

The steroid sulfatase inhibitor COUMATE attenuates rather than enhances access of dehydroepiandrosterone sulfate to the brain in the mouse

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ABSTRACT

Intraperitoneal injection of adult male mice with the neuroactive steroid dehydroepiandrosterone sulfate (DHEAS) at 1 and 40 mg/kg caused dose-dependent increases in the concentration of both this compound and its corresponding free steroid DHEA in brain within 1 h of injection.

Pretreatment of these animals for 24 h with the steroid sulfatase inhibitor COUMATE at a dose (10 mg/kg, p.o.) shown previously to cause almost complete inhibition of this enzyme in liver and brain was expected to increase the amount of the DHEAS dose reaching the brain.

Surprisingly however, the increases in brain concentrations of DHEAS and DHEA after injection of DHEAS i.p. were attenuated by pretreatment with COUMATE. The results suggest that the arylsulfamate based steroid sulfatase inhibitors such as COUMATE interfere with the influx of the DHEAS anion into the brain.

Section: Regulatory Systems

Keywords: COUMATE; Dehydroepiandrosterone; DHEAS; Mouse; Neurosteroid; Steroid sulfatase

Abbreviations:

DHEA, dehydroepiandrosterone

DHEAS, dehydroepiandrosterone sulfate

GABA, γ -aminobutyric acid

NMDA, N-methyl-D-aspartate

STS, steroid sulfatase

1. Introduction

Since the characterization of dehydroepiandrosterone sulfate (DHEAS) as a brain steroid (Corpéchet et al. 1981) there has been detailed investigation of the effects of this compound on neural function (see Gibbs et al. 2006). Of particular interest in view of the decline in plasma DHEAS of adrenal origin with human ageing is the ability of this steroid to improve retention of memory after subcutaneous or intracerebral injection in mice (Flood et al. 1988). Several studies have since shown DHEAS administered either systemically or directly into the brain to enhance the acquisition and consolidation of memory and reverse pharmacologically induced amnesia in a variety of species (see Wolf and Kirschbaum 1999). Possible neural sites of action for the above effects of DHEAS include antagonism of GABA_A receptors (Majewska et al., 1990) and stimulation of sigma receptors, the latter underlying an enhancement of NMDA receptor function (Monnet et al., 1995; see also Maurice et al., 2006). Additional modes of action are likely for DHEAS and its corresponding free steroid dehydroepiandrosterone (DHEA), in particular to explain the antigluco-corticoid and neuroprotective effects of these compounds (see Wolf and Kirschbaum 1999).

In addition to enhancement of memory, other behavioral effects of DHEAS in mice include anxiolysis on the elevated plus maze (Melchior and Ritzmann 1994) and increased attack behavior against a conspecific male (Nicolas et al., 2001). The latter is correlated with the expression of liver steroid sulfatase (STS; Le Roy et al., 1999), an enzyme which is also present in brain (Mortaud et al., 1996) and which catalyses the hydrolysis of 3 β -hydroxysteroid sulfates such as DHEAS to liberate the free steroid DHEA. Several irreversible inhibitors of this enzyme have become available in recent years, all of which feature an arylsulfamate moiety (see

Nussbaumer and Billich 2004). Although developed primarily as potential therapeutic agents for the treatment of estrogen- and androgen-dependent disorders of peripheral tissues, these STS inhibitors would be expected to increase the potency of steroid sulfates, at least for their direct actions at the receptor sites in the brain described above. Indeed, a previous study by one of us (Nicolas et al., 2001) showed that prior treatment of mice with the STS inhibitor COUMATE (7-O-sulfamoyl)-4-methylcoumarin) decreased the dose of intraperitoneally administered DHEAS needed to elicit aggressive behavior. At the time, this observation was interpreted as evidence for inhibition of STS allowing more of the circulating DHEAS to cross the blood-brain barrier. We now report that the opposite is the case and that pretreatment with the STS inhibitor COUMATE actually decreases the access of DHEAS to the brain in the mouse.

2. Results

As can be seen from Fig. 1, treatment with COUMATE had no significant effect on the concentrations of endogenous DHEAS or DHEA in the mouse brain after 24 h. The intraperitoneal administration of DHEAS to these mice caused a dose-dependent increase in the concentration of both this compound and its desulfated free steroid DHEA in the brain within 1 h of injection. However, such increases in the brain concentrations of DHEAS and DHEA were lower in animals which had been pre-treated with the STS inhibitor COUMATE. The ratios of DHEAS to DHEA were higher ($P < 0.05$) in mice injected with the 40 mg/kg compared to the 1 mg/kg dose of DHEAS, but these ratios were not altered significantly by COUMATE pretreatment (with methyl cellulose pretreatment DHEAS: DHEA 1.95 ± 0.60 after 1 mg/kg and 9.55 ± 1.50 after 40 mg/kg DHEAS; with COUMATE pretreatment DHEAS: DHEA 1.03 ± 0.12 after 1 mg/kg and 21.23 ± 8.62 after 40 mg/kg DHEAS).

