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EFFECTS OF STEROIDS ON β-ADRENERGIC BINDING SITES IN SHEEP PINEAL GLANDS

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Abstract—As an initial step in investigations of putative differences between central nervous system light-sensitive mechanisms in seasonally shedding and non-shedding breeds of sheep, some β-adrenoceptor characteristics of Merino sheep pineal glands were determined, using [^{3}H]dihydroalprenolol as the labelled ligand. Overall, a dissociation constant of 17.2 ± 2.6 nmoles/l and a daytime β-receptor density of 1.6 ± 0.3 pmoles/mg were determined at 37°. The binding sites exhibited stereospecificity, saturability and apparent homogeneity. 17β-Estradiol and progesterone implants that provided hormone concentrations in the physiological range had no significant effect on pineal β-receptors in male sheep castrated shortly after birth. Dexamethasone injections, on the other hand, in doses sufficient to loosen the attachment of wool fibres to the skin, resulted in decreased pineal β-receptor density and increased receptor affinity for dihydroalprenolol. This effect was apparently not mediated by altered plasma catecholamine concentrations, since the glucocorticoid treatment did not affect jugular venous noradrenaline, adrenaline or dopamine levels. The possible involvement of glucocorticoids in the regulation of wool growth could thus have a central neuronal component, mediated via action on pineal β-adrenoceptors in sheep; however, the existence of the putative gonadal steroid feedback on β-adrenoceptor-mediated pineal function remains to be demonstrated in this species.

The pineal gland and its enzymes, in particular serotonin-*N*-acetyltransferase (EC 2.3.1.5), are thought to be involved in regulation of circadian [1–3] and seasonal [1, 4] phenomena. Rhythms of physiological activity affected by either pinealectomy or melatonin administration include pelage changes in several species [5–8]. In recent studies, it was reported that the pineal gland as well as the anterior pituitary and the thyroid influence wool and horn growth in Soay sheep [9]. Further, pinealectomy was shown to change the timing of the annual moult in Wiltshire sheep [10].

Rhythms in pineal activity in response to light are regulated in part by neurotransmitter release from sympathetic fibres arising from the superior cervical ganglion [11], mediated by changes in pinealocyte β-adrenoceptor parameters [12–14]. Pineal function may also be regulated in part by gonadal and glucocorticoid steroid hormones. Gonadal steroids may regulate the pineal β-adrenoceptors as part of a putative negative feedback mechanism or may regulate pineal hydroxyindole-*O*-methyl transferase activity directly [15]. Glucocorticoids have also been shown to regulate β-adrenoceptor density and some β-receptor-mediated parameters in tissues other than the pineal [16–18].

Regulation of wool growth and shedding in breeds of sheep that undergo seasonal moulting (e.g. Soay and Wiltshire Horn) is an unresolved neurochemical problem of practical importance. The predominant stimulus which triggers regression of wool follicles in the Wiltshire Horn is believed to be changing day length [19]. Since the Merino does not respond in an identical manner to the same stimulus, the breeds probably differ in some aspects of their central nervous light sensing mechanisms. This paper examines some properties of pineal β-adrenoceptors in Merino sheep as a preliminary to comparative studies with other breeds.

Glucocorticoids inhibit wool growth in Merinos [20, 21] and cause changes in the morphology of the follicles [22] which resemble changes seen in naturally moulting Wiltshire Horn sheep (R. Chapman, personal communication). The second part of this study, therefore, deals with the effects of a small range of gonadal and glucocorticoid steroids on Merino pineal β-adrenoceptors.

MATERIALS AND METHODS

Animals

Merino sheep (females and castrated males, 1 to 48 months of age) were housed either indoors in pens, or outdoors under field conditions, subjected in both cases to natural lighting and temperature fluctuations (mean temperature range over year 4.7 to 28.4°). Sheep housed indoors were fed on a diet of 600 g of lucerne/oats chaff per day and water *ad lib*. The sheep were killed by cervical dislocation and exsanguination between 8:45 a.m. and 10:30 a.m. in all cases. Pineal glands were rapidly removed and placed into 25 vol. (w/v) of ice-cold Tris–Mg^{2+} buffer (pH 7.4) [23].

