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Metabolism of neuroactive steroids in day-old chick brain

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Abstract

Metabolism of the neuroactive steroids pregnenolone (PREG), progesterone (PROG), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) was investigated in day-old chick brain following direct injection of the ^{3}H-labelled compounds into the intermediate medial mesopallium and sampling at times known to be crucial for memory formation in this brain region. ^{3}H-label from these steroids was cleared rapidly from the brain, decreasing to barely detectable levels within 5 h. Following extraction and fractionation, the ^{3}H-labelled brain steroids were identified by TLC, coupled with acetylation and/or separation in different solvent systems. PREG and PROG were converted within 10 min mostly to 20β-dihydropregnenolone (20β-DHPREG) and 5β-dihydroprogesterone, respectively. There was no detectable metabolism of DHEA. Label from DHEAS persisted for longer (half-time 18.9 min) than the free steroid but with no detectable metabolism other than a small amount (4%) of desulphation to DHEA. Further investigation of chick brain steroid metabolism by incubation of subcellular fractions (1–3 h, 37°C) with PREG, PROG or DHEA plus NADPH led to the formation of the following compounds: 20β-DHPREG from PREG (particularly in cytosol); 5β-dihydroprogesterone and 3α,5β-tetrahydroprogesterone from PROG and no detectable metabolism of DHEA. Following incubation of the same brain fractions and labelled steroids with NAD^+, there was no detectable metabolism of PREG or PROG but some conversion of DHEA to androstenedione, especially in the nuclear fraction. The results suggest direct actions of DHEA(S) on the early stages of memory formation in the chick and introduce the possibility that PREG may act indirectly via 20β-DHPREG.

Keywords: brain, chicks, dehydroepiandrosterone, pregnenolone, progesterone, steroid.

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The one-trial passive avoidance task in day-old chicks can be used to investigate mechanisms of memory formation (see Rose 2000) and the steroid dehydroepiandrosterone (DHEA) and its sulphate ester (DHEAS), which are known to be present in chick brain, enhance learning in this model (Migues *et al.* 2002; Sujkovic *et al.* 2007). Other steroids shown to improve memory performance on this task include pregnenolone (PREG), 17β-estradiol, testosterone and corticosterone (CORT) (Gibbs *et al.* 1986; Johnston and Rose 1998; Migues 2001). However, little is known of the synthesis and metabolism of these and other steroids in chick brain, especially in relation to the time course of memory formation.

The steroids DHEA and DHEAS persist in adult male rat brain following adrenalectomy and gonadectomy (Corpéchot *et al.* 1981), indicating formation within this tissue and the definition of the term neurosteroid. Similar evidence has been presented (Corpéchot *et al.* 1983) for PREG and its sulphate ester (PREGS). Other steroids which appear to be synthesised within the adult rat brain include progesterone (PROG) and its reduced metabolites 5α-dihydroprogesterone (5α-DHPROG) and 3α,5α-tetrahydroprogesterone (3α,5α-THPROG, allopregnanolone; Corpéchot *et al.* 1993; Cheney

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Abbreviations used: 3α,5α/β-THPROG, 3α,5α/β-tetrahydroprogesterone; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; 5α/β-DHPROG, 5α/β-dihydroprogesterone; 17α-OHPREG, 17α-hydroxypregnenolone; 20-HSD, 20-hydroxysteroid dehydrogenase; 20β-DHPREG, 20β-dihydropregnenolone; ADIONE, androstenedione; CYP17, cytochrome P450 17α-hydroxylase/c17,20-lyase; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; DOC, 11-deoxycorticosterone; HST, hydroxysteroid sulphotransferase; i.c., intracranial; IMM, intermediate medial mesopallium; PREG, pregnenolone; PREGS, pregnenolone sulphate; PROG, progesterone.

et al. 1995). The 3β-, 11β-, 17α- and 20α-hydroxylated metabolites of 3α,5α-THPROG can also be found in adult rat brain, as can the 20α- and 20β-reduced metabolites of PREG plus 20α-DHPROG and 5α-DHPROG (Ebner *et al.* 2006). Other steroids detected in adult mammalian brain include 17β-estradiol and testosterone (Robel *et al.* 1973; Bixo *et al.* 1995), aldosterone, corticosterone and 3α,5α-tetrahydrodeoxycorticosterone (Corpéchot *et al.* 1981; Synguelakis *et al.* 1985; Yongue and Roy 1987; Purdy *et al.* 1991; Ebner *et al.* 2006) which, at least in the male rat brain, appear to be of endocrine origin.

From the above, a picture is emerging of the central and peripheral origins of adult mammalian brain steroids and their metabolism to neurally active or inactive compounds (see Ebner *et al.* 2006). In the day-old chick brain, there is immunocytochemical evidence for the steroidogenic cholesterol P450 side chain cleavage enzyme and the product of this enzyme PREG can be detected together with PREGS (Migues *et al.* 2005). As mentioned above, DHEA and its sulphate ester have been detected in the brains of day-old chicks (Migues *et al.* 2002). Steroidogenic enzymes and their products have also been demonstrated in the brains of adult quails and zebra finches. The enzymes present in these species include cholesterol P450 side chain cleavage, 3β-hydroxysteroid dehydrogenase/$\triangle^5$-$\triangle^4$-isomerase (3β-HSD), 5β-reductase, cytochrome P450 17α-hydroxylase/c17,20-lyase (CYP17), cytochrome P450 7α-hydroxylase, 17β-hydroxysteroid dehydrogenase and aromatase (Tsutsui *et al.* 2006; Schlinger and London 2006; see also Discussion). Consistent with the presence of these enzymes, accumulation of PREG has been demonstrated in the adult quail brain independently of endocrine sources (Tsutsui and Yamazaki 1995) and shown to be metabolised to 7α-hydroxypregnenolone, PROG, 3β,5β-THPROG, testosterone and 17β-estradiol (see Tsutsui *et al.* 2006, 2008).

In the present study and as a continuation of our investigation of the influence of steroids on learning and memory in the day-old chick, we have evaluated whether PREG, PROG, DHEA and DHEAS act directly or via metabolites by injecting them as ^{3}H-labels directly into the intermediate medial mesopallium (IMM, previously known as the intermediate hyperstriatum ventrale), a brain region known to be specifically involved in the early stages of memory formation in these animals (see Rose 2000). Functional relevance of any steroid metabolism was tested by sampling at times crucial for memory formation and the ^{3}H-labelled brain steroids extracted and fractionated for identification. In view of the difficulties in removing peripheral sources of steroid in these day-old chicks, we have further investigated possible pathways of steroid metabolism within the brain by incubating fractions of this tissue with the above ^{3}H-labelled steroids *in vitro*.

Materials and methods

Materials

[7-^{3}H(N)]-Pregnenolone (PREG, 22.5 Ci/mmol), [1,2,6,7,16,17-^{3}H(N)]-progesterone (PROG, 138 Ci/mmol), [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone (DHEA, 74 Ci/mmol), [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone sulphate (DHEAS, 60 Ci/mmol) and the Soluene 350 tissue solubiliser were obtained from PerkinElmer LAS Ltd. (Buckinhamshire, UK). Unlabeled reference steroid standards were purchased from Steraloids Inc. (Newport, RI, USA) or Sigma-Aldrich (Dorset, UK). The arylsulphatase enzyme (from Helix pomatia; type H1), β-NADPH and NAD^+ were also purchased from Sigma-Aldrich and the c*O*mplete© protease inhibitor cocktail tablets came from Roche Diagnostics Ltd. (West Sussex, UK). All solvents and chemical reagents were of analytical grade and obtained from VWR International (Leicestershire, UK) as were the TLC plates. Acetic anhydride was redistilled prior to use. For solid phase extraction we used Waters Oasis Hydrophobic-Lipophilic Balance and Mixed-Mode Anion Exchange cartridges, both at 3 cc, 60 mg and obtained from Waters Corporation, Milford, MA, USA. The scintillation fluid Ecoscint H was purchased from National Diagnostics (Yorkshire, UK). In order to avoid adsorptive losses of steroid, all glassware was deactivated by silanisation with 2% (vol/vol) dimethydichlorosilane in cyclohexane. Excess reagent and the hydrochloric acid produced during silanisation were removed by washing with cyclohexane then water, followed by baking for 2 h at 200°C.

