Identification of neuroactive steroids, their precursors and metabolites in adult male rat brain

M.J. Ebner, D.I. Corol, H. Havlíková, J.W. Honour and J.P. Fry

Department of Physiology (M.J.E., D.I.C., H.H., J.P.F.), University College London, Gower Street, London, WC1E 6BT, UK and SAS laboratory (J.W.H.), Clinical Biochemistry, University College London Hospitals, 60 Whitfield Street, London, W1T 4EU, UK

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Address all correspondence and requests for reprints to: Jonathan Fry, Department of Physiology, University College London, Gower Street, London, WC1E 6BT, UK. E-mail: j.fry@ucl.ac.uk

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Abbreviations: DHEA, dehydroepiandrosterone; GABA, γ-aminobutyric acid; GC-EIMS, gas capillary chromatography-electron impact mass spectrometry; HFBA, heptafluorobutyric acid anhydride; HLB, hydrophilic-lipophilic balance; HMDS, hexamethyldisilazane; MAX, mixed-mode anion exchange; MO, methoxyamine; P450scc, P450 side chain cleavage; Q, qualifier; RI, retention index; RRT, relative retention time; SIM, selected ion monitoring; T, target; TMSI, trimethylsilylimidazole.

Summary

Steroids in the brain arise both from local synthesis and from peripheral sources, and have a variety of effects on neuronal function. However, there is little direct chemical evidence for the range of steroids present in brain or of the pathways for their synthesis and inactivation. This information is a prerequisite for understanding the regulation and function of brain steroids. Following extraction from adult male rat brain, we have fractionated free steroids and their sulphate esters then converted them to heptafluorobutyrate or methyloxime-trimethylsilyl ether derivatives for unequivocal identification and assay by gas chromatography analysis and selected ion monitoring mass spectrometry. In the free steroid fraction, corticosterone, 3α,5α-tetrahydrodeoxycorticosterone, testosterone and dehydroepiandrosterone were found in the absence of detectable precursors usually found in endocrine glands, indicating peripheral sources and/or alternative synthetic pathways in brain. Conversely, the potent neuroactive steroid 3α,5α-tetrahydroprogesterone (allopregnanolone) was found in the presence of its precursors pregnenolone, progesterone and 5α-dihydroprogesterone. Further, the presence of 3β-, 11β-, 17α- and 20α-hydroxylated metabolites of 3α,5α-tetrahydroprogesterone implicated possible inactivation pathways for this steroid. The 20α-reduced metabolites could also be found for pregnenolone, progesterone and 5α-dihydroprogesterone, introducing a possible regulatory diversion from the production of 3α,5α-tetrahydroprogesterone. In the steroid sulphate fraction, dehydroepiandrostrone sulphate was identified but not pregnenolone sulphate. Although pharmacologically active, identification of the latter appears to be an earlier methodological artefact and the compound is thus of doubtful physiological significance in the adult brain. Our results provide a basis for elucidating the origins and regulation of brain steroids.

Introduction

Steroid hormones have long been known to enter the mammalian nervous system to influence its development and function. More recently, some steroids have also been shown to be present in brain tissue independently of peripheral sources (1-3) the so-called neurosteroids (4). In support of this concept, mRNA and protein have been detected in rodent brain for the steroidogenic cholesterol P450 side chain cleavage (P450scc or CYP11A1) enzyme and for certain other steroid metabolising enzymes (see 5, 6 and Discussion). However, there is a paucity of information on the actual steroid content of mammalian nervous tissue. Such information is essential for a proper understanding of the regulation and function of steroids in the brain.

The first compounds to be defined as neurosteroids were pregnenolone and dehydroepiandrosterone (DHEA), which had been detected in adult male rat brain as both the free steroids and their 3β-sulphate esters (1, 2). Both the free and sulphated forms of these steroids persisted at normal or only slightly reduced concentrations following adrenalectomy and gonadectomy, suggesting synthesis within the brain. Comparable evidence has since been provided for progesterone and its reduced metabolites 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone (allopregnanolone), although there appears to be more of a contribution from the gonads in the female rat (3, 7). Other steroids measured in adult mammalian brain include estradiol and testosterone (8, 9), aldosterone, corticosterone and 3α,5α-tetrahydrodeoxycorticosterone (10-12). In the male rat brain, testosterone, corticosterone, aldosterone and 3α,5α-tetrahydrodeoxycorticosterone appear to arise from endocrine sources (1, 11, 12).

Steroids in the brain are of profound physiological significance. There are well-established sites of action for estradiol, testosterone, progesterone, corticosterone and aldosterone through transcription factors in the mammalian brain (13, 14). More rapid, non-genomic effects of steroids, include those of the 3α,5α-reduced metabolites of progesterone and deoxycorticosterone, which are well known to modulate synaptic and extrasynaptic inhibition by acting at nanomolar concentrations as enhancers of γ-aminobutyric acid (GABA) at $GABA_A$ receptors (15, 16). By contrast and at lower (micromolar) potencies, pregnenolone sulphate and DHEA sulphate negatively modulate $GABA_A$ receptor function and pregnenolone sulphate also appears to enhance excitatory glutamate actions at the N-methyl-D-aspartate receptor. Other rapid and relatively low potency effects of brain steroids on neuronal membrane receptors include negative modulation by progesterone of the nicotinic acetylcholine receptor (17) and the strychnine-sensitive glycine receptor, positive modulation of σ-receptors by DHEA and DHEA sulphate and of voltage-gated K^+- and Ca^{2+}-channels by pregnenolone sulphate (18, 19). Moreover, there is increasing evidence for interactions between transcription factor mediated and non-genomic actions of steroids on the brain (see 19-23).

Despite the long-standing interest in steroid effects on the mammalian brain, there has to our knowledge been no detailed profiling of the steroid metabolites present in this tissue. This information could provide a foundation for identifying not only the origins of neuroactive steroids but also pathways for their inactivation in the brain. To a large extent, the lack of this information reflects technical difficulties. Concentrations of the above steroids have mostly been measured in brain tissue by immunoassay, which is generally accepted as the most sensitive method for the estimation of these compounds. However, problems with cross-reactivity of antibodies, especially at the low concentrations of steroid present and with

interference from the lipidic, cholesterol-rich tissue matrix, mean that brain extracts require fractionation by solvent partitioning and/or liquid chromatography before any confidence can be placed on the steroid measurements (24, 25). In some cases, further evidence of brain steroid identity has been obtained by gas capillary chromatography-mass spectrometry (GC-MS). With this technique, as for immunoassay, the steroids in brain extracts require fractionation before analysis and in addition need to be stabilised by derivatisation before injection onto the GC. However, GC-MS has the advantage that steroid derivatives can chemically be identified, both by their retention time on the chromatograph and also by their ion fragments in the mass spectrometer. Thus, pregnenolone, DHEA, testosterone, progesterone, 5α-dihydroprogesterone and 3α,5α- and 3β,5α-tetrahydroprogesterone have all been characterised in rat brain extracts by the appearance of at least two and sometimes more, diagnostic ions at the appropriate retention time on GC-MS (7, 8, 26-30). The sulphate esters of pregnenolone and DHEA were also identified by GC-MS as their corresponding free steroids following solvolysis of brain extracts (1, 2, 26).

Whether estimating brain steroids by immunoassay or by GC-MS, the above studies have each only focused on a few compounds at a time and revealed several discrepancies. Moreover, studies of steroidogenic enzyme expression suggest that further neuroactive metabolites could be produced in rat brain and/or enter from peripheral sources (see Discussion). In order to provide a framework in which the physiological actions of neurosteroids can be understood, and to facilitate further investigations on the regulation of steroid metabolism in the brain and the ways in which these pathways might interact with peripheral sources of steroid, we now report a detailed analysis of steroids in adult male rat brain, using GC-MS and applying the identification criteria of retention time and diagnostic ion ratios at a high level of stringency. The steroids to be screened were chosen from products

of enzymes known to be expressed in mammalian central nervous system and peripheral endocrine sources.

Materials and Methods

Chemicals

Reference steroids were obtained from Sigma-Aldrich, Dorset, UK or Steraloids, Inc., Newport, USA. ^{3}H-Progesterone (5106.0 GBq/mmol) was from PerkinElmer LAS Ltd., Buckinghamshire, UK All chemicals were analytical grade from VWR International, Leicestershire, UK or Sigma-Aldrich, unless stated otherwise and the solvents re-distilled before use. Water was double glass-distilled and silanised glassware was used throughout. Of the derivatisation reagents for gas chromatography-mass spectrometry (GC-MS), methoxyamine (MO) hydrochloride and heptafluorobutyric acid anhydride (HFBA) were from Sigma-Aldrich, while trimethylsilylimidazole (TMSI) and hexamethyldisilazane (HMDS) came from Pierce (Perbio Science Ltd., Cheshire, UK) Lipidex 5000® gel (PerkinElmer) was washed before use with cyclohexane and then stored in cyclohexane : HMDS : pyridine 98:1:1 (v/v/v). Oasis HLB® (5 ml, 200 mg) and MAX® (3 ml, 60 mg) solid phase extraction cartridges were obtained from Waters Corp., Milford, USA. Ecoscint H (National Diagnostics, Yorkshire, UK) was used for scintillation counting.