3. Discussion

The endogenous concentrations of both DHEA and DHEAS were close to previously reported values for mouse (Young et al., 1996; Le Goascogne et al., 2000; Tagawa et al., 2006) and rat (Corpéchet et al. 1981; Mathur et al., 1993; Ebner et al., 2006) brain. No other steroids have been reported in these tissues at concentrations likely to cross-react with the antibodies used in the present assays. The same may not be true after dosing with exogenous DHEAS. However, the major metabolites in rat and monkey brain after systemic administration of DHEAS appear to be DHEA and androstenediol (Knapstein et al., 1968; Kishimoto and Hoshi 1972) which, in view of the complete separation of free steroids and steroid sulfates prior to assay in the present study, would not cause interference with the DHEAS measurements reported here. Indeed, the surprising observation of the present study is that inhibition of STS with COUMATE results in reduced concentrations of DHEAS in the brain after the systemic administration of this steroid.

To the best of our knowledge, the only other study to investigate concentrations of DHEAS in brain after treatment with STS inhibitors has employed chronic dosing of rats (Johnson et al., 1997). Here, daily doses of the STS inhibitor (p-O-sulfamoyl)-N-tetradecanoyl tyramine (DU-14) over 15 days caused a 77.6% increase in whole brain DHEAS. In the present experiments, there was no significant change in endogenous DHEAS or DHEA 24 h after a single dose of COUMATE. However, a low level of 3β -hydroxysteroid sulfotransferase activity has been reported in brain tissue (Rajkowski et al., 1997) and accumulation of DHEAS formed from endogenous DHEA might be expected after chronic inhibition of STS. The dose of COUMATE used in the present experiments had been shown previously to inhibit STS activity in liver and

brain by 87% and 71%, respectively, by the time at which mice were treated with DHEAS (Nicolas et al. 2001). The increased sensitivity of these COUMATE-treated mice to the aggression enhancing effects of DHEAS now looks unlikely to be due simply to higher amounts of this sulfated steroid entering and/or accumulating in the brain, unless there are localized changes which have been missed in the present experiments. Interestingly, bell-shaped dose–response curves for intraperitoneally administered DHEAS have been observed both for the above aggression enhancing effects in mice (Nicolas et al. 2001) and for memory enhancing effects in rats (Li et al., 1997), with and without STS inhibition. Thus the rate as well as the absolute amount of DHEAS entering the brain could be a factor determining the effects of this steroid on behavior. Alternatively, the possibility cannot be excluded that DHEAS is enhancing aggression in mice by acting outside of the brain. Two likely such sites of action are the olfactory and vomeronasal sensory epithelia, which lie outside of the blood-brain barrier and which are known to be essential for attack behavior between male mice (Stowers et al., 2002; Mandiyan et al., 2005).

As expected, the present results show reduced formation of DHEA from DHEAS after STS inhibition with COUMATE. Little is known of the impact of COUMATE on other steroids although a recent clinical trial of the tricyclic coumarin sulfamate 667 COUMATE in women with breast cancer reported no change in endogenous circulating DHEAS or estrone sulfate but significant reductions in serum concentrations of DHEA, androstenedione, androstenediol, testosterone, estradiol and estrone (Stanway et al., 2006). As mentioned above, the major metabolites found in rat and monkey brain after systemic administration of DHEAS appear to be DHEA and androstenediol. Although both androgens, the latter has estrogenic properties (Poortman et al., 1975) and COUMATE itself is devoid of such activity (Purohit et al., 1996).

From the above evidence, we can suggest that pretreatment of mice with COUMATE is likely to have led to reduced stimulation of both androgen and estrogen receptors in the brain following dosing with DHEAS. The present results also indicate the COUMATE pretreatment would have led to reduced antagonism of GABA_A receptors and stimulation of sigma receptors in the brain (see Introduction) after systemic injection of DHEAS. Again, we are led to speculation that a possible site of action for DHEAS in enhancing aggression of these COUMATE-treated mice lies outside of the blood-brain barrier.