Animals

Fertile Ross Chunky eggs (*Gallus domesticus*) were obtained from Maurice Millard Chicks Ltd. (Trowbridge, UK) and incubated for 18 days with a cycle of 8 h light, 16 h dark at 38.0–38.5°C. On day 19 of incubation, the eggs were transferred to a hatching incubator maintained at 37°C with 12 : 12 h light/dark cycle. Once hatched, chicks were transferred to a group brooder (at 29–30°C heated by a 150 W ceramic dull emitter) and supplied with food (Wm Lillico & Son Ltd., UK) and water *ad libitum*. All experimental procedures were approved by the UK Animal (Scientific Procedures) Act 1986 under personal and project Home Office licence.

Collection of brain and plasma samples

Chicks were killed by decapitation immediately after removal from the holding pen. Whole brains were removed rapidly, frozen on dry ice and stored at −70°C until analysis. Trunk blood samples were collected into heparinised tubes and plasma obtained by centrifugation (1000 *g*, 10 min, 4°C) then stored at −20°C. Sex was determined by inspection of gonads *post-mortem*.

Intracranial injection of steroids for the investigation of metabolism *in vivo*

To minimise isolation stress, randomly selected chicks were housed in pairs and kept undisturbed for at least 1.5 h prior to injection. The soft, unossified skull of the chick permits intracranial (i.c.) injection without the use of implanted cannulae or anaesthesia (Rose 2000).This was achieved in the present study by using a head holder (Davis *et al.* 1979) to direct injections to the IMM through a Hamilton microsyringe, fitted with a plastic sleeve to allow repeated injection to a depth of 4 mm beneath the skull and left in place for

Table 1 Percentage recoveries of radioactivity in free steroid and steroid sulphate fractions of chick brain at different times after intracranial injection of ^{3}H-pregnenolone (PREG), ^{3}H-progesterone (PROG), ^{3}H-dehydroepiandrosterone (DHEA) or ^{3}H-dehydroepiandrosterone sulphate (DHEAS) to the intermediate medial mesopallium

	PREG		PROG		DHEA		DHEAS		
Time points	Free	*n* (F/M)	Free	*n* (F/M)	Free	*n* (F/M)	Free	Sulphate	*n* (F/M)
5 min	34.4 ± 3.5	*n* = 5 (1/4)	40.1 ± 0.7	*n* = 5 (5/0)	79.3 ± 3.3	*n* = 5 (4/1)	3.7 ± 1.0	43.2 ± 16.7	*n* = 5 (5/0)
10 min	44.4 ± 4.7	*n* = 4 (3/1)	22.9 ± 2.8	*n* = 5 (2/3)	69.6 ± 2.0	*n* = 5 (2/3)	4.6 ± 0.9	38.3 ± 8.2	*n* = 5 (3/2)
30 min	ND	*n* = 5 (3/2)	ND	*n* = 5 (2/3)	ND	*n* = 5 (4/1)	3.3 ± 0.4	41.5 ± 9.9	*n* = 4 (2/2)
1 h	ND	*n* = 5 (2/3)	ND	*n* = 5 (2/3)	ND	*n* = 5 (3/2)	2.7 ± 0.5	31.7 ± 4.0	*n* = 5 (5/0)
5 h	ND	*n* = 5 (1/4)	ND	*n* = 5 (2/3)	ND	*n* = 5 (2/3)	ND	ND	*n* = 5 (3/2)

Recoveries are given as percentage of total brain homogenate radioactivity (mean ± SEM) and when not detectable (ND) < 15 DPM/sample. There was no detectable radioactivity in the steroid sulphate fractions at any time point after injection of either PREG or DHEA. The number of animals used (*n*) and the proportions of female (F) to male (M) chicks are presented for each treatment. For radioactive doses see legend to Fig. 1.

5 s. Accuracy of the procedure was established by prior injections of methylene blue and dissection of the IMM *post-mortem*. For injection, the ^{3}H-steroids in ethanol were dried under N_2, and re-suspended in 0.9% (w/v) sterile sodium chloride. Chicks were then injected bilaterally into the IMM with 2 µL of one the ^{3}H-steroids at total doses determined by scintillation counting to be as follows: ^{3}H-PREG 0.043 µCi, ^{3}H-PROG 0.077 µCi, ^{3}H-DHEA 0.080 µCi and ^{3}H-DHEAS 0.075 µCi. The numbers of male and female chicks used are indicated in Table 1.

Extraction and fractionation of steroids from brain tissue

Metabolites of ^{3}H-steroids produced after i.c. injection of the labelled substrates were identified following extraction and fractionation from individual brain samples, as described previously (Ebner *et al.* 2006; Nicolas and Fry 2007). Briefly, each brain was homogenised in 5 vol of ice cold 5 mM KH_2PO_4 buffer (pH 7). A 1 mL sample of each homogenate was retained for measurement of total radioactivity (see below) and the remainder extracted by sonication in 20 vol of 3% (vol/vol) acetic acid in 96% ethanol. After centrifugation, (28 000 *g*, 30 min, 4°C), the supernatant extract was dried under N_2 then redissolved in 20 vol of 20% vol/vol ethanol in KH_2PO_4 buffer. Separation of free from sulphated steroids was carried out on Oasis Mixed-Mode Anion Exchange cartridges, which permit both hydrophobic interaction and anion exchange chromatography. After loading of the extract, cartridges were washed with 5 mL 20% vol/vol ethanol in 20 mM ammonium acetate (pH 7) before eluting free steroids with 4 mL ethyl acetate. Steroid sulphates were then eluted with 13 mL 1% (wt/vol) ammonium carbonate in 60% ethanol. Fractions were dried under N_2 and stored in ethanol (containing 0.2% vol/vol ammonia for the steroid sulphates) at −20°C until analysis by TLC.

Solubilisation of tissue and plasma samples for measurement of total radioactivity

Samples of brain and plasma were solubilised in 3 vol of Soluene-350 for 15 min at 50°C then bleached with hydrogen peroxide (final concentration 1% wt/vol) for 2 h at 50°C. This bleaching process was repeated for a further 2 h with the plasma samples. Radioactivity could then be measured by scintillation counting in Ecoscint H containing Triton X-100 6% (vol/vol; scintillation grade), glacial acetic acid 0.6% (vol/vol) and butylated hydroxytoluene 2% (wt/vol). All samples were assayed in triplicate.

Subcellular fractionation of brain for the investigation of steroid metabolism *in vitro*

Single whole chick brains were homogenised using a Dounce homogeniser (five strokes loose clearance, followed by five strokes tight clearance) in 10 vol of ice-cold 5 mM HEPES buffer (pH 7.4) containing 0.32 M sucrose, one c*O*mplete© EDTA-free protease inhibitor cocktail tablet per 250 mL plus phenylmethyl sulphonyl fluoride (0.1 mM). The homogeniser was rinsed with a further 10 vol of the above buffer solution which was then added to the rest of homogenate. A sample of this crude homogenate denoted P_0 was retained for further analyses. The remaining homogenate was centrifuged at 1000 *g* for 10 min at 4°C to yield the supernatant fraction S_1 and pellet fraction P_1 (nuclear). The pellet was resuspended in 5 vol of ice-cold 5 mM HEPES buffer (pH 7.4) containing 0.32 M sucrose and centrifuged at 1000 *g* at 4°C for a further 10 min. The resulting supernatant was pooled with S_1. The pooled supernatant fraction was then centrifuged at 8000 *g* for 10 min at 4°C to yield a supernatant fraction S_2 and pellet fraction P_2 (mitochondria and synaptosomes). The remaining supernatant was centrifuged at 110 000 *g* for 60 min at 4°C to produce a supernatant cytosolic fraction S_3 and pellet fraction P_3 (microsomal). Each brain sample was processed separately. Fractions were frozen in liquid nitrogen and stored at −80°C until assayed for enzyme activity. The protein content of each fraction was determined using the dye-binding method of Bradford (1976) with bovine serum albumin as a standard.