Brain samples

Adult male Sprague-Dawley rats (250-450 g) from the breeding colony at Biological Services, University College London were kept in a 12 h lighting regimen (lights on at 8.00 h) and fed rat chow and water *ad libitum*. For experiments to test the extraction of endogenous ^{3}H-progesterone and its metabolites from brain tissue, rats were killed by CO_2 inhalation. All other rats were killed between 11.00 h and 15.00 h by cervical dislocation. The whole brain

(including cerebellum but excluding olfactory bulbs) was rapidly removed and stripped of meninges then stored at -70°C until extraction. All animal procedures were given local ethical committee approval and performed under licence from the UK Government Home Office.

Tissue extraction and fractionation

Pooled tissue samples from 2-4 rats were homogenised in 5 volumes of ice-cold potassium phosphate buffer (5 mM, pH 7) with a Polytron® blender. A 0.5 ml portion of the homogenate was removed to determine blood contamination of the brain samples as described below and stored at -20^{o}C. The remaining homogenate was added dropwise to 20 volumes of acetic acid (3%, v/v) in 96% ethanol in polypropylene tubes in an ultra-sonicating bath. After further sonication for 10 min followed by incubation at -20^{o}C overnight, the extracts were sonicated on ice with an MSE Soniprep probe. After a further 30 min on ice, extracts were centrifuged at 28000×g for 30 min at 25 ^{o}C. Lipid was removed from the supernatants by partitioning three times against 10 volumes of isooctane (previously saturated with 77% ethanol in potassium phosphate buffer (5 mM, pH 7) plus acetic acid (2.4%, v/v). After drying down under vacuum, the extracts were resuspended in 4 volumes 60% ethanol (in H_2O, v/v) then centrifuged at 1000×g for 12 min, the supernatant (corresponding to up to 8 g of original tissue) was passed through a 200 mg reverse phase Oasis hydrophilic-lipophilic balance HLB® cartridge for the further removal of lipidic material. An additional 4.4 volumes of 60% ethanol in potassium phosphate buffer (5 mM, pH 7, v/v) were passed through the cartridge to ensure elution of steroids and the combined eluate dried down under vacuum. For separation of free and conjugated steroids, the extracts were then redissolved in 3.75 volumes 20% ethanol in potassium phosphate buffer and passed through 60 mg reverse phase Oasis mixed-mode anion exchange MAX® cartridges. After a wash with 5 ml 20% ethanol in ammonium acetate buffer (20 mM, pH 7, v/v), free steroids were eluted in 4 ml ethyl acetate. Any steroid

glucuronides present could then be eluted with 20 ml 60% ethanol in formate/pyridine buffer (20 mM, pH 3, v/v). Finally, after a wash with 2 ml ethyl acetate (dried over Na_2SO_4), the steroid sulphates were eluted in 15 ml 50 mM benzene sulphonic acid in ethyl acetate saturated with 2 M H_2SO_4. The free steroid eluates were dried under nitrogen and derivatised as described below. Steroid sulphate fractions were solvolysed before derivatisation by incubation at 40^{o}C in the acidified ethyl acetate eluant for 16 h in the presence of Na_2SO_4. After neutralisation with pyridine, ethyl acetate was evaporated from these solvolysed fractions and the residue extracted three times with 2 ml ether. The combined ether phase was dried down for derivatisation by MO-TMSI or HFBA. If the latter, the ether phase was washed beforehand three times with 1 ml of H_2O. Reagent blank samples were carried alongside each tissue sample throughout the above procedures.

Extraction efficiency of ethanol/acetic acid for brain steroids was estimated after ip injection of two rats with 0.7 MBq/kg ^{3}H-progesterone in phosphate buffered saline (5 ml/kg). These rats were killed after 2.5-3h by CO_2 inhalation and their brains quickly removed before storage at -70^{o}C. As described above, brains were homogenised in 5 volumes of ice-cold potassium phosphate buffer (5 mM, pH 7). The total brain radioactivity was then estimated by removing portions of homogenate for solubilisation in 3 volumes Soluene 350. These solubilised samples were then bleached with hydrogen peroxide (final concentration of 1% w/v) for 2 h at 50^{o}C, before counting in Ecoscint H containing Triton X-100 (6% v/v), glacial acetic acid (0.6% v/v) and butylated hydroxytoluene (2% w/v). The remaining homogenate was extracted into ethanol/acetic acid as already described. Radioactivity contained in these brain extracts was found to be 88.2% of total brain radioactivity.

Blood contamination of the brain samples was estimated by the spectrophotometric determination of haemoglobin. Portions (0.5 ml) of brain homogenates which had been withheld from steroid extraction and stored at -20°C were thawed and centrifuged (28000×g, 30 min, 4°C). Haemoglobin was then measured in these supernatants by the change in absorbance between 560 and 578 nm upon reduction by the addition of sodium dithionite (to a final concentration of 10 mM). Changes in absorbance were calibrated with respect to haemoglobin standards (Sigma) and the blood content of the original brain samples then calculated to be 1.26 ± 0.10% (v/v; mean ± SEM, n=12), assuming a haemoglobin concentration in rat blood of 157 g/l (31).

Sample derivatisation

For samples to be derivatised with MO and TMSI, the internal standards 16-dehydropregnenolone, 6α-methyl-17-hydroxyprogesterone and prednisolone were added beforehand at 50-100 ng. These samples were then dried under nitrogen and redissolved in 200 µl MO in pyridine (2%, w/v). In the case of the steroid sulphate fractions, improved yields were obtained if samples were reduced in volume to approximately 50 µl after dissolving in MO/pyridine (32). Both steroid sulphates and free steroids were incubated with MO/pyridine at 60°C for 1 h. The TMSI was then added at 100 µl and the mixture heated a further 3 h at 100°C. At the end of this derivitisation, pyridine was evaporated under nitrogen and the residue dissolved in cyclohexane : HMDS : pyridine 98:1:1 (v/v/v) and passed through a Lipidex 5000® gel column (0.5 cm diameter and 8 cm high). After further elution with 2 ml of the same solvent, the pooled eluates were dried under nitrogen and dissolved in cyclohexane for injection onto the GC.

For samples to be derivatised with HFBA, the internal standard 16-dehydropregnenolone was added at 50 ng. After drying under nitrogen, these samples were then redissolved in 30 µl benzene and 30 µl HFBA for incubation at 60 ^{o}C for 30 min. Following this incubation, samples were dried under nitrogen and the residue redissolved in 1 ml cyclohexane : pyridine 98:2 (v/v) for passage through Lipidex 5000® gel columns. Columns were eluted with a further 2 ml of the same solvent and to the pooled eluates the internal standards tetracosane and octacosane were then added at 25 ng each. Finally, the samples were dried under nitrogen and dissolved in cyclohexane for injection into the GC.

Gas chromatography-mass spectrometry analysis

All analyses were carried out on a Shimadzu 17A GC coupled to a QP 5050A MS (Shimadzu, Milton Keynes, UK), equipped with autosampler AOC-20s. The system was controlled and data processed by the Shimadzu Class 5000 software. A 30 m long Zebron ZB1 wall coated open tubular column (Phenomenex, Macclesfield, UK) with 0.25 mm inner diameter and 0.25 µm film thickness was used for GC with helium as the carrier gas at a constant flow rate of 0.7 ml/min. All analyses were performed in the splitless mode and the injector purge valve was opened after 4 min or 2 min for analysis of MO-TMS- or HFB-derivatives, respectively. For the analysis of MO-TMS-derivatives, the injector temperature was kept constant at 280^{o}C and the pressure at 400 kPa for 5 min. Thereafter the pressure was decreased to 34.2 kPa, followed by a rise of 6.5 kPa/min to 81 kPa. After 0.33 min at this pressure, the gradient was set at 1.6 kPa/min to a final pressure of 111.7 kPa, which was held for 4 min. The oven temperature was at 70^{o}C for 5 min, then rose at 20^{o}C/min to 220^{o}C. After 0.33 min, the gradient was 5^{o}C/min to 315^{o}C, which was held for 4 min. The interface temperature was constant at 315^{o}C. For analysis of HFB-derivatives, the injector temperature was constant at 250^{o}C and the pressure at 400 kPa for 2 min. Thereafter, the pressure was decreased to 34.2

kPa followed by a rise of 5.9 kPa/min to 79 kPa. After 0.33 min at this pressure, the gradient was set at 1.5 kPa/min to 98 kPa, which was held for 5 min. Oven temperature was at 70°C for 2 min followed by a rise of 20°C/min to 220°C then, after 0.33 min, a rise of 5°C/min to 285°C, which was held for 5 min. The interface temperature was constant at 285°C. Samples were ionised by electron impact ionisation with an energy of 70 eV. The detector voltage was at 1.7 kV. For initial characterisation, retention indices after Kovats (33) were determined for derivatised reference steroids according to the following equation:

$$RI = 100 \times N + \frac{100 \times n \times \left[\log t(A) - \log t(N)\right]}{\left[\log t(N+n) - \log t(N)\right]}$$

where N is the number of carbon atoms in the alkane eluting before the compound of interest, n the increment in number of carbon atoms from this alkane eluting to the one eluting after the compound of interest, t(A) the retention time (min) of the compound of interest, t(N) the retention time of the alkane eluting before the compound of interest and t(N+n) the retention time of the alkane eluting after the compound of interest. For highest possible sensitivity, the MS was run in selected ion monitoring (SIM) mode. In two ion SIM of MO-TMS-derivatives, pairs of target and qualifier ions for each steroid were monitored in groups of 4 to 6 ions at a time such that potentially overlapping pairs did not coincide. The ions were monitored in 4 different injections with the detector settings changed according to RI as indicated in Table 1.

