, UK), were infused with normal saline vehicle, ACTH (40 ng/day) or dexamethasone (10 μg/day) via Alzet osmotic minipumps model 2002 (pump rate 0.5 μl/h, Alzet osmotic minipumps, Cupertino, CA, USA) for 7 days. Blood pressure was recorded by tail cuff plethysmography and rats were weighed before and at the end of treatment.

Adrenalectomy and tissue collection

Adrenal glands were removed from 12-week-old male WKY rats (N=6). Another group of six animals was subjected to sham operation as a control. Post-operation, the animals were maintained for 7 days on normal chow (Special Diet Services Ltd) and were given 0.154 M sodium chloride to drink. On completion of experiments, the animals were killed by decapitation and trunk blood was collected for the assay of plasma renin activity (PRA). The adrenal glands, hippocampus, cerebellum, hypothalamus, brain stem, and cerebral cortex were removed, cleaned, weighed, and frozen at −70 °C for RNA isolation.

Plasma and fecal analyses

PRA and steroid concentration were measured as previously described (Ye et al. 2005). Fecal steroids were measured by a modified version of the method of Pihl & Hau (2003). Briefly, fecal pellets were collected daily into preweighed 50 or 100 ml tubes and stored at −20 °C until analyzed. Each 24 h sample was homogenized by a hand-held disperser, Ultra-Turrax T8 (IKA Werke GmbH & Co. KG, Janke & Kunkel Str. 10, D-79219 Staufen, Germany) in four volumes of H2O. Approximately 1 g of homogenate was extracted twice with five volumes of dichloromethane (CH2Cl2) and then washed once with one volume of NaOH (0.1 M) and twice with one volume of dH2O. The dichloromethane extract was dried down under a stream of nitrogen and reconstituted in assay buffer. Corticosterone (B) and aldosterone were measured by RIA.

Total RNA isolation

Tissues were homogenized using a RiboLyzer cell disruptor (Hybaid, Middlesex, UK) and total tissue RNA was isolated using RNaBee (200 mg/ml; Biogenesis, Poole, UK). RNA was treated with DNase (DNA-free, Ambion, Austin, TX, USA) and its quality was confirmed by electrophoresis on agarose gels. RNA concentration was measured using the RiboGreen RNA Quantitation Kit (Molecular Probes, Leiden The Netherlands) and a Wallac Victor2 1420 Multilabel Counter (Perkin–Elmer, Waltham, MA, USA).

Real-time quantitative reverse transcriptase-PCR (qRT-PCR)

CYP11B1 and CYP11B2 mRNA levels were measured using the Roche LightCycler and external homologous RNA standards as previously described (Ye et al. 2003). The lower limit of detection of each assay was 100 copies/μg total RNA. Each qRT-PCR run consisted of up to 45 PCR cycles; adrenal and brain sample crossing points were all in the range of 15–36 cycles. The intra- and inter-assay coefficients of variation of each assay, calculated using the RT-PCR crossing point, was <5%.

Data analysis

ACTH infusion increased adrenal weight significantly. This was presumably mainly due to zona fasciculata hypertrophy and hyperplasia. The calculated CYP11B1 copy numbers for the infusion experiments were therefore corrected for changes in tissue mass. The mRNA quantities are presented both as uncorrected and as copy number/μg total RNA/g fresh tissue weight. Zona glomerulosa volume would be expected to decrease but it was not possible to quantify this accurately. Therefore, adrenal CYP11B2 mRNA expression

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and brain CYP11B1 and CYP11B2 mRNA expression are expressed per unit weight of tissue.

Data were analyzed by ANOVA or by Kruskal–Wallis ANOVA on ranks, when data were not normally distributed or variance was different between groups. Differences between specific mean pairs were analyzed by Tukey’s test for multiple comparisons or by the Holm–Sidak method. For all analyses, \( P<0.05 \) was required for statistical significance. Data are expressed as the mean ± S.E.M.