Investigation of steroid metabolism *in vitro*

Subcellular fractions containing 100 µg of protein from each chick brain were assayed in duplicate in KH_2PO_4 (50 mM, pH 7.4). Following addition of substrate ^{3}H-PREG, ^{3}H-PROG or ^{3}H-DHEA (20 nM), samples were warmed at 37°C for 2 min. The reaction was started by the addition of β-NADPH or β-NAD^+ to a final concentration of 1 mM, followed by further incubation at 37°C. Based on pilot studies, incubation times of 1 h or of 3 h were used with ^{3}H-PROG or with ^{3}H-PREG and ^{3}H-DHEA as substrates, respectively. The total assay volume was 500 µL. Boiled tissue fractions (100°C, 5 min) and buffer blanks (containing all the above

reagents except a tissue sample) were used as controls and included in all enzyme assays. The reaction was stopped by placing samples on ice. Steroids were then extracted twice into ethyl acetate:isooctane (1 : 1, vol/vol, 1 mL) and the resulting supernatants pooled, dried under N_2 and redissolved in 96% ethanol for storage at 4°C until analysis by TLC. Initial investigations were performed with subcellular brain fractions derived from male chicks ($n = 2$). Whenever metabolic activity was detected, further investigations were made using subcellular brain fractions from female chicks ($n = 2$).

TLC

Samples were applied to aluminium backed, silica gel 60 coated TLC plates and developed in one of the following solvent systems: A. cyclohexane:n-butyl acetate (1 : 2, vol/vol), B. chloroform:ether (10 : 3, vol/vol), C. isooctane:ethyl acetate (13 : 7, vol/vol), D. chloroform:ethyl acetate (3 : 1, vol/vol) or E. ethyl acetate:ethanol:ammonia (2.5 : 1 : 0.2, vol/vol). Separated ^{3}H-labeled steroids could be visualised by exposing these TLC plates for 2 days to Fujifilm BAS-TR2040S imaging plates (Raytek Scientific Ltd., Sheffield, UK) which were then scanned in a Typhoon 9410 Variable Mode phosphorimager (Molecular Dynamics, Amersham, UK). Unlabeled steroid reference standards (50 μg) were visualised by exposure to iodine vapour.

Identification of ^{3}H-steroid metabolites

Initial identification of the ^{3}H-steroid metabolites was based on comparison of their mobility on TLC relative to the solvent front (Rf) with that of unlabeled steroid standards. Confirmation of steroid identity was then attempted wherever possible by acetylation alongside the appropriate non-radioactive standard. For the free steroids, each region of a chromatogram containing the ^{3}H-steroid under investigation was cut out and eluted with chloroform:ethyl acetate 3 : 1, vol/vol (5 mL). Non-radioactive standards were then added (50 μg) before drying down the eluates under N_2. These dried samples were redissolved in 200 μL pyridine and acetylated by the addition of an equal volume of acetic anhydride followed by incubation at 37°C for 2 h. The reaction was stopped by the addition of distilled water (1 mL) and the products extracted three times with ethyl acetate (1 mL). The acetylated products were then separated by TLC in solvent system C and the positions of ^{3}H-labelled and unlabelled derivatives visualised by phosphorimaging and exposure to iodine vapour, respectively, as described above.

Sulphated steroids were desulphated by incubation with arylsulphatase prior to acetylation, as follows. Following their separation on TLC in solvent system E, samples were eluted from the plate in ammoniated ethanol (2 × 4 mL), evaporated under N_2 and redissolved in 5 mL of 0.5 M sodium acetate buffer (pH 4.6) to which was added 5 mg of the arylsulphatase enzyme followed by incubation for 16 h at 40°C then 3 h at 55°C. The desulphated free steroids were then recovered by loading the incubation mixture onto an Oasis Hydrophobic-Lipophilic Balance cartridge and washing with a further 5 mL of sodium acetate buffer (0.5 M, pH 4.6) before elution in 4 mL ethyl acetate. This desulphated steroid fraction was dried under N_2 and stored at −20°C in ethanol prior to acetylation as above.

The identity of steroid metabolites which could not be acetylated was resolved by running them together with a carrier in at least two different solvent mixtures. Identity was considered confirmed if both the radioactive and carrier steroid migrated again to the same positions on TLC in other solvent mixtures.

Analysis of results

Half-times for the loss of radioactivity from brain following injection of the ^{3}H-labelled steroids were estimated from linear regression of semi-log plots of tissue radioactivity against time after injection. Comparisons between the half-time for loss of radioactivity after injection of ^{3}H-DHEAS and that of free steroid ^{3}H-DHEA were done using the Student's *t*-test.

Phosphoimages from TLC plates were analysed using ImageQuant software (version 5.2 Molecular Dynamics) with corresponding background activity subtracted from the final values. A value of 5000 dpm was established as the minimum amount of radioactivity required for detection on the phosphorimager following TLC. This is given as % of substrate in Results.

Results

Metabolism of ^{3}H-steroids by chick brain *in vivo*

Following i.c. injection of ^{3}H-PREG, ^{3}H-PROG, ^{3}H-DHEA or ^{3}H-DHEAS directly to the IMM, there was a rapid loss of radioactivity from the brain to barely detectable levels within 5 h (Fig. 1a). Half-times for disappearance of brain radioactivity after injection of labelled steroids into the IMM were as follows: ^{3}H-PREG 14.5 ± 2.0, ^{3}H-PROG 15.6 ± 0.9, ^{3}H-DHEA 9.1 ± 0.3 and ^{3}H-DHEAS 18.9 ± 2.8 min (all values mean ± SEM). The half-time for loss of radioactivity after injection of ^{3}H-DHEAS was significantly longer ($p < 0.01$) than for the corresponding free steroid ^{3}H-DHEA. In plasma, only a small proportion of the injected radioactivity (< 5000 dpm/mL) could be detected (Fig. 1b) and insufficient for subsequent TLC, hence no metabolic analyses could be carried out on these samples.

Steroids were extracted from the brain homogenates of the chicks injected i.c. into the IMM with ^{3}H-PREG, ^{3}H-PROG, ^{3}H-DHEA or ^{3}H-DHEAS and then separated into free steroid and steroid sulphate fractions. Table 1 shows the percentage of the total label in the brain homogenate which was recovered into each of these fractions. Following injection of ^{3}H-PREG, ^{3}H-PROG or ^{3}H-DHEA into the IMM, radioactivity could be recovered in the brain free steroid fraction for up to 10 min but there was no detectable sulphation of PREG or DHEA. By contrast, radioactivity persisted in the steroid sulphate fraction for up to 1 h following i.c. injection of ^{3}H-DHEAS and there was also evidence of desulphation of this label giving rise to radioactivity in the free steroid fraction. By 5 h post-injection, there was no detectable radioactivity in any fraction.