For confirmation of compound identities, three ions were monitored for MO-TMS-derivatives or two ions for HFB-derivatives, in groups of 3 to 6 ions. These ions are shown in Table 2.

Analysis of results

Peaks were integrated manually and retention time and integration data were further processed

in Microsoft Excel®. For identification, qualifier to target ion ratios (Q/T) were calculated from their areas. Compounds had to meet target values of Q/Ts of reference compounds run alongside tissue samples within 0.67 and 1.5 (± 20% of relative abundance of target and qualifier ions). Further identification was obtained from relative retention times (RRT). These RRTs were calculated as the ratio of retention times of analyte and the closest of one of three internal standards. For positive identification in SIM, RRTs of analytes had to lie within ±0.5% of the RRTs of reference compounds run alongside tissue samples.

For calibration, increasing amounts of reference steroids (0.5-10 ng) were run together with fixed amounts (25-100 ng) of internal standards. The ratios of reference to standard target ion areas could then be plotted against amount ratios. Regression lines were fitted to these plots, which were linear up to 10 ng. Endogenous brain steroids could then be quantified by using the area ratio of their target ions to those of the closest internal standard.

Accuracy of this quantitation was examined by assaying mixtures of standards at 0.5, 1.6, 4.0 and 10.0 ng injected mass per steroid (results shown only for 0.5 ng). The amount of each steroid in each sample was determined and the % accuracy calculated as the amount measured over the calculated amount. The intra- and inter-assay reproducibilities were calculated from the assays carried out for determination of accuracy. The % coefficients of variation (CV) were calculated from the standard deviations and means within and between assays (results shown only for 0.5 ng).

Detection limits of the overall extraction and assay procedure were determined for brain steroids from area ratios of peaks in reagent blanks extracted and assayed alongside the brain samples. For detection, area ratios had to be three times those of peaks at the same RRT in the

corresponding extraction blank. The concentrations at those three times blank values were calculated using the calibration curves. Detection limits shown in Table 3 were the minimal values found from all extracts.

Results

Two ion selected ion monitoring of steroids in rat brain

The first stage of the present investigation was to screen for a wide range of compounds in both the free steroid and sulphate conjugate fractions of rat brain extracts. For this purpose, we used gas capillary chromatography-electron impact mass spectrometry (GC-EIMS) in two ion selected ion monitoring (SIM) mode after derivatisation of these fractions with MO and TMSI. The steroids for which we screened are listed in Table 1. Pure standards of these steroids were first used to adjust sample injection, temperature and pressure conditions of the gas chromatograph for their optimum resolution as MO-TMS derivatives (see Methods). The MO-TMS derivatives of each steroid were then individually analysed for their mass spectrometric behaviour. An example of a mass spectrum is shown in Figure 1 for MO-TMS-pregnenolone. Diagnostic ions for each compound were chosen from their mass spectra according to high relative abundance and selectivity, excluding those which might originate from overlapping elutions of steroid derivatives. Choice of these diagnostic ions then allowed SIM for endogenous brain steroids at increased sensitivity. Usually the ion of higher relative abundance was chosen as the target (T) ion for quantitation and the second ion as a qualifier (Q) to aid in identification. These ions are also listed for the non-biological steroids which were used in the present study as internal standards for quantitation. In order to maintain high analytical sensitivity during SIM, the T and Q ions were monitored in groups of no more than 6 and changed at set times during the elution. To monitor all T and Q ions of the MO-TMS derivatives of the compounds listed in Table 1, four separate injections were performed for

each sample and altogether four different brain extracts screened in this way. This initial screen tentatively identified 32 free steroids and 23 steroid sulphates in the rat brain extracts, as indicated in Table 1. Also shown are the accuracy, intra- and inter-assay reproducibilities determined at the 0.5 ng level for all compounds in this screening procedure.

Confirmation and quantitation of brain steroids

Confirmation of the identities of the compounds revealed in the above two ion SIM screening was attempted using three ion SIM of their MO-TMS-derivatives and/or two ion SIM of their HFB-derivatives. The additional ions for the former derivatives were chosen following the same principles as for the two ion SIM methods. Likewise, specific ions were identified for two ion SIM of the HFB-derivatives. Figures 2 and 3 illustrate typical chromatograms obtained from three ion SIM of MO-TMS-derivatives and from two ion SIM of HFB-derivatives, respectively. In both Figure 2 and Figure 3, the trace from a brain extract is shown below that of the appropriate reference steroid. Examples are shown not only for identified brain steroids but also for some of those steroids which were not confirmed as present in the brain extracts.

Table 2 lists all 17 free steroids together with one steroid sulphate, DHEA sulphate, which met the criteria for unequivocal identification in the present study. This Table 2 also shows the diagnostic ions for three ion SIM of MO-TMS-derivatives and for two ion SIM of HFB-derivatives for each compound and allows comparison of the RRTs and Q/T ratios of brain analytes with those of standard steroids. Overall definitive identification of an endogenous brain steroid is achieved, if coelution of three ions of the compound derivatised with MO and TMSI or of two ions from both the MO-TMS- and HFB- derivatives occurred within the RRT- and Q/T-limits of the standard. For such confirmation, the RRTs and Q/T ratios of brain

analytes had to be within ±0.5% and 0.67 and 1.5, respectively, of those of standards analysed on the same day (see Discussion). Confidence limits at the 99.9% level for RRTs and Q/T ratios were also calculated from series of standard samples and found to coincide well with the above pragmatic limits (not shown).

Identified rat brain steroids were quantified using two or three ion SIM and their concentrations are shown in Table 3. Also shown in this Table are the recoveries for all of the steroids screened by two ion SIM in the present study, after their addition as pure standards to rat brain homogenates and subsequent extraction, fractionation and derivatisation with MO-TMSI. In addition, for each steroid, we give the minimal detection limits of the overall extraction and fractionation procedure, as determined from reagent blank samples run alongside the tissue samples.

Discussion

The present study has employed ethanolic extraction of steroids from adult male rat brain followed by mixed mode hydrophobic interaction and anion exchange chromatography to completely separate free steroids and steroid sulphates, prior to their identification and assay by gas capillary chromatography-electron impact mass spectrometry (GC-EIMS). Others have also used methanol or ethanol to extract free and sulphated steroids from brain tissue (12, 30, 34, 35). With the addition of acetic acid as a denaturant, Liere *et al.* (28) obtained comparable apparent extraction efficiencies for pregnenolone to those reported here for progesterone. As for previous studies, we were unable to estimate extraction efficiencies for steroid sulphates because these undergo desulphation after systemic injection and do not remain in the brain in significant quantities as the original ester (7, 36). However, the polar steroid sulphates are soluble in aqueous ethanol and so should extract more efficiently than the free steroids.

Lipoidal steroid conjugates (26) were not analysed in the present study. These would not extract well into ethanol and in any case should have been removed during the initial clean-up of the extracts by isooctane partitioning and then passage through the HLB cartridges.

After extraction and fractionation, the free steroids and steroid sulphates from rat brain were derivatised for identification and assay by GC-EIMS. Identification was based on comparison with steroid standards and relied on two criteria: relative retention time (RRT; with respect to the nearest internal standard) on the GC and diagnostic ions from the MS. For the former, we adopted the European Commission recommendation (37) of only accepting RRTs which fell within ± 0.5% of a reference standard analysed under the same conditions. As for diagnostic ions in the MS, it was not possible to obtain complete spectra for endogenous brain steroids and selected ion monitoring had to be employed in order to increase sensitivity. An initial screen of rat brain extracts monitoring only two diagnostic ions for each compound indicated the possible presence of 32 free steroids and 23 steroid sulphates. A more rigorous analysis then confirmed 17 free steroids plus one steroid sulphate, DHEA sulphate, as present in adult male rat brain. To be considered as identified, the brain steroid derivatives had to meet not only the ± 0.5% RRT criteria but also to have relative ion abundance for three diagnostic ions within ± 20% of the standard. Previous evaluations of mass spectral databases have shown such limits on the 3 ion criterion alone to provide an unambiguous identification of target compounds (38, 39).

With reference to previous analyses of steroids in nervous tissue (see Introduction), the present results confirm the identification of pregnenolone, progesterone, 5α-dihydroprogesterone, 3α,5α- and 3β,5α-tetrahydroprogesterone, DHEA, DHEA sulphate and testosterone in adult male rat brain extracts. The GC-MS analysis reported here has also

provided chemical identification for the 3α,5α-tetrahydrodeoxycorticosterone and corticosterone previously measured by immunoassay in rat brain. Whole brain concentrations of the above steroids were found to be in the same ranges as given in the previous reports cited here apart from one study (26), which found approximately 10- to 40-fold higher concentrations of pregnenolone, 3α,5α- and 3β,5α-tetrahydroprogesterone and DHEA. In addition to the above steroids, we have also identified 20α- and 20β-dihydropregnenolone, 20α-dihydroprogesterone, 5α,20α-tetrahydroprogesterone, 5α-pregnane-3α,20α-diol, 5α-pregnane-3α,17-diol-20-one and 5α-pregnane-3α,11β-diol-20-one as present in adult male rat brain. None of the steroids identified here has been reported to occur in plasma at concentrations which would contribute significantly to the amounts measured in the present brain extracts and contamination with blood can be excluded as a source.