## Results

### Effects of ACTH and dexamethasone infusion

ACTH infusion significantly decreased body weight (\( P<0.01 \)) and increased blood pressure (\( P<0.01 \)) and adrenal gland weight (\( P<0.01 \)) at the time of killing, relative to vehicle animals (Table 1). PRA in the ACTH-treated animals was reduced to almost undetectable levels (\( P<0.05 \)). In ACTH-treated animals, plasma aldosterone and corticosterone were increased relative to vehicle animals (\( P<0.01 \), Table 1), similar to fecal corticosterone and aldosterone levels (\( P<0.001 \), Fig. 1a and b).

Dexamethasone infusion reduced body weight (\( P<0.05 \)) and increased blood pressure (\( P<0.05 \)) relative to vehicle animals, but had no effect on adrenal gland weight (Table 1). Dexamethasone infusion significantly suppressed fecal corticosterone levels (\( P<0.001 \), Fig. 1a). Although there was a trend towards an increase in PRA with dexamethasone, this was not statistically significant (Table 1).

Table 2 summarizes changes in adrenal gene expression rates. ACTH infusion increased CYP11B1 mRNA copy number in adrenal glands (\( P<0.05 \)), but only when correction was made for increased adrenal weight. ACTH decreased CYP11B2 copy number (\( P<0.01 \)). Dexamethasone infusion decreased CYP11B1 mRNA, both corrected and uncorrected (\( P<0.05 \)), relative to vehicle-infused animals but had no significant effect on CYP11B2 mRNA.

### Effects of adrenalectomy

Fecal aldosterone and corticosterone were significantly reduced in adrenalectomized animals (both \( P<0.01 \)), confirming the success of the procedure (Fig. 3a and b). Adrenalectomy increased CYP11B1 expression in the hypothalamus (\( P<0.05 \)) and hippocampus (\( P<0.05 \)), and increased CYP11B2 expression in the cerebellum (Fig. 4a and b), although the magnitude of the effect was much smaller with 20–25% increase in copy number.

### Discussion

These results confirm the widespread expression of CYP11B1 and CYP11B2 transcripts in the brain. Although the levels of mRNA are several orders of magnitude lower than those seen in the adrenal gland and their abundance varies significantly throughout the rat brain, central CYP11B1 and CYP11B2 expression are subject to transcriptional regulation.

Three experimental manipulations were used in order to examine this regulation: (i) systemic infusion of ACTH which mimics stress-induced hypothalamo-pituitary axis (HPA) activation; (ii) suppression of ACTH by infusion of dexamethasone; and (iii) elimination of adrenal corticosteroids through adrenalectomy.

The effects of these treatments on the adrenal gland were largely predictable. ACTH infusion significantly increased blood pressure, adrenal weight, and the fecal levels of corticosterone. Adrenal CYP11B1 transcript number

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### Table 1 Blood pressures, weights, renin activities, and plasma corticosteroids of vehicle, dexamethasone- and adrenocorticotrophin (ACTH) treated animals

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n=8)</th>
<th>ACTH (n=8)</th>
<th>Dexamethasone (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial blood pressure (mmHg)</td>
<td>124±1±2.9</td>
<td>120.5±3±0</td>
<td>132.0±3±3</td>
</tr>
<tr>
<td>Final blood pressure (mmHg)</td>
<td>134±8±3.7</td>
<td>176.5±6±9</td>
<td>152.5±5±5†</td>
</tr>
<tr>
<td>ΔBlood pressure (mmHg)</td>
<td>10.7±3.8</td>
<td>56.0±6.7†</td>
<td>20.5±4.7*</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>294±5±4.6</td>
<td>302±1±6.0†</td>
<td>284.8±3±7</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>332±0±5.0</td>
<td>287.5±9.2</td>
<td>310.2±6.8</td>
</tr>
<tr>
<td>ΔBody weight (g)</td>
<td>37.4±3±9</td>
<td>-14.6±5.7†</td>
<td>-25.3±5±6*</td>
</tr>
<tr>
<td>Initial adrenal weight (mg)</td>
<td>68±3±9.1</td>
<td>231±7±20†</td>
<td>68±3±9.5</td>
</tr>
<tr>
<td>Final adrenal weight (mg)</td>
<td>68±3±9.1</td>
<td>0.03±0.02‡</td>
<td>0.78±0.20</td>
</tr>
<tr>
<td>PRA (mg Al/m per min)</td>
<td>0.29±0.11</td>
<td>975±126†</td>
<td>412±166</td>
</tr>
<tr>
<td>Plasma aldosterone (pM)</td>
<td>117±65</td>
<td>3539±680†</td>
<td>200±77</td>
</tr>
<tr>
<td>Plasma corticosterone (nM)</td>
<td>285±157</td>
<td>200±77</td>
<td>200±77</td>
</tr>
</tbody>
</table>