Composition of the radioactivity present in the above free and sulphated steroid fractions following injection of the ^{3}H-steroids to the IMM was investigated by TLC. Phosphorimaging of the TLC plates enabled visualisation of

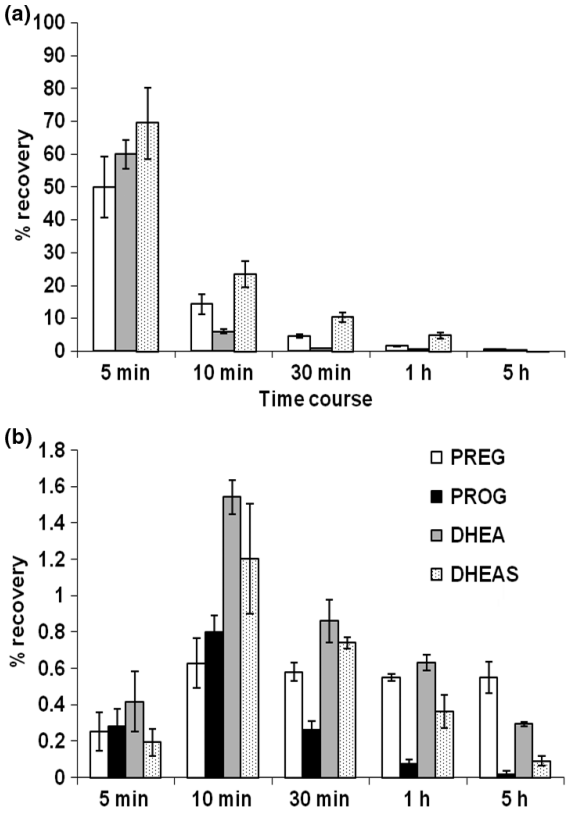

Fig. 1 Total radioactivity recovered in (a) brain homogenates and (b) plasma at different times following the intracranial injection of ^{3}H-pregnenolone (PREG; 0.043 μCi), ^{3}H-progesterone (PROG; 0.077 μCi), ^{3}H-dehydroepiandrosterone (DHEA; 0.080 μCi) or ^{3}H-dehydroepiandrosterone sulphate (DHEAS; 0.075 μCi) to the intermediate medial mesopallium. Results are shown as % of the injected dose (mean ± SEM; n = 4–5) and the numbers of male and female chicks used given in Table 1.

^{3}H-labelled material at a detection limit equivalent to the following percentages of injected label for each steroid: ^{3}H-PREG, 5.2%; ^{3}H-PROG, 2.9%; ^{3}H-DHEA, 2.8%; ^{3}H-DHEAS, 3.0%. The distribution of this radioactivity is presented below for each of these labels.

After i.c. injection of ^{3}H-PREG, most of the label had been metabolised to a compound which ran to the same position (Rf 0.39 ± 0.01) as standard 20β-dihydropregnenolone (20β-DHPREG; Fig. 2a and b). This identification was confirmed by eluting the radioactivity from the product peaks on the chromatograms for acetylation together with standard 20β-DHPREG. Acetylated products were separated by TLC in solvent system C, which yielded the following Rf values (n = 4): 0.68 ± 0.01 for the radioactive metabolite and 0.69 ± 0.01 for the carrier 20β-DHPREG.

Following i.c. injection of ^{3}H-PROG, clearance of the label was rapid and on TLC of the free steroid fraction in solvent system A, significant quantities could only be detected up to the 5 min time interval. As illustrated in Fig. 2(c), the distribution of the radioactivity along the TLC plate showed most of the ^{3}H-PROG to have been metabolised during this period. Comparisons with Rf values for non-radioactive steroid standards previously separated in solvent system A, suggested formation of 5β-dihydroprogesterone (5β-DHPROG; Rf 0.59 ± 0.01, mean ± SEM, n = 4). As 5β-DHPROG could not be acetylated, confirmation that this was the metabolic product formed from ^{3}H-PROG after i.c. injection was carried out by running further TLC of free steroid fractions from these brains alongside radioinert reference steroids in two solvent systems, A and C. The Rf values for the radioactive metabolite and for 5β-DHPROG respectively were 0.58 ± 0.01 and 0.63 ± 0.01 in solvent system A and 0.40 ± 0.01 and 0.42 ± 0.01 in solvent system C (all values mean ± SEM, n = 4).

After i.c. injection of ^{3}H-DHEA, radioactivity could only be detected on TLC of the free steroid fraction for up to 5 min. Figure 2(d) shows there was no evidence for metabolism of the label during this period *in vivo*.

In contrast to the free steroid labels and as already shown in Fig. 1 and Table 1, radioactivity persisted in the brain for up to 1 h following the i.c. injection of ^{3}H-DHEAS. This was reflected on TLC in solvent system E of the steroid sulphate fractions from these brains, when label could be detected for up to 30 min after injection (see Fig. 3). However, there was no evidence for metabolism within the steroid sulphate fraction during this period. Indeed, elution of the ^{3}H-DHEAS peak from these TLC plates followed by desulphation and re-chromatography in system A gave Rf values of 0.40 ± 0.01 for the 5 min and 0.40 ± 0.01 for the 10 min interval (both mean ± SEM, n = 5) which corresponded to that of free DHEA. Positive confirmation for DHEA was obtained by eluting the label from these peaks for acetylation together with standard DHEA and further TLC in solvent system C, which yielded the Rf values for the radioactive product of 0.57 ± 0.01 at 5 min and of 0.57 ± 0.01 at 10 min post-injection (both mean ± SEM, n = 5). The corresponding value for carrier acetylated DHEA was: 0.57 ± 0.01 (mean ± SEM, n = 6).

As shown in Table 1, radioactivity could also be detected in the free steroid fraction for up to 1 h after i.c. injection of ^{3}H-DHEAS. On TLC in solvent system A, this radioactivity gave an Rf value 0.40 ± 0.01 (mean ± SEM, n = 4) for both the 5 min and the 10 min time intervals, suggesting the formation of DHEA. Confirmation of the identity of this material was attempted by elution and acetylation together with standard DHEA but because of the small amounts of radioactivity present, some samples were pooled before TLC in solvent system C. The Rf values obtained were 0.55 ± 0.01 for the acetylated ^{3}H-metabolite and 0.55 ± 0.01 for acetylDHEA (both values mean ± SEM, n = 3).