In contrast to one previous report which employed two ion SIM on GC-MS (26), epiandrosterone was not identified in either the free or the sulphated steroid fractions of male rat brain. This steroid was detected in two ion SIM of the sulphate fraction in the present study but not confirmed with three ion SIM. The present results also differ from several previous reports (see, for example, 2, 26, 28) in failing to detect pregnenolone sulphate in rat brain. These previous studies have employed solvent phase partitioning and/or hydrophobic interaction chromatography to separate free and sulphated steroids and relied on indirect measurements of pregnenolone sulphate in which the ester is solvolysed to yield pregnenolone for identification and assay. In retrospect, these earlier measurements of pregnenolone sulphate seem likely to have assayed contaminating free steroid or another solvolysable, possibly lipoidal, conjugate from the original extract (40). Direct immunoassay of pregnenolone sulphate in adult male rat brain reported this conjugate at less than 0.4 ng/g adult male rat brain (41) and direct LC-MS failed to detect it at a limit of 0.3 ng/g tissue (35).

The present study relied on the solvolysis of sulphate esters but employed prior anion exchange chromatography for the complete separation of free steroids from steroid sulphates and failed to detect pregnenolone sulphate at a limit of 0.05 ng/g brain (uncorrected for procedural losses). This is consistent with recent functional studies in rat hippocampus which suggest a role for pregnenolone sulphate as a retrograde synaptic messenger, but only during development and not older than postnatal day 5 (42). Nevertheless and in contrast to the lack of pregnenolone sulphate in adult male rat brain, we were able to detect DHEA sulphate in this tissue. The latter was found at concentrations close to those given in the initial characterisation of this rat brain steroid (1).

Steroids identified as present in adult male rat brain by the present study are shown in Figure 4 in relation to the possible metabolic conversions which could give rise to these compounds. The rats used in our study had not been adrenalectomised or gonadectomised and so endocrine sources cannot be excluded for some of these brain steroids. Indeed, 3α,5α-tetrahydrodeoxycorticosterone, corticosterone and testosterone were found in the absence of detectable concentrations of their respective precursors, 5α-dihydrodeoxycorticosterone, 11-deoxycorticosterone and androstenedione, suggesting endocrine origins. Previous studies in adrenalectomised and/or castrated rats have suggested endocrine sources for tetrahydrodeoxycorticosterone, corticosterone and testosterone in male rat brain (1, 11, 12). For corticosterone, the 11-keto metabolite could not be detected, confirming that the 11β-hydroxysteroid dehydrogenase in brain works predominantly as a reductase and so would not attenuate the glucocorticoid receptor actions of this steroid (43). Figure 4 illustrates that DHEA was also found in the present survey in the absence of detectable concentrations of its known precursor in endocrine tissues. In these tissues, 17α-hydroxypregnenolone is an intermediate produced by the enzyme CYP17, which catalyses the conversion of

pregnenolone to DHEA and possesses both steroid 17-hydroxylase and 17,20-lyase activities (44). The absence of 17α-hydroxypregnenolone in the present extracts is consistent with previous investigations which have failed to detect activity of the CYP17 enzyme in adult mammalian brain (5, 6). Nevertheless and unlike the above corticosteroids and testosterone, DHEA persists in male rat brain after adrenalectomy and castration (1) and is therefore thought to arise within this tissue.

For the remaining steroids identified as present in adult male rat brain and shown in black in Figure 4, enzymes previously identified in terms of mRNA, protein and/or activity (5, 6) can account for their presence. Thus, pregnenolone can be converted to 20α-dihydropregnenolone or progesterone by the brain enzymes 20α-hydroxysteroid dehydrogenase or 3β-hydroxysteroid dehydrogenase/isomerase, respectively. It is unclear at present whether the 20α-reduced metabolites are formed by a specific 20α-hydroxysteroid oxidoreductase or by one of the 3α-hydroxysteroid dehydrogenases (6, 43). The latter can also give rise to 20β-reduced metabolites which would account for the presence of 20β-dihydropregnenolone, although we were unable to detect 20β-dihydroprogesterone. By contrast, the 20α-reduced metabolite of progesterone was detected, although only a small proportion of progesterone appears to undergo this conversion in rat brain homogenates *in vitro* (45).

For progesterone, the major metabolic route in rat brain appears to be 5α-reduction to yield 5α-dihydroprogesterone, on the pathway to the $GABA_A$ receptor enhancing metabolite 3α,5α-tetrahydroprogesterone (see Introduction). Consistent with the 5α-reductase enzyme being the highest affinity, rate limiting step on this pathway (46), we could not detect 3α- or 3β-dihydroprogesterone. The present study also failed to detect 5β-reduced metabolites of progesterone, in agreement with the reported lack of 5β-reductase activity in mammalian

brain tissue. However, we did find 3β,5α-tetrahydroprogesterone. Unlike their respective 3α,5α-reduced compounds, the 3β,5α-reduced steroids are not active at the $GABA_A$ receptor (47). Nevertheless, they would be substrates for the 3β-diol hydroxylase enzyme, which hydroxylates at positions 6 or 7, and is found throughout the brain. Competitive inhibition of 3β-diol hydroxylase increases the duration of anaesthesia induced by 3α,5α-tetrahydroprogesterone in the rat and this enzyme has therefore been suggested to provide an inactivation pathway for 3α,5α-reduced pregnanes and androstanes in the central nervous system (48). The 3β,5α-tetrahydroprogesterone found in the present study could have arisen through epimerisation of 3α,5α-tetrahydroprogesterone (49) or direct from 5α-dihydroprogesterone. The latter would serve as a diversion of substrate from the formation of neuroactive 3α,5α-tetrahydroprogesterone.

The present results also identify 11β-, 17α- and 20α-hydroxylation as possible routes for the inactivation of 3α,5α-tetrahydroprogesterone. The first two metabolites appear to be inactive at the $GABA_A$ receptor, whereas the latter has partial agonist properties at the 3α,5α-tetrahydroprogesterone-modulated site (47, 50). An 11β-hydroxylase (CYP11B) is widely expressed in brain and usually denoted as the final step in the production of corticosterone from deoxycorticosterone. However, the lack of evidence in this and previous studies for 21-hydroxylase (CYP21) activity and thus deoxycorticosterone formation in brain suggests that the 11β-hydroxylase may actually be more important in this tissue for the inactivation of 3α,5α-tetrahydroprogesterone. Formation of 5α-Pregnane-3α,17-diol-20-one is harder to explain in view of the apparent lack of CYP17 activity in brain (see above), although 17-hydroxylation could result from variable site specificity of one of the 3α-hydroxysteroid dehydrogenases (43) or another as yet unknown mechanism. As mentioned above, 20α-dihydroprogesterone was also detected in the present survey. This steroid is known to be a

better substrate than progesterone for rat brain 5α-reductase (51), which would explain the presence of 5α, 20α-tetrahydroprogesterone. Further reduction to 5α-pregnane-3α,20α-diol may therefore indicate an alternative metabolic route rather than a true inactivation pathway for 3α,5α-tetrahydroprogesterone. Likewise, the formation of 20α-dihydropregnenolone may serve to divert pregnenolone from the formation of neuroactive progesterone and its 3α,5α-reduced metabolites.

To conclude, the present study has chemically identified several neuroactive free steroids in adult male rat brain. Some of these compounds were found together with precursors suggesting they could be formed within this tissue, whereas others appear to enter from peripheral sources, although further studies will be necessary to confirm such origins. For 3α,5α-tetrahydroprogesterone, which is known to enhance the interaction of GABA with the $GABA_A$ receptor, 11β-, 17α- and 20α-hydroxylated metabolites were identified, thereby implicating inactivation pathways for this potent neuromodulatory compound. Our results also confirm the suggestion that epimerisation could serve as a first step on the inactivation of 3α,5α-reduced steroids in brain. In addition, the presence of 20α- and 20β-reduced pregnenolone indicates a possible regulation on the production of progesterone and 3α,5α-tetrahydroprogesterone. For the steroid sulphates, only DHEA sulphate was confirmed and pregnenolone sulphate not detected at limits below those quoted by previous authors. Despite its reported effects on $GABA_A$ and NMDA receptors, pregnenolone sulphate is therefore of doubtful physiological significance in the adult CNS. Further studies are now needed to elucidate the regulation of steroid production within the brain and to evaluate how these processes interact with peripheral sources of steroid.

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Authors' present addresses

M.J. Ebner: Procognia Limited, Unit 4, The Switchback, Gardner Road, Maidenhead, Berkshire, SL6 7RJ, UK; D. Corol: Crop Performance Improvement Division, Rothamsted Research, Harpenden, Herts, AL5 2JQ, UK; H. Havlíková: Institute of Endocrinology, Národní 8, 116 94. Prague 1, Czech Republic.