*†P<0.05 relative to vehicle; †P<0.01 relative to vehicle.

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increased significantly after correction for the increased adrenal mass, which was predominantly zona fasciculata. This is in agreement with a previous study which also found no overall adrenal response to ACTH unless adrenal growth was taken into account (Holland & Carr 1993). While it is possible that ACTH stimulates CYP11B1 transcription acutely within each zona fasciculata cell over 7 days ACTH also causes zona fasciculata cell proliferation (Mitani et al. 1996), thereby reducing the expression rate required of each individual cell and probably explaining why we saw only a proportionate increase in CYP11B1 mRNA after adrenal mass was taken into account.

Chronic ACTH infusion increased fecal and plasma aldosterone levels while decreasing CYP11B2 transcript levels in the adrenal gland. Our steroid measurements are in contrast to those from other studies that showed aldosterone to rise sharply after ACTH administration but then gradually decrease over time. However, the present study used lower concentrations of ACTH and we have verified that our results are not attributable to cross-reactivity of the RIA antibody with other steroids likely to be increased by ACTH, as has been suggested in other studies (Abayasekara et al. 1993).

ACTH excess is known to reduce zona glomerulosa mass but this is difficult to quantify (Mazzocchi et al. 1986, Mitani et al. 1996). Therefore, it is likely that the apparent reduction in CYP11B2 expression is due in part to a lower proportion of zona glomerulosa-derived RNA relative to zona fasciculata-derived RNA in ACTH-treated animals. Purification of RNA from isolated adrenal capsules may have given a clearer indication of zona glomerulosa CYP11B2 expression.

In the CNS, ACTH increased CYP11B1 expression in the hypothalamus and cerebral cortex. It is possible that this subserves a need for altered local glucocorticoid provision in these regions or that expression is controlled differently in other regions. ACTH significantly increased CYP11B2 mRNA levels in almost all of the brain regions we studied. ACTH acts through the various forms of the melanocortin (MC) receptor and, although the adrenocortical form, MC2, has not been detected in the adult rat brain, there is evidence for the expression of MC3, MC4, and MC5 throughout the brain, including areas of brain stem, cortex, amygdala, hippocampus, and cerebellum (reviewed in Adan & Gispen 1997). However, it is not immediately clear how systemically administered ACTH could influence brain function as the blood–brain barrier is assumed to be impermeable to peptides. Some studies suggest that the circumventricular organs, which lack a blood–brain barrier, play an important role in conveying blood-borne substances and may provide a port for peptide access (Kastin et al. 1984, Banks et al. 1993, Chikhale et al. 1994, Ganong 2000). Alternatively, ACTH-induced stimulation of 11β-hydroxylase may raise circulating levels of DOC and corticosterone, which can enter the brain and act as substrates for aldosterone synthase (MacKenzie et al. 2000a).

Dexamethasone had no significant effect on CYP11B1 or CYP11B2 transcription in the brain. Dexamethasone suppresses adrenal corticosterone production which is expected to lead to reduced occupancy of the brain MR. However, this so-called hypocorticoid brain state does not appear to influence central expression. Dexamethasone is known to be excluded from the brain by P-glycoproteins in all but the very highest amounts (Karssen et al. 2001). As the dosage administered here was not excessive, it is doubtful that sufficient dexamethasone entered the brain to influence local CYP11B1 expression directly.