LOW RESOLUTION FIG

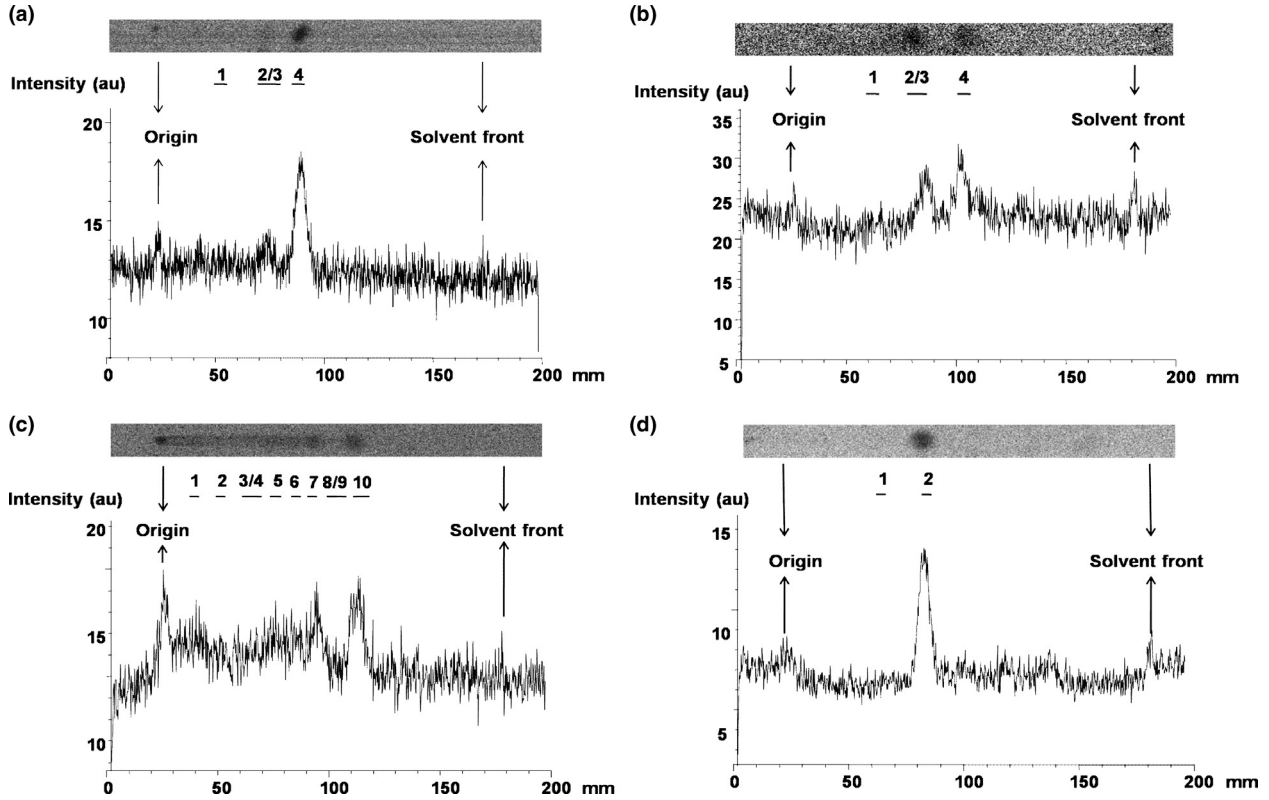

Fig. 2 Typical chromatographic profiles and phosphorimages from TLC of free steroid fractions extracted from brains after intracranial injection of ^{3}H-pregnenolone (PREG), ^{3}H-progesterone (PROG) or ^{3}H-dehydroepiandrosterone (DHEA) to the intermediate medial mesopallium. Traces (a) 5 min and (b) 10 min after injection of ^{3}H-PREG, (c) 5 min after injection of ^{3}H-PROG and (d) 5 min after injection of ^{3}H-DHEA. The positions of unlabelled reference steroids are indicated by horizontal bars and numbered as follows. In (a) and (b): (1) 20α-dihydropregnenolone, (2) 17α-hydroxypregnenolone, (3) 20β-dihydropregnenolone and (4) PREG. In (c): (1) Corticosterone, (2) 11-deoxycorticosterone, (3) 20β-dihydroprogesterone, (4) 17α-hydroxyprogesterone, (5) 20α-dihydroprogesterone, (6) 3α,5β-tetrahydroprogesterone, (7) PROG, (8) 3α,5α-dihydroprogesterone, (9) 5α-dihydroprogesterone, and (10) 5β-dihydroprogesterone. In (d): (1) androstenedione and (2) DHEA. All TLC was in solvent system A.

LOW RESOLUTION FIG

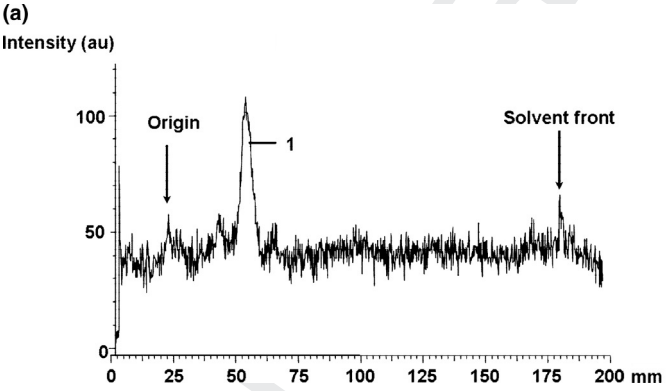

Fig. 3 Typical chromatographic profile (a) from TLC of steroid sulphate fraction extracted from chick brain at 5 min after an intracranial injection of ^{3}H-dehydroepiandrosterone sulphate (DHEAS) to the intermediate medial mesopallium and (b) phosphorimage of TLC for steroid sulphate fractions at: (i) 5 min, (ii) 10 min, (iii) 30 min, (iv) 1 h and (v) 5 h after injection of this label. The peak for DHEAS is labelled as 1 and all TLC was in solvent system E.

Metabolism of ^{3}H-steroids by chick brain homogenates *in vitro*

Whole chick brain homogenates and subcellular fractions were incubated with the substrates ^{3}H-PREG, ^{3}H-DHEA or ^{3}H-PROG for 1–3 h at 37°C in the presence of either NAD^{+} or NADPH as a cofactor. Products were identified by TLC as described below (detection limits for each substrate and percentage conversion by different subcellular fractions are summarised in Table S1). These investigations did not include ^{3}H-DHEAS as a substrate because our pilot studies revealed no detectable desulphation of this label by chick brain homogenates *in vitro* (< 33 fmol/mgprotein/h; Sujkovic *et al.* 2007).

Incubation of ^{3}H-PREG with NAD^{+} was carried out to investigate the possible conversion of this steroid to PROG. However, the radioactivity recovered from such incubations (following separation on TLC in solvent system B) indicated that most of this remained as the unmetabolised substrate (Fig. S1a). By contrast and as can be seen from Fig. 4(a), incubation of ^{3}H-PREG with NADPH followed by separation of the products on TLC (in solvent system A), revealed a single product with an Rf value of 0.32 ± 0.01 (n = 4). Comparisons with Rf values for non-radioactive steroid standards previously run in solvent system A suggested the metabolic product was either 20β-DHPREG or 17α-hydroxypregnenolone (17α-OHPREG). Optimal separation of these two steroids was then achieved using solvent system C to give Rf values for standard 17α-OHPREG and 20β-DHPREG of 0.35 ± 0.01 (n = 3) and 0.54 ± 0.01 (n = 3), respectively. Using the same solvent system C, the ^{3}H-metabolic product was found to migrate to a similar position (Rf 0.54 ± 0.01, n = 4) to that of 20β-DHPREG. Identification of 20β-DHPREG as the product of incubations of ^{3}H-PREG plus NADPH was confirmed by acetylation; the mean Rf value ± SEM for the ^{3}H-acetylated product was 0.72 ± 0.01 (n = 4) and that of the acetylated 20β-DHPREG carrier steroid 0.72 ± 0.01 (n = 4). The activity producing this metabolite was enriched in the cytosolic fractions and not detected in either boiled fractions or the buffer blank (Table S1 and Fig. S1b).

No evidence of enzyme activity could be detected when ^{3}H-DHEA was incubated with chick brain homogenate and subcellular fractions in the presence of NADPH for 3 h at 37°C followed by separation on TLC in solvent system D (Fig. S2b). However, under the same assay conditions but using NAD^{+} as a co-factor, a single product with Rf value of 0.29 ± 0.01 (n = 4) could be detected following separation in solvent system A, indicative of androstenedione (ADIONE) formation (Fig. 4b). The enzyme activity producing this metabolite was found to be enriched within the nuclear fraction (see Table S1). A product of similar Rf value to that obtained for the putative ADIONE appeared in the boiled tissue controls. However, the % conversion from ^{3}H-DHEA substrate in nuclear fractions (11.7 ± 3.8%) was more than twice that seen in their respective boiled controls (3.7 ± 1.1%; both values mean ± SEM, n = 4) confirming enzyme activity.