Table 1. Screen for steroids in rat brain by GC-EIMS using two ion selected ion monitoring of their MO-TMS derivatives

Systematic name	Trivial name	RI	T	Q	Injection number	Ion group number	Tentatively identified	Accuracy (%)	Intra-assay CV (%)	Inter-assay CV (%)
5-Androsten-3β-ol-17-one	Dehydroepiandrosterone	2631	358	268	3	1	FS, SS	124.4±3.8	12.7	6.9
5α-Androstan-3β-ol-17-one	Epiandrosterone	2645	360	270	3	1	SS	109.6±2.5	13.2	5.1
4-Androsten-3,17-dione	Androstenedione	2690*	344	313	4	1		108.5±3.6	10.0	7.3
4-Androsten-17β-ol-3-one	Testosterone	2713*	389	268	4	1	FS, SS	114.7±3.3	8.2	6.4
5β-Pregnan-3α,17-diol-20-one	17(5β)-OH-Pregnanolone	2740	476	188	2	1	SS	109.3±7.4	8.1	15.1
4-Pregnen-3α-ol-20-one	3α-Dihydroprogesterone	2746	417	244	1	1	FS,SS	107.7±6.2	16.4	12.9
5β-Pregnan-3β-ol-20-one	3β,5β-Tetrahydroprogesterone	2752	388	298	1	1	FS, SS	111.9±2.7	12.2	5.5
5α-Pregnan-3α-ol-20-one	3α,5α-Tetrahydroprogesterone	2757	388	298	1	1	FS, SS	97.6±3.2	6.9	7.3
5β-Pregnan-3α-ol-20-one	3α,5β-Tetrahydroprogesterone	2764	388	298	1	1	FS, SS	118.5±2.8	12.0	5.2
5α-Pregnan-3α,17-diol-20-one	17(5α)-OH-Pregnanolone	2766	476	188	2	1	FS, SS	103.0±7.0	7.0	15.1
5β-Pregnane-3α,20β-diol	Pregnanediol	2779	284	269	2	1	FS, SS	103.0±7.0	NM	7.5
5,16-Pregnadien-3β-ol-20-one	16-Dehydropregnenolone	2802	415	384	1,2,3,4	2	NA	NA	NA	NA
5α-Pregnane-3α,20α-diol	Allopregnanediol	2803	346	269	1	2	FS, SS	125.5±5.0	6.8	9.0
5β-Pregnane-3α,20α-diol	5β-Pregnane-3α,20α-diol	2811	284	269	2	2	SS	110.2±2.1	11.4	4.3
4-Pregnen-3β-ol-20-one	3β-Dihydroprogesterone	2818	386	244	3	2	SS	118.8±6.4	9.7	12.1
5β-Pregnan-3,20-dione	5β-Dihydroprogesterone	2820	343	275	4	2	FS	126.1±5.0	12.1	8.9
5-Pregnen-3β-ol-20-one	Pregnenolone	2834	402	386	1	3	FS, SS	95.0±1.5	14.8	3.6
5α-Pregnan-3β-ol-20-one	3β,5α-Tetrahydroprogesterone	2847	388	100	1	3	FS, SS	121.7±3.2	9.7	5.9
5α-Pregnan-3,20-dione	5α-Dihydroprogesterone	2859*	343	275	2	3	FS	110.2±3.3	9.4	6.6
5-Pregnene-3β,20β-diol	20β-Dihydropregnenolone	2860	372	462	2	3	FS,SS	120.2±3.3	2.5	6.2
5α-Pregnan-3α-ol-11,20-dione	Alfaxalone	2880	402	433	1	4		117.0±1.8	10.1	3.5
5-Pregnene-3β,20α-diol	20α-Dihydropregnenolone	2882	372	462	1	4	FS	119.4±2.7	12.2	5.0
4-Pregnen-3,20-dione	Progesterone	2900	372	341	4	3	FS	115.6±1.9	18.8	3.7
5β-Pregnan-3α,21-diol-20-one	3α,5β-Tetrahydrodeoxy-corticosterone	2907*	476	188	2	4	FS	118.2±8.0	10.1	15.1
5α-Pregnan-20α-ol-3-one	5α,20α-Tetrahydroprogesterone	2907*	303	289	4	3	FS, SS	107.8±2.5	22.4	5.2
5α-Pregnan-3α,11β-diol-20-one	5α-Pregnan-3α,11β-diol-20-one	2921	386	296	1	5	FS, SS	181.1±4.9	14.5	6.1

Table 1 continued.

5-Pregnen-3β,17α-diol-20-one	17α-Hydroxypregnenolone	2921	362	474	1	5	FS	118.6±2.8	14.6	5.2
5β-Pregnan-3β,21-diol-20-one	3β,5β-Tetrahydrodeoxy-corticosterone	2924*	507	358	3	3	FS, SS	110.1±2.6	14.2	5.4
4-Pregnen-20β-ol-3-one	20β-Dihydroprogesterone	2927	417	286	2	4	FS	NM	NM	NM
5α-Pregnan-3α,21-diol-20-one	3α,5α-Tetrahydrodeoxy-corticosterone	2929*	507	358	3	3	FS, SS	112.7±5.4	11.5	10.8
4-Pregnen-20α-ol-3-one	20α-Dihydroprogesterone	2947	417	286	3	3	FS, SS	117.5±3.8	13.7	7.3
4-Pregnen-17α-ol-3,20-dione	17α-Hydroxyprogesterone	2967*	429	339	1	6	FS, SS	112.5±2.7	11.4	5.4
6α-Methyl-pregnen-17α-ol-3,20-dione	6α-Methyl-17-hydroxyprogesterone	2970*	443	474	1,2,3,4	6,5,4,4	NA	NA	NA	NA
5β-Pregnan-21-ol-3,20-dione	5β-Dihydrodeoxy-corticosterone	2987	431	462	2	4	FS	122.5±6.7	20.2	12.3
5α-Pregnan-3β,21-diol-20-one	3β,5α-Tetrahydro-deoxycorticosterone	3045	476	188	3	4	FS, SS	115.4±6.0	6.8	11.6
5α-Pregnan-21-ol-3,20-dione	5α-Dihydrodeoxy-corticosterone	3058*	462	431	4	4	FS	103.5±24.3	25.5	47.0
4-Pregnen-21-ol-3,20-dione	11-Deoxycorticosterone	3098	273	286	2	6	FS, SS	208.5±8.5	10.2	9.1
4-Pregnen-11β-ol-3,20-dione	11β-Hydroxyprogesterone	3105*	370	339	2	6	FS	130.3±9.0	10.4	15.4
4-Pregnen-17α,21-diol-3,20-dione	Reichstein's Substance S (or 11-Deoxycortisol)	3124*	517	427	3	5	SS	119.6±2.6	17.6	4.8
4-Pregnen-21-ol-3,11,20-trione	11-Dehydrocorticosterone	3196	443	474	3	5	FS	297.3±14.2	43.3	10.7
4-Pregnen-17α,21-diol-3,11,20-trione	Cortisone	3223*	531	441	4	5		116.2±3.1	6.7	5.9
4-Pregnen-11β,21-diol-3,20-dione	Corticosterone	3247*	548	517	2	7	FS	110.7±2.7	5.6	5.4
1,4-Pregnadien-11β,17,21-triol-3,20-dione	Prednisolone, 1-Dehydrocortisol	3280	513	603	1,2,3,4	7,7,6,5	NA	NA	NA	NA
11β,17α,21-Trihydroxy-4-pregnen-3,20-dione	Hydrocortisone, Cortisol	3290	605	515	1	7		247.1±9.4	16.9	14.3

Retention index (RI, * of first peak in the case of double peaks) plus target (T) and qualifier (Q) ions (m/z) for screening of steroids in adult male rat brain by two-ion SIM of their MO-TMS-derivatives in GC-EIMS. Four different rat brain extracts were screened and each injected 4 times into the GC with the MS set selectively to monitor different groups of 4-6 diagnostic ions along the elution profile. Steroids are tentatively identified as present in free steroid (FS) and/or steroid sulphate (SS) fractions. Also shown are accuracy (%, mean±SEM), intra- and inter-assay coefficients of variation (CV, %) of two ion SIM analyses of mixtures of MO-TMS-derivatised steroid standards (for 0.5 ng injected mass). NM: Not measured; NA: not applicable.

Table 2. Confirmation of identity for free and sulphate conjugated steroids in adult male rat brain.