How might the STS inhibitor COUMATE reduce entry of systemic DHEAS into the brain? As a charged conjugate, DHEAS is thought to be less permeable through the blood-brain barrier than its corresponding free steroid DHEA and experiments in monkeys (Knapstein et al., 1968) and rats (Kishimoto and Hoshi 1972) have shown only a small proportion of circulating DHEAS to enter the brain. This entry is likely to be through an anion transporter. Such transporters have been found in the blood-brain barrier, although investigated for their role in the efflux rather than the influx of DHEAS (Asaba et al., 2000; Lee et al., 2005). The possibility arises that anion transporters can also facilitate the influx of DHEAS into the brain and are inhibited by COUMATE and other aryl-sulfamate based STS inhibitors. This would limit access of DHEAS to the brain, especially under conditions of raised circulating concentrations. Interestingly, in view of our speculation above that DHEAS might be enhancing aggression in mice through acting peripherally to influence olfaction, organic anion transporters have recently been identified in both the main olfactory and vomeronasal epithelial layers and shown to transport a steroid sulfate (Kaler et al., 2006). Moreover, one of the adverse events reported from the clinical trial of 667 COUMATE (Stanway et al., 2006) was taste disturbance.

To conclude, the present observations indicate that STS inhibitors such as COUMATE cannot always be assumed to increase the concentrations of DHEAS in the brain. This caution is likely to be even more apposite in those animals such as primates with higher levels of circulating DHEAS of adrenal origin and has implications for the neurological health of patients treated with STS inhibitors for steroid-dependent tumours and other disorders.

4. Experimental Procedures

Treatments of male mice with COUMATE and DHEAS were chosen according to the previous study of aggressive behavior (Nicolas et al, 2001) and using the same CBA/H colony. Animals were kept in a 12:12 photoperiod (lights on at 8am) at $23.5 \pm 0.5^{\circ}\text{C}$ with food and water available ad libitum. Each male was individually housed with a female, generally a littermate and experiments took place at 12-16 weeks of age. Animal care followed C.N.R.S. protocols and guidelines. The STS inhibitor COUMATE (10 mg/kg) or its vehicle of 0.5% w/v methylcellulose in 0.9% NaCl was administered p.o. (5 ml/kg) 24 h before mice were killed for brain samples. Then, 1 h before they were killed, the animals were injected with DHEAS (1 or 40 mg/kg, i.p., 10 ml/kg) or the water vehicle alone. Mice were killed by cervical dislocation and decapitation. Their brains (including olfactory bulbs and cerebellum) were rapidly removed and frozen in liquid nitrogen before storage at -80°C . The COUMATE was synthesized by Gérald Guillaumet (I.C.O.A, Orléans, France) according to Purohit et al. (1996) and DHEAS purchased as the sodium salt from Sigma® Chemical Co (St Louis, MO, USA).

Brain steroid extraction and fractionation was essentially as described by Ebner et al. (2006).

Each brain was homogenized in 5 volumes (w/v) of ice-cold potassium phosphate buffer (5 mM,

pH 7) with a Polytron® blender then added dropwise to 20 volumes of acetic acid (3%, v/v) in 96% ethanol in an ultra-sonicating bath. The extract was deproteinised by centrifugation (28000×g, 30 min) and delipidated by partitioning three times against 10 volumes of isooctane followed by passage (in 60% ethanol) through a 60 mg reverse phase Oasis hydrophilic-lipophilic balance HLB® cartridge. Extracts were then dried down under vacuum, redissolved in 20% ethanol and loaded onto 60 mg reverse phase Oasis mixed-mode anion exchange MAX® cartridges for separation of free and sulfated steroids. After a wash with 5 ml of 20% ethanol in ammonium acetate (20 mM, pH 7), the free steroids were eluted with 4 ml ethyl acetate. Passage of 20% ethanol in formate/pyridine (20 mM, pH 3) removed any steroid glucuronides present and the steroid sulfates could then be eluted with 15 ml of 1% w/v ammonium carbonate in 60% ethanol. Silanised glassware was used throughout and all solvents redistilled from AR grade (VWR International, Leicestershire, UK). The Oasis HLB® and MAX® cartridges were purchased from Waters Corp., Milford, USA. One brain from each of the six different treatments was included in every batch of samples extracted and fractioned for steroid radioimmunoassay and each batch also included normal mouse brain homogenates to which 2 pmol of either DHEAS or DHEA had been added to check their recoveries through the above procedure.