Binding studies

Preparation. Individual sheep pineals were hom-

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ogenised in Tris–Mg^{2+} buffer (pH 7.4) (Ultraturrax homogeniser, 10 sec, 0°), and aliquots of the homogenate were placed directly into 2.0 ml capped microfuge incubation tubes. In some experiments, a portion of the homogenate was centrifuged (20,000 *g*, 4 min, 4°), and the membrane pellet after washing was resuspended in the original volume of Tris–Mg^{2+} buffer.

Incubation. To 20–50 μl of the crude homogenate or of the washed membrane fraction at 0° was added, in order, Tris–Mg^{2+} buffer (pH 7.4), 200–230 μl; propranolol (5×10^{-4} moles/l) or water, 30 μl; and other drugs as appropriate. The incubation, in a final volume of 300 μl for 10 min (25° or 37°), was commenced by the addition of [^{3}H]dihydroalprenolol (DHA: 15 μl containing a maximum of 0.15 μCi of tritium label). Final concentration of [^{3}H]DHA in the incubation fluid was in the range of 1–32 nmoles/l.

Separation of free and bound ligand. At the conclusion of the incubation, the incubation tubes were rapidly placed in ice prior to centrifugation (20,000 *g*, 4°). The supernatant fraction was removed by aspiration, and the pellet was rapidly washed in ice-cold Tris–Mg^{2+} buffer (500 μl). The supernatant fraction was again aspirated. The entire washing procedure was completed within 5–10 sec. The pellet was dissolved in Soluene-350 tissue solubiliser at 60° prior to determination of the bound radioactivity by liquid scintillation spectrophotometry. The counting efficiency for the solubilised samples was determined to be approximately 50% by spiking with [^{3}H]hexadecane. Preliminary experiments indicated a negligible (positive) change in the extent of specific binding of [^{3}H]DHA with increased time of standing in ice from 1 to 70 min prior to centrifugation.

Metabolism of the radioligand. Simultaneous thin-layer chromatography of [^{3}H]DHA standard and of the label recovered in the supernatant fraction following incubation of pineal homogenates with [^{3}H]DHA at 25° or 37° (silica gel plates; butanol–acetic acid–water, 25:4:10, by vol.) showed identical R_f values for both incubated and non-incubated radiolabelled species, with no significant contaminant peaks. This indicates that no appreciable metabolism of the radioligand occurred during the incubation period.

Tissue linearity. Specific binding increased linearly with increasing amount of homogenate incubated, up to at least 300 μg of tissue protein: the binding curve had a linear regression coefficient of 1.00.

Steroid treatment

Male Merino sheep castrated at an early age received subcutaneous implants containing 17β-estradiol or progesterone by means of a small (1–2 cm) incision in the inside skin of one foreleg.

The 17β-estradiol implants consisted of four 5-mm segments of silastic tubing (2.41 mm o.d., 1.57 mm i.d.) filled with the steroid. The total amount of steroid implanted was 35.0 ± 1.5 mg in each of seven sheep, total release rate being 19 ± 3 μg/24 hr. This rate is greater than the *in vivo* release rates of 1.6 to 5.0 μg/24 hr in ewes [24]. The estradiol implants gave rise to jugular venous plasma steroid levels in the range 4.4 to 7.3 pg/ml (N = 7) which, while in the physiological range, was not significantly above the limit of detection of the radioimmunoassay used. In untreated control sheep, the range was 2.5 to 7.1 pg/ml (N = 7).

Progesterone ("Sil-estrus", 375 mg) was similarly implanted in another group of seven castrated male sheep, resulting in jugular venous plasma progesterone levels of 2.95 ± 0.17 ng/ml 14 days post-implantation. Control progesterone levels at this time were 0.62 ± 0.05 ng/ml.

Treated and paired untreated control animals were killed 14 days after implantation, and their pineals were assayed as above. Jugular venous blood samples for plasma catecholamine and steroid determinations were taken immediately prior to killing the animals.

Dexamethasone (Dex, 400 mg), or the vehicle only, was injected intramuscularly into the hind legs of castrated Merino sheep as a single dose. Dex powder (0.9 g) was suspended in a gelatin gel [20% (w/w), 18 ml] at 30° and dispersed uniformly by repeated passage through an 18 gauge needle. The suspension was cooled to the gel point, and extruded through the needle into a stirred solution of glutaraldehyde [25% (w/v), 8 ml] in phosphate buffer (0.1 mole/l, pH 7.5, 400 ml) at 5°. After stirring for 60 min, the crosslinked gel was collected by filtration, washed with distilled water, suspended in saline (30 ml), and homogenised to facilitate injection of the required steroid dose.