	MO-TMS-derivatives									HFB-derivatives					
	Diagnostic ions			Steroid standards			Rat brain			Diagnostic ions		Steroid standards		Rat brain	
Steroid	T	Q1	Q2	RRT	Q1/T	Q2/T	RRT	Q1/T	Q2/T	T	Q	RRT	Q/T	RRT	Q/T
Dehydroepiandrosterone	358	268	260	0.911	1.436	1.363	0.909	2.148	0.700	270	255	1.051	0.189	1.050	0.195
Dehydroepiandrosterone sulphate	358	268	260	0.915	1.442	1.048	0.914	1.934	0.825	270	255	1.050	0.197	1.051	*1.327*
Testosterone	389	358	268	0.954*	0.190	0.357	0.953	0.220	0.258	680	451	1.038	0.272	1.038	*3.595*
3α,5α-Tetrahydroprogesterone	388	241	298	0.977	0.311	NM	0.976	0.292	NM	496	514	0.959	0.453	0.960	0.439
5α-Pregnan-3α,17-diol-20-one	476	386	364	0.982	0.231	0.299	0.981	*1.955*	0.235	442	487	1.032	2.176	1.032	2.207
5α-Pregnane-3α,20α-diol	117	269	346	0.999	0.022	0.012	1.000	*0.057*	0.017	712	697	0.969	0.693	0.968	0.550
Pregnenolone	402	386	312	1.016	1.842	1.071	1.015	1.680	0.901	298	283	1.024	0.201	1.023	0.186
3β,5α- Tetrahydroprogesterone	243	298	404	1.022	0.482	0.615	1.022	0.624	0.404	467	514	1.036	5.326	1.035	5.101
5α-Dihydroprogesterone	343	275	288	1.028*	0.497	0.595	1.028	0.602	0.402	NM	NM	NM	NM	NM	NM
20β-Dihydropregnenolone	462	372	332	1.028	2.687	0.855	1.027	2.510	1.039	496	481	1.001	0.115	1.000	0.115
20α-Dihydropregnenolone	462	372	332	0.955	2.360	0.866	0.954	3.167	0.944	496	481	1.032	0.069	1.030	0.074
Progesterone	372	341	273	0.965	0.924	0.549	0.964	0.973	0.656	510	495	1.023	0.137	1.023	0.187
5α,20α-Tetrahydroprogesterone	303	288	386	0.968*	0.409	1.666	0.966	*0.678*	1.464	499	514	0.952	1.583	0.955	1.682
5α-Pregnan-3α,11β-diol-20-one	386	476	296	0.974	0.379	0.406	0.974	*0.623*	0.679	469	512	0.977	0.941	0.976	1.156
3α,5α-Tetrahydrodeoxycorticosterone	476	507	404	0.979	0.318	0.209	0.978	0.278	0.188	499	257	1.060	3.250	1.059	*5.876*
20α-Dihydroprogesterone	417	301	296	0.988	0.410	0.330	0.984	0.559	*1.802*	708	693	1.030	0.204	1.030	0.166
Corticosterone	548	517	427	0.987	1.070	1.284	0.986	1.317	1.407	738	491	1.110	1.253	ND	ND

Qualifier (Q) and target (T) diagnostic ions, relative retention times (RRT, * of first peak in the case of double peaks), Q/T ratios of standard compounds and brain extracts from three ion SIM of their MO-TMS-derivatives or two ion SIM of their HFB-derivatives. Values outside identification limits are shown in italic. ND: not detected; NM: not monitored.

Table 3. Quantitation of steroids in adult male rat brain

	Free steroids			Steroid sulphates		
Steroid	Concentration (ng/g)	Detection limit (ng/g)	Recovery %	Concentration (ng/g)	Detection limit (ng/g)	Recovery %
Dehydroepiandrosterone	0.27±0.20	0.004	59.9±0.7	1.04±0.83	0.14	51.3±2.3
Testosterone	2.24±0.95	0.007	53.0±1.2	ND	0.17	NM
Pregnenolone	1.67±0.45	0.05	39.8±1.0	ND	0.05	28.6±2.2
20α-Dihydropregnenolone	0.27±0.08	0.05	38.1±1.1	ND	0.04	NM
20β-Dihydropregnenolone	0.17±0.06	0.03	36.8±0.7	ND	0.17	NM
Progesterone	0.70±0.14	0.09	63.1±3.4	NA	NA	NA
5α-Dihydroprogesterone	1.01±0.30	0.12	31.5±3.9	NA	NA	NA
3α,5α-Tetrahydroprogesterone	0.35±0.09	0.06	39.3±1.1	ND	0.21	NM
3β,5α-Tetrahydroprogesterone	0.13±0.05	0.05	36.4±0.8	ND	0.16	NM
20α-Dihydroprogesterone	0.19±0.08	0.002	46.5±0.7	ND	0.19	NM
5α,20α-Tetrahydroprogesterone	0.25±0.11	0.01	40.9±2.6	ND	0.09	NM
5α-Pregnane-3α,20α-diol	0.25±0.09	0.07	55.9±3.2	ND	0.11	NM
5α-Pregnan-3α,17-diol-20-one	0.06±0.02	0.02	49.9±1.8	ND	0.18	NM
5α-Pregnan-3α,11β-diol-20-one	0.77±0.70	0.005	43.3±2.5	ND	0.08	NM
3α,5α-Tetrahydrodeoxycorticosterone	0.20±0.05	0.006	41.4±2.1	ND	0.02	NM
Corticosterone	4.63±1	1.02	43.7±5.8	ND	0.32	NM
3β,5α-Tetrahydrodeoxycorticosterone	ND	0.03	37.0±2.4	ND	0.03	NM
Epiandrosterone	ND	0.006	57.8±0.5	ND	0.02	NM
Androstenedione	ND	0.03	71.2±1.3	NA	NA	NA
3α-Dihydroprogesterone	ND	0.09	21.0±2.3	ND	0.16	NM
3β-Dihydroprogesterone	ND	0.06	27.3±3.1	ND	0.06	NM
5β-Dihydroprogesterone	ND	0.27	35.2±2.0	NA	NA	NA
11β-Hydroxyprogesterone	ND	0.33	63.8±4.7	ND	0.55	NM
17α-Hydroxypregnenolone	ND	0.07	47.6±1.4	ND	0.06	NM
17α-Hydroxyprogesterone	ND	0.12	58.5±1.4	ND	0.15	NM
20β-Dihydroprogesterone	ND	0.01	NM	ND	0.01	NM
3β,5β-Tetrahydroprogesterone	ND	0.07	42.2±1.1	ND	0.21	NM
3α,5β-Tetrahydroprogesterone	ND	0.04	39.4±1.1	ND	0.02	NM
Alphaxalone	ND	0.009	48.5±1.9	ND	0.02	NM
5β-Pregnan-3α,17-diol-20-one	ND	0.04	52.0±4.9	ND	0.03	NM
5β-Pregnane-3α,20β-diol	ND	0.14	36.3±3.8	ND	0.06	NM
5β-Pregnane-3α,20α-diol	ND	1.02	29.5±0.8	ND	1.11	NM
11-Deoxycorticosterone	ND	0.35	109.9±33	ND	0.81	NM
5α-Dihydrodeoxycorticosterone	ND	0.44	45.0±1.1	ND	0.25	NM
5β-Dihydrodeoxycorticosterone	ND	0.03	35.9±0.8	ND	0.01	NM
3α,5β-Tetrahydrodeoxycorticosterone	ND	0.005	35.4±2.7	ND	0.005	NM
3β,5β-Tetrahydrodeoxycorticosterone	ND	0.03	44.3±4.0	ND	0.004	NM
Cortisol	ND	0.04	12.8±2.3	ND	0.04	NM
11-Deoxycortisol	ND	0.06	49.0±4.4	ND	0.05	NM
11-Dehydrocorticosterone	ND	3.06	34.0±9.3	ND	1.50	NM
Cortisone	ND	0.10	21.9±3.4	ND	0.05	NM

Concentrations of MO-TMS- or HFB-derivatives of steroids in pooled male rat brain extracts analysed by 2 and 3 ion SIM are given in ng/g tissue (means ± SEM, n≥3). Detection limits (ng/g tissue) were determined from reagent blanks carried alongside tissue samples through the whole extraction and fractionation procedure. Recoveries (%, mean±S.E.M., n=4) are also shown for pure standards added to brain homogenates and carried through entire procedure. ND: not detectable; NA: not applicable; NM: not measured.

Figure 1. EI-mass spectrum (99-800 m/z) of pregnenolone derivatised with MO and TMSI and proposed fragmentation pathways.

Ion abundance is shown in arbitrary units.

Figure 2. Typical chromatograms of male rat whole brain extracts analysed in GC-EIMS by selected ion monitoring of MO-TMS-steroid derivatives.