Free and sulfated steroid fractions were dried down under nitrogen at 60°C and redissolved in phosphate buffered saline containing 3% w/v bovine serum albumin and 0.01% thimerosal w/v for radioimmunoassay. All assay components were dissolved in the same buffer to a total volume of 0.25 ml. The measurement of DHEA in the free steroid fraction employed ³H-DHEA at 0.25 nM and anti-DHEA antibodies at a dilution of 1:12500. In order to assay DHEAS in the steroid sulfate fraction, we employed ³H-DHEAS at 0.25 nM and anti-DHEAS antibodies at 1:14300. After incubation for at least 2 h at room temperature, both sets of immunoassays were terminated

by the addition of 25 µl of a heat-killed, formalin-fixed 1% v/v suspension of *Staphylococcus aureus* cells (Pansorbin®, Calbiochem, USA), followed by 3 ml of ice-cold PBS and rapid filtration through glass-fibre filters (GF/C, Whatman, UK) under vacuum. Filters were then placed in 3 ml of scintillation fluid (Ecoscint H, National Diagnostics, UK) for the measurement of antibody bound ³H-label. All measurements were made in triplicate and a range of unlabelled standard concentrations included in each assay for the construction of calibration curves. Both antibodies were purchased from ICN Biomedicals Inc. USA and supplied with the following % cross reactions for anti-DHEA and anti-DHEAS, respectively: DHEA 100.00 and 58.50, DHEAS 1.18 and 100.00, androstenedione 0.32 and 39.70, androsterone 0.02 and 30.52, pregnenolone 0.02 and <0.01, estrone <0.01 and 0.67, testosterone <0.01 and 0.61. In addition, the following compounds were given as <0.01% cross-reaction with either antibody: aldosterone, cholesterol, corticosterone, cortisol, desoxycorticosterone, 17β-estradiol, estriol, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 5α-dihydrotestosterone. The label employed was [1,2,6,7-³H(N)]-DHEA (PerkinElmer, UK; 3422.5 GBq/mmol). For use in the assays of DHEAS, this label was sulfated with triethylamine sulfur trioxide (Dusza et al. 1968), cleaned by solvent extraction (Corsan et al. 1997) and then purified by celite chromatography in the solvent system (v/v) isooctane (20); tert-butanol (40): water (39): ammonia (1). Thin layer chromatography of the final product on silica gel 60 coated plates in ethyl acetate/ethanol/ammonia (25/10/2 v/v) revealed a purity of greater than 99%. Student's t-test was used to evaluate the significance of differences in brain DHEAS and DHEA concentrations between mice pretreated with COUMATE and those given the vehicle alone.