Androstenedione would not be expected to be acetylated by acetic anhydride. Nevertheless for comparison with other ^{3}H-labelled enzyme products in the present study, the above ^{3}H-metabolite produced from ^{3}H-DHEA by the chick brain nuclear fractions in the presence of NAD^{+} was treated with acetic anhydride together with carrier standard ADIONE. Separation of the reaction products by TLC (in solvent system C) yielded Rf values of 0.21 ± 0.01 (n = 3) for the ^{3}H-metabolite and 0.21 ± 0.01 (n = 3) for the carrier

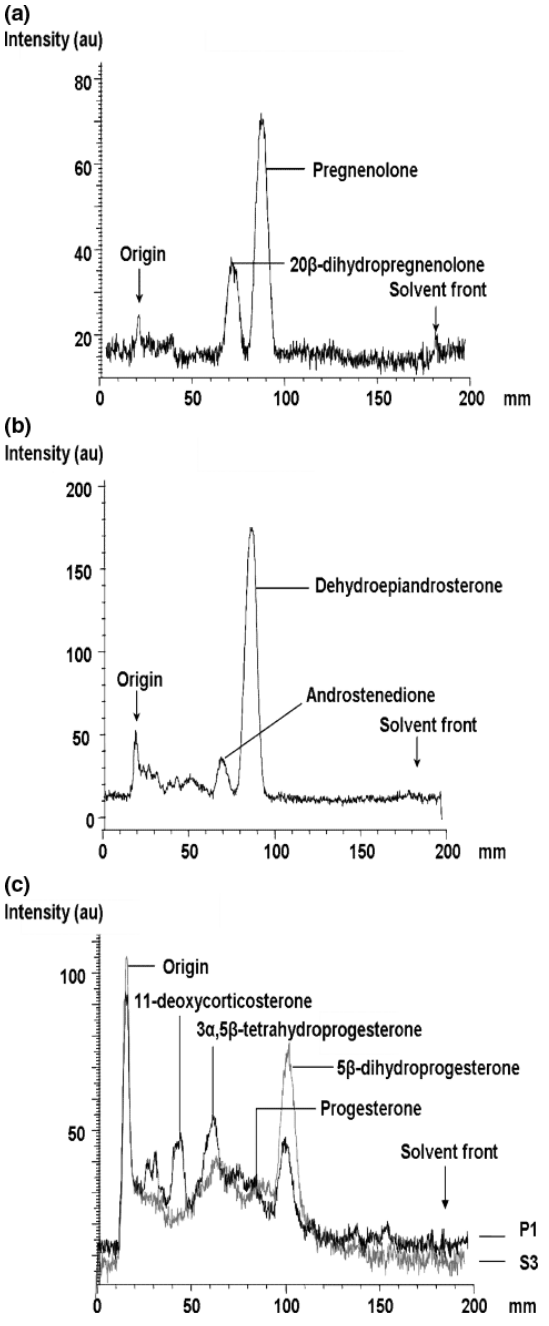

Fig. 4 Typical TLC profiles illustrating the metabolism of ^{3}H-steroids (all at 20 nM) by male chick brain homogenate fractions *in vitro*: (a) formation of 20β-dihydropregnenolone from pregnenolone by the cytosolic (S3) fraction in the presence of NADPH (1 mM; 3 h, 37°C), (b) formation of androstenedione from dehydroepiandrosterone by the nuclear (P1) fraction in the presence of NAD^{+} (1 mM; 3 h, 37°C) and (c) formation of 11-deoxycorticosterone, 3α,5β-tetrahydroprogesterone and 5β-dihydroprogesterone from progesterone by the nuclear or cytosolic fractions in the presence of NADPH (1 mM; 1 h, 37°C). All TLC was in solvent system A and comparable results obtained from other brains, both male and female. Results from all homogenate fractions are summarised in Table S1 and the TLC illustrated under Supporting Information.

ADIONE. As expected, both the above values were close to the Rf value (0.20 ± 0.01; $n = 3$) for ADIONE run in the same solvent system without prior treatment with acetic anhydride.

When chick brain homogenate and subcellular fractions were incubated with ^{3}H-PROG and NADPH as a cofactor, most of the substrate was metabolised within 1 h of incubation at 37°C. Separation by TLC in solvent system A revealed three metabolites with the following Rf values: 0.18 ± 0.01, 0.35 ± 0.01 and 0.62 ± 0.01 (all values mean ± SEM, $n = 4$) which, in comparison with the migration of non-radioactive standards, suggested the formation of 11-deoxycorticosterone (DOC), 3α,5β-tetrahydroprogesterone (3α,5β-THPROG) and 5β-DHPROG, respectively (Fig. 4c). Boiled controls contained mainly unmetabolised ^{3}H-PROG (Fig. S3). Several breakdown products were also visible on TLC, however, their positions did not coincide with those of any of the metabolic products present in the unboiled samples. Comparison of the enzyme activity present in the different subcellular fractions indicated slight enrichment of putative 5β-DHPROG production within the cytosol and of DOC and 3α,5β-THPROG production in the nuclear fraction (Table S1).

Acetylation confirmed formation of 3α,5β-THPROG in the nuclear fraction, as the migration of the ^{3}H-metabolite on subsequent TLC in solvent system C coincided (Rf 0.61 ± 0.01; $n = 4$) with that of the carrier standard steroid converted to acetyl-3α,5β-THPROG (0.62 ± 0.01; $n = 5$). Identification of the putative DOC could not be confirmed in the present study, as the acetylation process yielded several breakdown products of the radioactive metabolite, concealing its position on TLC (not shown). Owing to its chemical structure, 5β-DHPROG could not be identified by acetylation. Instead, the regions of the chromatograms from cytosolic fractions corresponding with standard 5β-DHPROG were eluted and run again by TLC alongside reference steroids in the two solvent systems A and C. In these two systems, Rf values were as follows for the ^{3}H-metabolite and standard 5β-DHPROG, respectively: system A, 0.59 ± 0.01 and 0.63 ± 0.01, system C, 0.38 ± 0.01 and 0.38 ± 0.01 (all values mean ± SEM, $n = 4$).

Discussion

The present study has employed ^{3}H-labelled PREG, PROG, DHEA and DHEAS to investigate pathways of metabolism for these steroids in day-old chick brain, both after i.c. injection to the IMM *in vivo* and in whole brain fractions *in vitro*. Our results can be discussed not only in relation to studies of brain steroid metabolism in other species but also with regard to the known effects of these steroids on learning and memory in the chick.

Chick brain steroid metabolism *in vivo*

Following injection of the free steroids ^{3}H-PREG, PROG, DHEA into the IMM, a rapid clearance of the radioactive label from the brain was observed, to undetectable levels within 30 min. The ^{3}H-label from DHEAS persisted in brain for longer than that from the corresponding free steroid DHEA and could still be detected for up to 1 h post-injection. For all ^{3}H-steroids, only a small amount of radioactivity was detectable in plasma and this peaked at 10 min following i.c. injection. We were unable to determine whether these steroids left the brain in their original injected form or subsequent to prior metabolism because there was insufficient ^{3}H-label in the plasma samples for the analysis of chemical identity. Nevertheless, sufficient ^{3}H-label persisted in brain for us to extract and separate the material into free steroid and steroid sulphate fractions for the identification of possible metabolites.

Although the ^{3}H-label could be detected in brain for up to 1 h after injection of ^{3}H-DHEAS into the IMM, analysis of the sulphated steroid fraction of brain extracts by TLC showed no evidence of ^{3}H-DHEAS metabolism in the esterified state. This is different to the results of a previous study (Kishimoto and Hoshi 1972) following i.c. injection of ^{3}H-DHEAS in adult rats, when the majority of the label was found to be metabolised to ~~ADIOL~~ sulphate without prior desulphation. However and consistent with this previous study, a small proportion of the label was recovered in the free steroid fraction and identified as DHEA, suggesting activity of the steroid sulphatase enzyme. In chick (Migues *et al.* 2002) as well as mouse and rat brain (Corpéchot *et al.* 1981; Ebner *et al.* 2006; Nicolas and Fry 2007), concentrations of the ester DHEAS are higher than its corresponding free steroid. Taken together with the lack of evidence for CYP17 activity in chick brain (see below), our present findings suggest that DHEA in this tissue may originate from its sulphate ester.

For both ^{3}H-PREG and ^{3}H-DHEA, there was no evidence of sulphation following i.c. injection into the IMM, although we cannot exclude that this may have taken place at time points later than 10 min, beyond which the label was below detection limits. Sulphation of PREG has previously been detected at a low level in rat brain *in vitro* (Rajkowski *et al.* 1997). The hydroxysteroid sulphotransferase (HST) enzyme catalysing this sulphation showed highest activity during foetal life and declined at birth. Whether a similar pattern of developmentally regulated HST activity occurs in the chick brain remains to be investigated. However, the low affinity and activity of the rat brain HST is consistent with the lack of detectable product within 10 min of injection of substrates to chick brain *in vivo*. In addition to the lack of sulphation, we could also detect no further metabolism of ^{3}H-DHEA within the free steroid fraction. By contrast, ^{3}H-PREG showed rapid metabolism to 20β-DHPREG and to no other product. The clearance of label from the brain after i.c. injection of ^{3}H-PROG was also rapid and most of the radioactivity

recovered during the 5-min period post-injection, with 5β-DHPROG being the major metabolite.