In the adrenalectomy studies, the success of the surgery was confirmed by the low post-operative corticosterone levels.

Table 2: Effect of adrenocorticotrophin (ACTH) and dexamethasone infusion on adrenal CYP11B1 and CYP11B2 mRNA levels

<table>
<thead>
<tr>
<th></th>
<th>Adrenal CYP11B1</th>
<th>Adrenal CYP11B2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copies/µg</td>
<td>Copies/µg×adrenal weight (g)</td>
</tr>
<tr>
<td>ACTH</td>
<td>3–0×10⁸±0·3×10⁸</td>
<td>6·9×10⁷±0·4×10⁷*</td>
</tr>
<tr>
<td>Dex</td>
<td>1·4×10⁸±0·3×10⁸*</td>
<td>9·1×10⁷±0·3×10⁷*</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2·8×10⁸±0·4×10⁸</td>
<td>1·9×10⁷±0·4×10⁷</td>
</tr>
</tbody>
</table>

*P<0·05; †P<0·01.

Extra-adrenal production of corticosterone from intestinal and possibly other tissues might account for the extremely low but nonetheless detectable levels of fecal steroid (Mueller et al. 2007). Adrenalectomy caused small but significant increases in CYP11B1 mRNA levels in the hippocampus and hypothalamus and CYP11B2 mRNA levels in the cerebellum. It is expected that adrenalectomy would result in increased plasma levels of ACTH. However, these effects are clearly different from those of ACTH infusion in both their region specificity and the magnitude of response. The overall lack of change in CYP11B2 expression may be due to the provision of saline following surgery, which would maintain normal PRA and angiotensin II, although these were not measured. However, as with dexamethasone, it seems apparent that induction of a hypocorticotoid state has a lesser effect on the CYP11B1 and CYP11B2 expression in the CNS.

When analyzing levels of mRNA within a tissue, studies are open to the criticism that variations in transcription may not feed through to differences in enzyme level, or that the real limiting factor in the pathway may lie elsewhere. An elegant study by Gomez-Sanchez et al. (2005) demonstrated that providing DOC to adrenalectomized rats did not result in any detectable levels of brain corticosterone; the authors logically took this as an indication that levels of 11β-hydroxylase, and not substrate, was the factor limiting corticosterone production in these animals. However, if local corticosteroid biosynthesis is a real phenomenon within the CNS, that study did raise the question of why no corticosterone could be detected at all, rather than simply a reduction relative to the intact animals. We suspect that the reason the authors could not see any corticosterone following adrenalectomy was twofold. First, the low local production of steroid within discrete brain regions will be further diluted when analyzed in a whole brain extract such as that employed by Gomez-Sanchez et al. Second, our studies on the brain tend to suggest that aldosterone synthase and 11β-hydroxylase are co-expressed within the brain. Since both enzymes have a common substrate, DOC, and any corticosterone produced by 11β-hydroxylase is a potential substrate for aldosterone synthase, then one might expect to see a more significant rise in aldosterone which was detected by Gomez-Sanchez et al. in the brains of adrenalectomized rats. It further follows that there will be marked regional variation in brain tissue levels of aldosterone and corticosterone if output is related to the relative ratios of these two enzymes.

This study confirms the differential expression of CYP11B1 and CYP11B2 genes in various regions of the CNS and supports
a wealth of immunocytochemical and biochemical evidence that aldosterone and corticosterone can be synthesized de novo from cholesterol in the CNS (Gomez-Sanchez et al. 1996, 1997, MacKenzie et al. 2000a, b). Although the levels of gene expression and the steroidogenic capacity are much lower than those of the adrenal gland (Gomez-Sanchez et al. 1997, Ye et al. 2003), the presence of MR and GR in the immediate vicinity may facilitate autocrine- or paracrine-mediated physiological effects.