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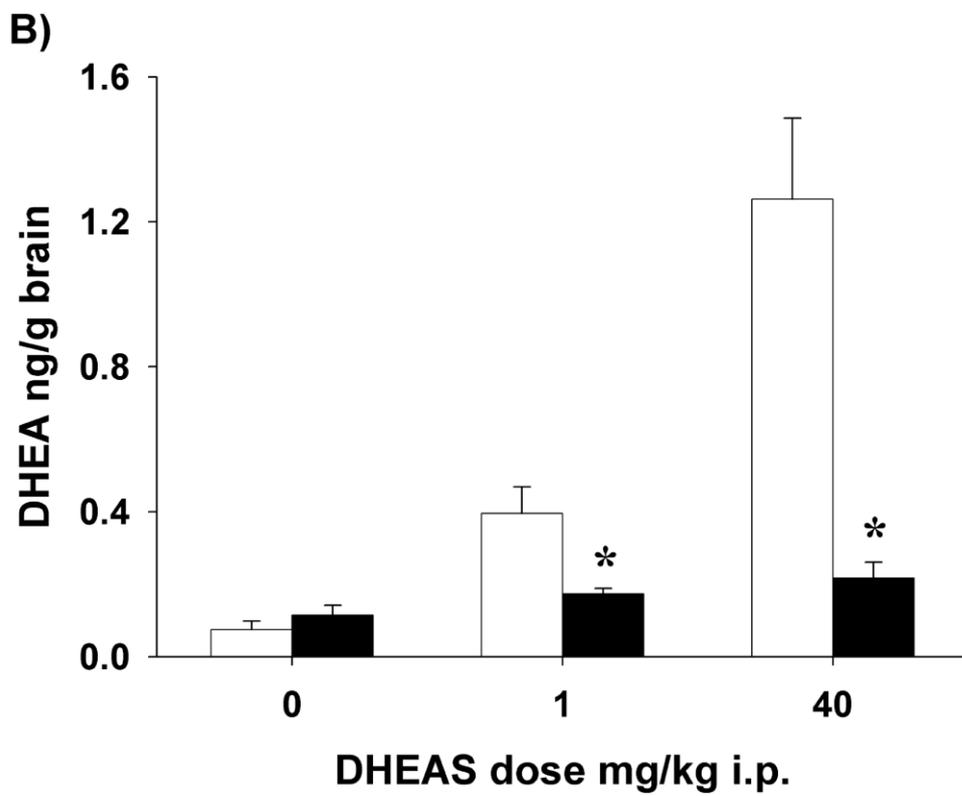
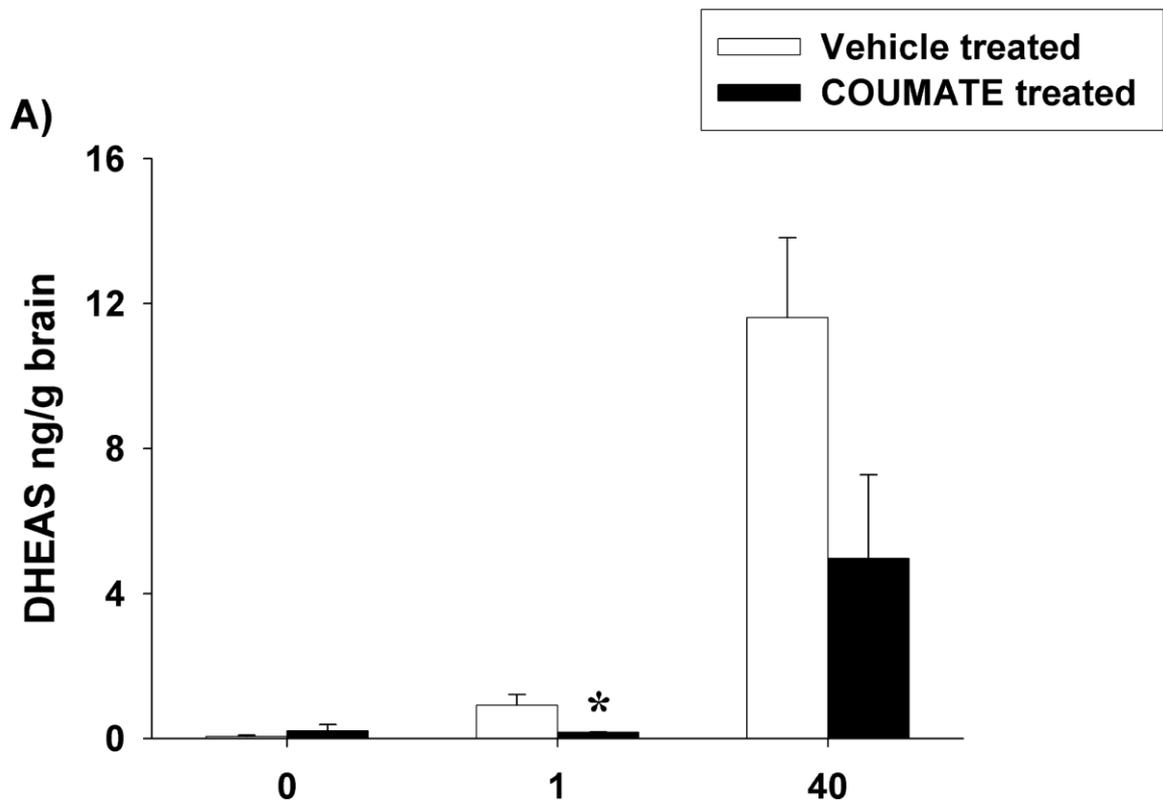


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Fig. 1 Effects of steroid sulfatase inhibition with COUMATE (10 mg/kg, p.o., 24 h) on the concentrations of (A) DHEAS and (B) DHEA in adult male mouse brain following intraperitoneal injection of DHEAS (1 or 40 mg/kg, 1 h). Animals not pre-treated with COUMATE were instead given a control injection of the vehicle alone (0.5% w/v methylcellulose in 0.9% NaCl) and the vehicle for DHEAS was distilled water. All values shown as mean \pm SEM (n = 5 mice) and uncorrected for recoveries in the extraction and fractionation of steroids from brain homogenates, which were as follows: DHEAS $30.2 \pm 4.6\%$; DHEA $107.1 \pm 7.1\%$ (both values mean \pm SEM; n = 10). * $P < 0.05$ for significance of difference between mice pre-treated with COUMATE or the methylcellulose vehicle alone then injected with the same dose of DHEAS.