What implications do the above results have for the likely modes of action of PREG, DHEA and DHEAS, which are known to enhance learning and memory in the chick and a variety of other species? Administration of PREG has been found to facilitate memory formation in day-old chicks following direct injection into the IMM (Migues 2001) and in mice after intracerebroventricular administration (Flood *et al.* 1992). The present observations raise the possibility that the effect of PREG at least in the chick may not be mediated by PREG itself, but rather via its major and rapidly produced metabolite 20β-DHPREG. Possible neural sites of action for this steroid or its metabolites remain to be elucidated. Nevertheless, the present results allow us to exclude the possibility that PREG is acting after conversion to either PREGS or PROG. The latter has been shown here to be converted to 3α,5β-THPROG, which is known to potentiate the actions of GABA at the $GABA_A$ receptor (Lambert *et al.* 2001) and which would therefore be expected to be amnestic rather than memory enhancing in the IMM (Clements and Bourne 1996). For DHEA, memory enhancing properties are well documented for several learning paradigms in rodents (reviewed in Vallée *et al.* 2001; Wolf and Kirschbaum 1999). In the day-old chick, direct injection of DHEA into the IMM enhances memory formation when given 5–15 min pre- or 30- to 60-min post-training but not at 180-min post-training (Johnston and Migues 2001; Migues *et al.* 2002). Taken together with present evidence for the clearance of DHEA without further metabolism from the brain within 10 min of injection into the IMM, these results suggest rapid and direct effects of this steroid on the early events of memory formation. The lack of ^{3}H-DHEAS conversion to other steroid sulphates following injection to the IMM suggests that the memory enhancing effects of this steroid in the day-old chick (Migues *et al.* 2002; Sujkovic *et al.* 2007) might also be a direct action of this steroid. However, DHEAS persisted in brain for longer than DHEA following i.c. injection and a small proportion of the sulphate ester was found to be hydrolysed to free DHEA throughout the different time points in this study. Previous investigations (Migues *et al.* 2002) have shown DHEAS to be more potent than DHEA at enhancing memory following injection into the IMM of day-old chicks and the present observations introduce the possibility that this might be because of the slower clearance of DHEAS than DHEA from the brain rather than differences in potency at receptor sites.

Chick brain steroid metabolism *in vitro*

When chick brain homogenates and subcellular fractions were incubated with ^{3}H-PREG, DHEA or PROG in the presence of either NADPH or NAD^+, the following metabolites were detected: 20β-DHPREG from PREG (with NADPH); ADIONE from DHEA (with NAD^+); 3α,5β-THPROG, 5β-DHPROG and DOC from PROG (with NADPH). These results can now be discussed in relation to our observations following i.c. injection of the ^{3}H-steroid labels to the IMM and with reference to possible pathways of steroid metabolism in chick brain (see Fig. S4).

Consistent with the results following i.c. injection of ^{3}H-PREG, incubation of chick brain fractions with this label plus NADPH revealed no detectable formation of 17α-OHPREG and/or DHEA indicative of CYP17 activity. Although CYP17 mRNA and protein can be detected in fetal and neonatal rodent brain (see Mensah-Nyagan *et al.* 1999; Compagnone and Mellon 2000), there is no evidence for the actual production of DHEA from PREG in these tissues. Evidence for such enzyme activity has been provided for adult quail brain, where CYP17 mRNA expression is accompanied by the ability of brain slices to convert a small proportion of ^{3}H-PROG to ^{3}H-ADIONE (Matsunaga *et al.* 2001). Transcripts of CYP17 can also be detected in adult chicken brain (Nomura *et al.* 1998), although at a lower level than other steroidogenic enzymes. As mentioned above, our present observations suggest that DHEA is more likely to arise in day-old chick brain through the desulphation of DHEAS rather than CYP17 activity and are consistent with the emerging view (see Soma 2006) that circulating DHEAS provides a major source of androgen in avian brain. Both DHEAS and DHEA have been found in the plasma of day-old chicks (Migues *et al.* 2002).

As for DHEA itself, incubation of this ^{3}H-labelled steroid with chick brain fractions in the presence of NAD^+ indicated the formation of ADIONE, especially in the nuclear fraction. The subcellular distribution of this activity was unexpected as the 3β-HSD enzyme which would catalyse this conversion is generally thought to reside in mitochondria and the endoplasmic reticulum. Nevertheless, endogenous nuclear 3β-HSD activity has been described previously in guinea pig adrenal cortex, albeit with PREG as the substrate (Demura *et al.* 1990). The conversion of DHEA to ADIONE has also been reported in the brains of zebra finches (Vanson *et al.* 1996) and this conversion shown to be the first step in the formation of active androgens and estrogens in avian brain (see Schlinger *et al.* 2008). Some production of ADIONE from DHEA has been detected in foetal rat forebrain astrocytes (Akwa *et al.* 1993) but this activity does not appear to be significant in adult mouse (Young *et al.* 1994) or rat (Ebner *et al.* 2006) brain. The present study has identified further differences in DHEA(S) metabolism between chick and rodent brain in that the former reveals no 7α/β-hydroxylation of DHEA, as seen in rat and mouse brain microsomes (Akwa *et al.* 1992; Doostzadeh *et al.* 1997), or 17β-reduction of DHEA and DHEAS to androstenediol and its sulphate, respectively, as seen in rat brain both *in vivo* (Kishimoto and Hoshi 1972) and *in vitro* (Kishimoto 1973).

Our results following incubation of ^{3}H-PREG with chick brain fractions in the presence of NAD^+ were also consistent

with those seen after i.c. injection of this label in that there was no detectable production of PROG. This suggests a lack of 3β-HSD activity and conflicts with the above evidence for the formation of ADIONE from DHEA in chick brain. Studies of 3β-HSD isoforms in mammalian tissues (see Labrie *et al.*1992) have revealed no such differences in substrate specificity between PREG and DHEA. However, a chicken gene has been identified (Nakabayashi *et al.* 1995) and shown to be expressed in both adult and embryonic brain (Nomura *et al.* 1998, 1999) for a form of 3β-HSD which displays more than twice the affinity for DHEA over PREG. In contrast to our present observations in the chick, production of PROG from PREG has been observed in embryonic, early post-hatch and adult quail brain homogenates and was significantly greater in the presence of excess non-labelled PROG, because of a high level of PROG metabolism in these brains (Ukena *et al.* 2001). This cannot be the explanation for the lack of 3β-HSD activity on PREG in chick brain as there was no evidence from TLC of the ^{3}H-labelled PREG metabolites for the formation of either PROG or its metabolites. Consistent with the experiments employing ^{3}H-DHEA as substrate, we could also detect no 7α-hydroxylation of PREG, as reported to occur in quail brain (Tsutsui *et al.* 2006). Indeed, the present study has shown 20β-DHPREG to be the major metabolic product of PREG in the brain of day-old chick, suggesting activity of a 20-hydroxysteroid dehydrogenase (20-HSD) enzyme. Expression of this enzyme has been reported previously for chicken brain (Bryndová *et al.* 2006) and the present study showed such activity to be located predominantly in the cytosol, as described previously for neonatal pig testes (Ohno *et al.* 1992). The enzyme product 20β-DHPREG can be found in adult rat brain (Ebner *et al.* 2006). However, we cannot be clear as to the function of such 20β-HSD activity in the brain. In mammalian gonads, 20α/β- reduction is thought to divert C21 steroids from androgen synthesis (see Ohno *et al.* 1992; Bryndová *et al.* 2006), but this may not be significant in day-old chick brain where there is no evidence of CYP17 activity (see above).