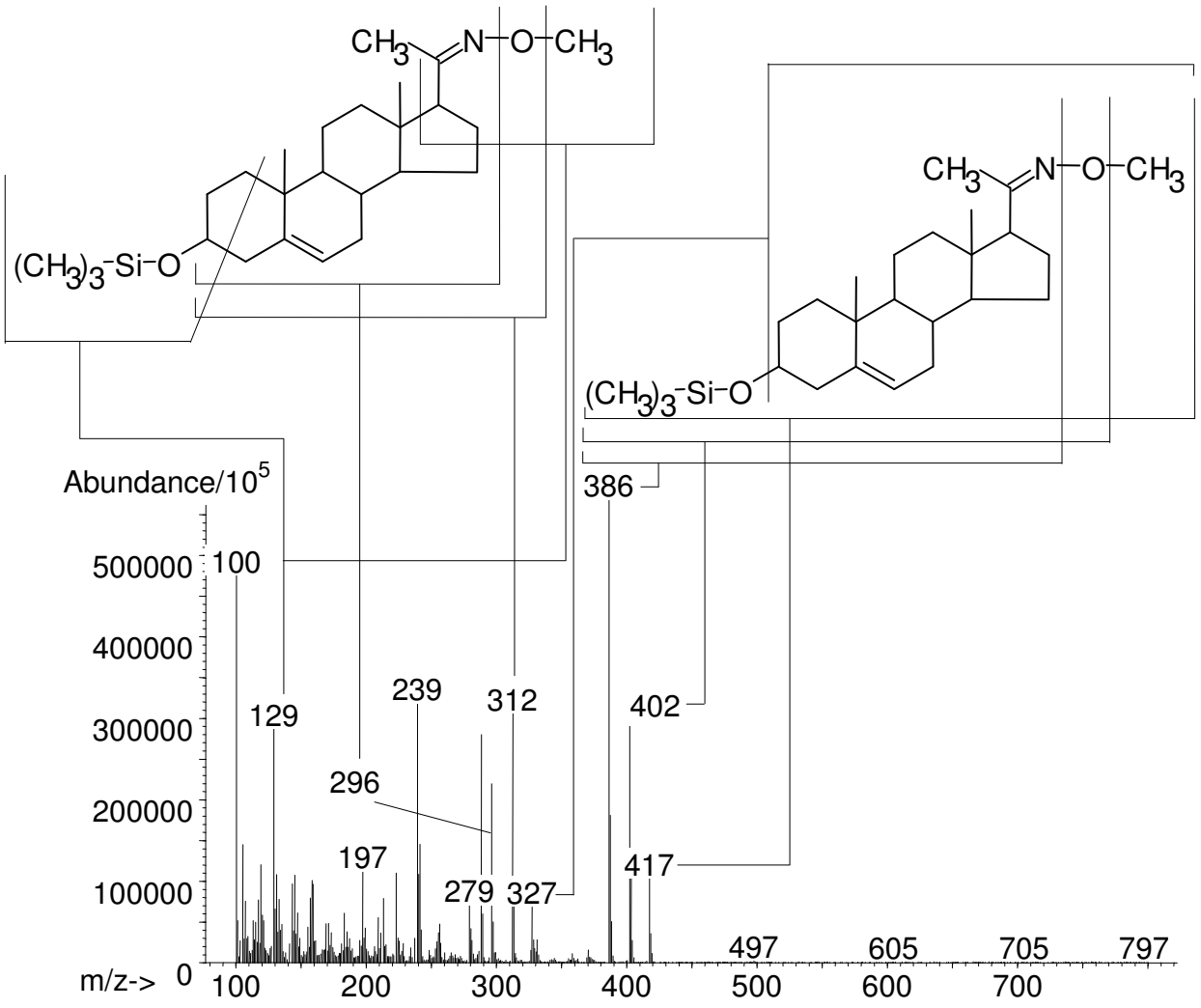
Traces from brain extracts are shown below those of reference compounds. Examples are given of both positively identified and non-detectable brain steroids. Ion (m/z as shown) abundance is plotted against relative retention time (RRT) to one of three internal standards as indicated in parentheses: 16-dehydropregnenolone (1), 6α-methyl-17-hydroxyprogesterone (2), prednisolone (3). The * indicates expected RRT. For clarity of presentation, most traces are shown base-shifted (no y-axis drawn).

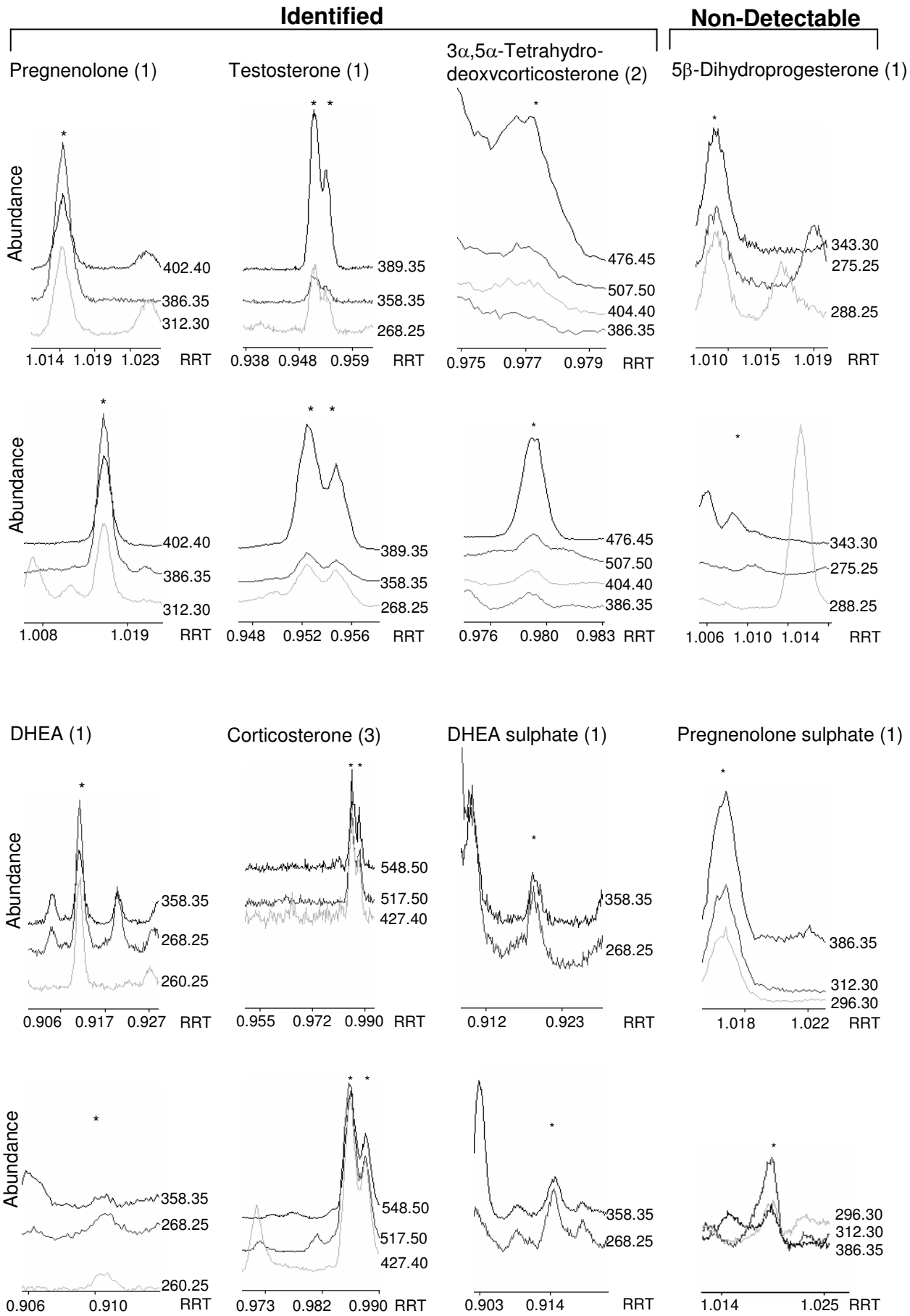
Figure 3. Typical chromatograms of male rat whole brain extracts analysed in GC-EIMS by selected ion monitoring of HFB-steroid derivatives.

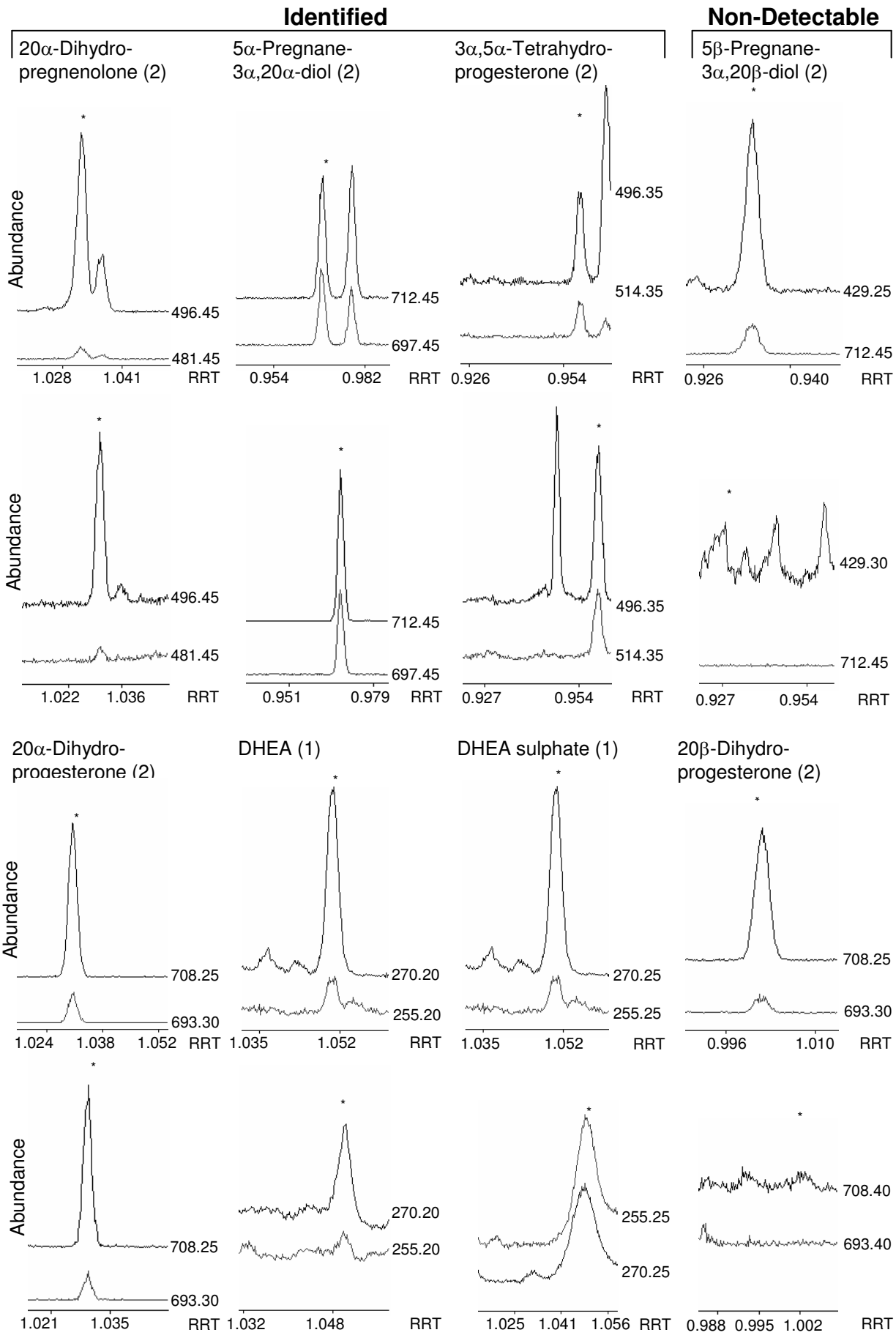
Traces from brain extracts are shown below those of reference compounds. Examples are given of both positively identified and non-detectable brain steroids. Ion (m/z as shown) abundance is plotted against RRT to one of three internal standards as indicated in parentheses: tetracosane (1), 16-dehydropregnenolone (2), octacosane (3). The * indicates expected RRT. For clarity of presentation, most traces are shown base-shifted (no y-axis drawn).