What might these effects be? GR are widely distributed throughout the brain and are associated with a range of functions. For example, appetite and cognitive function are controlled by GR, which is expressed at high levels in the hypothalamus and hippocampus respectively, and both of these areas mediate feedback control of the HPA axis. Although all CNS regions that we examined express CYP11B1, it is significant that the lowest levels of transcription are associated with the hypothalamus and hippocampus. Arguably, this mitigates against the importance of local CYP11B1 transcription in brain glucocorticoid action. However, as previous immunocytochemical studies of 11β-hydroxylase indicate that expression is localized to particular cell types within a region, it follows that the CYP11B1 levels that we report may be diluted by RNA from the majority of non-expressing cells. The expression of CYP11B1 is highest in the cortex and brain stem. In the brain stem, GR is present in the nerve cells nuclei associated with norepinephrine, epinephrine, and serotonin. The subcellular translocation of GR from cytoplasm to nucleus takes place after receptor activation and might therefore indicate that cells are tonically exposed to high levels of glucocorticoid hormone which, theoretically, could be supplied autonomously by local 11β-hydroxylase activity.

MR is found in high concentrations in the hippocampus, septum, and in some brain stem nuclei and is associated with the regulation of fluid homeostasis through effects on thirst and salt appetite. Central actions of aldosterone have been proposed to play a role in blood pressure homeostasis. In this respect, intracerebroventricular (ICV) administration of systemically ineffective levels of aldosterone significantly increases blood pressure (Gomez-Sanchez 1986). Whether modulation of blood pressure is mediated by locally synthesized aldosterone is unclear. However, ICV administration of 19-ethynyl-DOC, an inhibitor of aldosterone synthase, prevents the increase in blood pressure following sodium treatment in the Dahl salt-sensitive rat suggesting that locally synthesized aldosterone may be important (Gomez-Sanchez et al. 1996).

How aldosterone mediates these central effects is unclear. The enzyme 11β-HSD2 which inactivates corticosterone and protects the MR from occupation by glucocorticoids is only expressed in the subcommissural organ and nucleus tract solitarius (Robson et al. 1998). This means that the vast majority of brain MR is non-selective for mineralocorticoid and glucocorticoid. However, the hypertensive response to ICV aldosterone can be inhibited by MR blockade, suggesting that mineralocorticoids can access the MR even in the absence of 11β-HSD2. It is possible that ICV administration of aldosterone generates a high local concentration of steroid, allowing it to compete on more equal terms with glucocorticoid for MR occupancy. In this way, aldosterone could occupy sufficient MR in order to generate a specific mineralocorticoid response. Extra-adrenal aldosterone biosynthesis could be a way of generating similar localized, high concentrations of mineralocorticoid within specific brain regions.

We have previously shown that the CYP11B2 gene is expressed in specific cell types within discrete brain regions and that its transcription is regulated by angiotensin II and sodium (MacKenzie et al. 2002). Using different stimuli, this study confirms that both CYP11B1 and CYP11B2 gene expression in the CNS can be regulated, suggesting a biological role for 11β-hydroxylase and aldosterone synthase within the CNS. While the central role of these enzymes remains largely unknown, this work represents important progress in the elucidation of this complex system and demonstrates the need for further detailed physiological studies.
Acknowledgements

We wish to thank Dr Megan Holmes, Dr Joyce Yau, and Mrs June Noble for their assistance with the collection of brain tissue. P Y was supported by a UK Overseas Research Student’s (ORS) Award. S M M is a Hypertension Trust Research Fellow and an MRC Research Fellow. J M C C and E D are supported by and MRC Programme Grant G0400874. C J K is supported by MRC Programme Grant G9306883. J R S is supported by a Wellcome Trust Programme Grant. The Roche LightCycler was financed by a grant from the National Heart Research Fund. We declare that there is no conflict of interest that would prejudice the impartiality of this research.

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