Our experiments identified 5β-DHPROG as the major metabolite of ^{3}H-PROG, both *in vivo* and *in vitro*. Activity of the 5β-reductase enzyme responsible for its synthesis has been detected in the nervous system of dove (Hutchison and Steimer 1981), chicken (Massa and Sharp 1981), quail (Balthazart and Schumacher 1984) and zebra finch (Balthazart *et al.* 1986) using testosterone as a precursor. The conversion of PROG to 5β-DHPROG has also been established in quail brain (Ukena *et al.* 2001) and the present results are consistent in showing 5β-reduction of PROG in avian brain, rather than the 5α-reduction which predominates in adult mammalian brain (see Compagnone and Mellon 2000; Ebner *et al.* 2006). The present study also showed that 5β-DHPROG could be further reduced to 3α,5β-THPROG in chick brain. The presence and activity of 3α-HSD enzymes which would catalyse this conversion have been demonstrated in rodent and human brain (see Mensah-Nyagan *et al.* 1999; Compagnone and Mellon 2000) and this activity is also displayed by the 20-HSD chicken enzyme discussed above (Bryndová *et al.* 2006).

In conclusion, the present study has identified pathways of steroid metabolism in the brain of the day-old chick and suggested ways in which these might influence the actions of steroids on learning and memory. With further resolution of the regional and subcellular localisation of such metabolic pathways, investigations will be facilitated into the mechanisms by which steroids influence memory formation and other aspects of development in the brain of the chick.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Distribution of activity producing identified metabolites from the substrates 3Hpregnenolone (PREG), 3H-progesterone (PROG) or 3H-dehydroepiandrosterone (DHEA) in whole homogenates and subcellular fractions of chick brain.

Figure S1 Typical phosphorimages of TLC plates obtained following incubation (3 h, 37°C) of 3H-pregnenolone (20 nM) with whole chick brain homogenate (P0) or nuclear (P1), supernatant 1 (S1), mitochondrial (P2), supernatant 2 (S2), microsomal (P3) and cytosol (S3) fractions, in the presence of either (a). NAD+ or (b). NADPH (both at 1 mM).

Figure S2 Typical phosphorimages of TLC plates obtained following incubation (3 h, 37°C) of 3H-dehydroepiandrosterone (20 nM) with whole chick brain homogenate (P0) or nuclear (P1), supernatant 1 (S1), mitochondrial (P2), supernatant 2 (S2), microsomal (P3) and cytosol (S3) fractions, in the presence of either (a). NAD+ or (b). NADPH (both at 1 mM).

Figure S3 Typical phosphorimage of a TLC plate obtained following incubation (1 h, 37°C) of 3H-progesterone (20 nM) with whole chick brain homogenate (P0) or nuclear (P1), supernatant 1 (S1), mitochondrial (P2), supernatant 2 (S2), microsomal (P3) and cytosol (S3) fractions, in the presence of NADPH (1 mM).

Figure S4 Identified pathways of pregnenolone, progesterone, dehydroepiandrosterone and dehydroepiandrosterone sulphate metabolism in day-old chick brain *in vitro* and/or *in vivo*

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USING E-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

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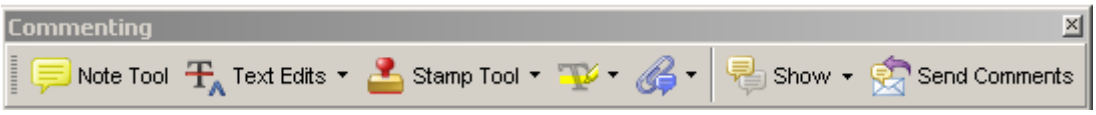
Adobe Acrobat Professional or Acrobat Reader (version 7.0 or above) is required to e-annotate PDFs. Acrobat 8 Reader is a free download: http://www.adobe.com/products/acrobat/readstep2.html

Once you have Acrobat Reader 8 on your PC and open the proof, you will see the Commenting Toolbar (if it does not appear automatically go to Tools>Commenting>Commenting Toolbar). The Commenting Toolbar looks like this:



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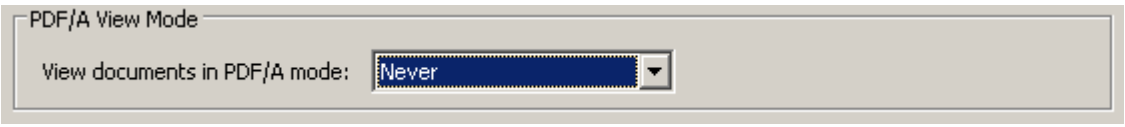
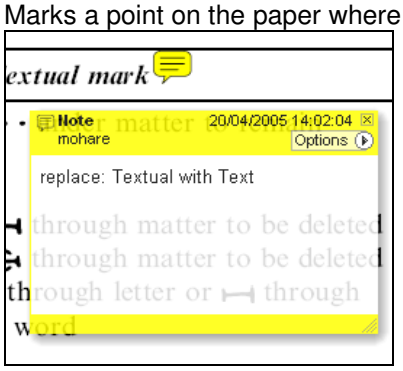


Note Tool — For making notes at specific points in the text

Marks a point on the paper where a note or question needs to be addressed.

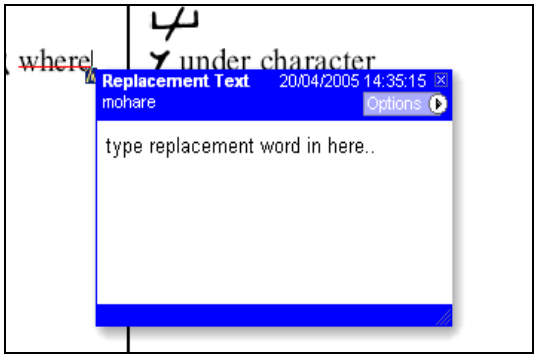


How to use it:

1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
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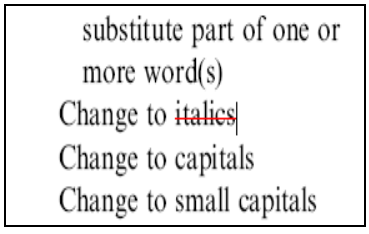
Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

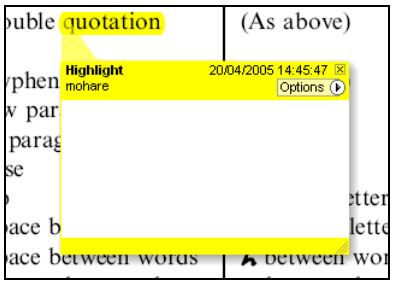
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the 'standard business' selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

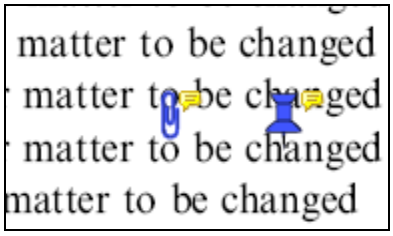


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.



How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
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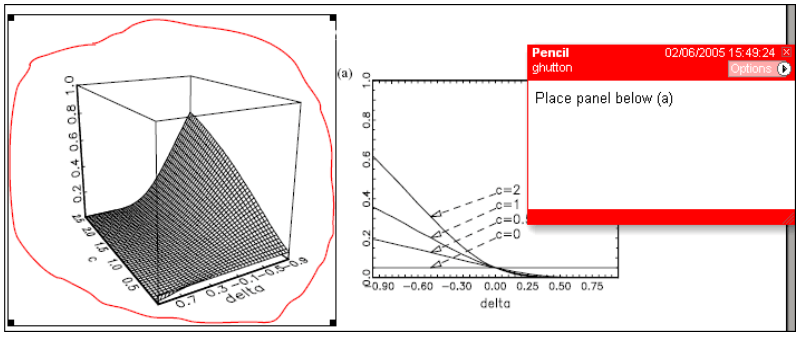
Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

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