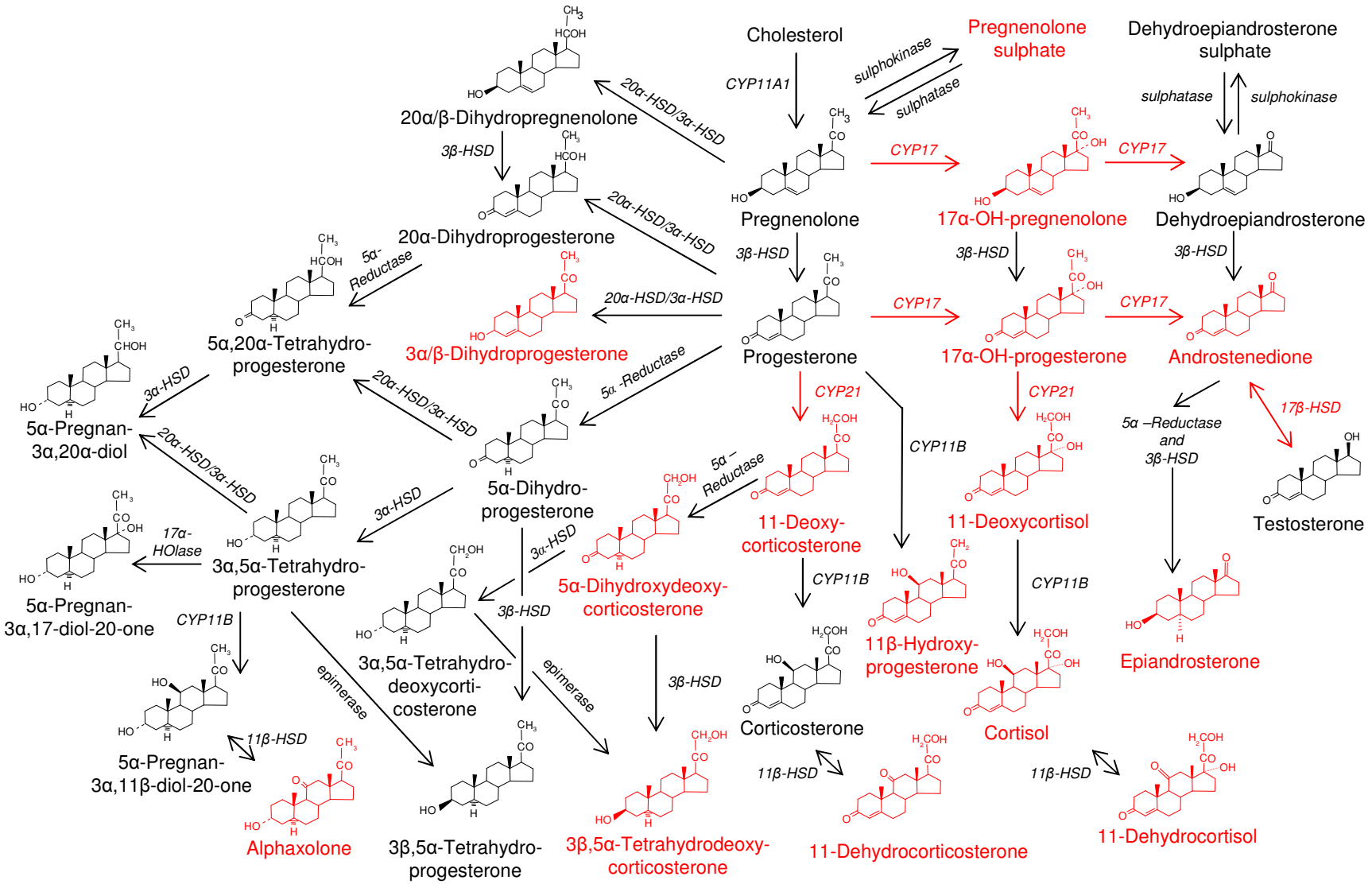
Figure 4. Identified pathways of steroid synthesis and metabolism in adult male rat brain.

Compounds identified in the present study are shown in black and those not detected left in red. For clarity, the 5β-pregnanes not detected in the present study have been omitted from the scheme. Enzymes previously identified at activity, protein or mRNA levels by others (see Discussion) are also shown in black and again, those not found shown in red. Abbreviations are as follows: CYP11A1, cholesterol side chain cleavage; CYP17, 17α-hydroxylase/c17,20-lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/isomerase; CYP21, 21-hydroxylase; 11β-HSD, 11β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3α/β-HSD, 3α/β-hydroxysteroid dehydrogenase; 17α-HOlase, 17α-hydroxylase; 20α-HSD, 20α-hydroxysteroid dehydrogenase; CYP11B, 11β-hydroxylase.


CH3 N O CH3
(CH3)3-Si-O
CH3 N O CH3
(CH3)3-Si-O
Abundance/10^5
500000
400000
300000
200000
100000
0
m/z->
100
200
300
400
500
600
700
100
129
197
239
279
296
312
327
386
402
417
497
605
705
797


Identified
Non-Detectable
Pregnenolone (1)
Testosterone (1)
3α,5α-Tetrahydro-deoxvcorticosterone (2)
5β-Dihydroprogesterone (1)
Abundance
402.40
386.35
312.30
389.35
358.35
268.25
476.45
507.50
404.40
386.35
343.30
275.25
288.25
RRT
DHEA (1)
Corticosterone (3)
DHEA sulphate (1)
Pregnenolone sulphate (1)
358.35
268.25
260.25
548.50
517.50
427.40
296.30
312.30


Identified
Non-Detectable
20α-Dihydro-pregnenolone (2)
5α-Pregnane-3α,20α-diol (2)
3α,5α-Tetrahydro-progesterone (2)
5β-Pregnane-3α,20β-diol (2)
Abundance
496.45
481.45
712.45
697.45
496.35
514.35
429.25
712.45
429.30
1.028
1.041
RRT
0.954
0.982
0.926
0.954
0.926
0.940
1.022
1.036
0.951
0.979
0.927
0.954
0.927
0.954
20α-Dihydro-progesterone (2)
DHEA (1)
DHEA sulphate (1)
20β-Dihydro-progesterone (2)
708.25
693.30
270.20
255.20
270.25
255.25
708.40
693.40
1.024
1.038
1.052
1.035
1.052
1.035
1.052
0.996
1.010
1.021
1.035
1.032
1.048
1.025
1.041
1.056
0.988
0.995
1.002


Cholesterol
CYP11A1
sulphokinase
sulphatase
Pregnenolone sulphate
Dehydroepiandrosterone sulphate
sulphatase
sulphokinase
20α/β-Dihydropregnenolone
20α-HSD/3α-HSD
3β-HSD
Pregnenolone
CYP17
17α-OH-pregnenolone
CYP17
Dehydroepiandrosterone
20α-Dihydroprogesterone
20α-HSD/3α-HSD
3β-HSD
3β-HSD
3β-HSD
5α-Reductase
20α-HSD/3α-HSD
Progesterone
CYP17
17α-OH-progesterone
CYP17
Androstenedione
5α,20α-Tetrahydro-progesterone
3α/β-Dihydroprogesterone
3α-HSD
20α-HSD/3α-HSD
5α-Reductase
CYP21
CYP21
5α –Reductase and 3β-HSD
17β-HSD
5α-Pregnan-3α,20α-diol
20α-HSD/3α-HSD
5α-Dihydro-progesterone
5α –Reductase
CYP11B
11-Deoxy-corticosterone
11-Deoxycortisol
Testosterone
3α-HSD
3α-HSD
17α-HOlase
3α,5α-Tetrahydro-progesterone
5α-Dihydroxydeoxy-corticosterone
5α-Pregnan-3α,17-diol-20-one
CYP11B
3β-HSD
CYP11B
CYP11B
11β-Hydroxy-progesterone
Epiandrosterone
3α,5α-Tetrahydro-deoxycorti-costerone
epimerase
epimerase
3β-HSD
Corticosterone
Cortisol
11β-HSD
11β-HSD
11β-HSD
5α-Pregnan-3α,11β-diol-20-one
Alphaxolone
3β,5α-Tetrahydro-progesterone
3β,5α-Tetrahydrodeoxy-corticosterone
11-Dehydrocorticosterone
11-Dehydrocortisol