Jugular venous sampling for plasma catecholamine and Dex assays was done at 1, 3 and 5 hr post-treatment on the day of injection and at 24 hourly intervals thereafter until sacrifice at 8 days.

Steroid assays

All three steroids were determined by radioimmunoassay. For Dex, a modification of the method of Farmer and Pierce [25] was used, the limit of sensitivity being 100 pg/ml. For the gonadal steroids, the limit of sensitivity was approximately 5 pg/ml (17β-estradiol) and 100 pg/ml (progesterone).

Catecholamine assay

Noradrenaline, adrenaline and dopamine were individually determined in each plasma sample by the radioenzymic assay of Da Prada and Zurcher [26].

Depilatory force measurement

Depilatory force, a measure of the strength of attachment to the skin of a staple of wool of known thickness, was measured at daily intervals in Merino sheep following Dex treatment, according to the method of Gordon [27] with minor modifications. Results were expressed in Newton/kiloTex instead of g/kiloTex* (1 Newton ≈ 120 g force), and the apparatus for measuring linear density was calibrated with rovings which had been conditioned at 20° and 65% relative humidity.

Protein estimation

Protein was routinely assayed by the method of

* The kiloTex is a unit of wool staple linear density; 1 kiloTex = area staple cross section (mm^2) × wool specific gravity (1.3).

Lowry *et al.* [28] using bovine serum albumin as standard.

Statistical methods

Lines of best fit for catecholamine standard curves and for binding analysis results were fitted to data points by linear regression analysis. Significance of results was determined using Student's paired or unpaired *t*-test, as appropriate.

Materials

DHA ([^{3}H]dihydroalprenolol) (approximately 47.4 Ci/mmole) and [^{3}H]*S*-adenosyl methionine (12.9 Ci/mmole) were purchased from New England Nuclear (Searle Nucleonics), Australia, and their radiochemical purities were monitored by thin-layer chromatography. Propranolol (*d, l* and *dl* isomers) was a gift from I.C.I. Australia Operations Pty. Ltd., Melbourne. 17β-Estradiol was obtained from the Sigma Chemical Co., St. Louis. MO, U.S.A., progesterone (SIL-ESTRUS) implants from Abbott Laboratories, N.S.W., Australia, and dexamethasone from Roussel-UCLAF, Paris, France. Soluene-350 tissue solubiliser was purchased from the Packard Instrument Co., Downer's Grove, IL, U.S.A. Other reagents were of commercially available analytical grade.

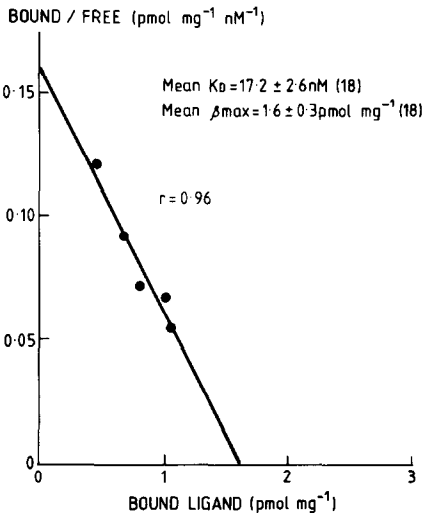

Fig. 1. Scatchard plot for Merino pineal homogenate β-adrenoceptors. An example of a Scatchard plot (bound/free vs bound ligand) is shown for [^{3}H]DHA binding to pineal β-adrenoceptors at 37°. The intercept on the abscissa yields the maximum β-receptor density (β_{max}), while the negative reciprocal of the gradient yields the dissociation constant (K_D). The means and standard errors of eighteen determinations of these two parameters are also shown.

RESULTS

Partial characterization of Merino pineal β-adrenoceptors

Specific binding of [^{3}H]DHA to β-adrenoceptors in pineal homogenates (and washed membrane fractions) was shown to be saturable, whereas total binding was linear over the range of DHA concentrations tested. In all of these studies, total binding was 5–9% of added counts. The specific binding of DHA to the receptors reached maximum values within 1 min of incubation, both at 25° and at 37°, indicating a very rapid $T_{\frac{1}{2}}$ for association.

The DHA-binding sites exhibited stereospecificity, 50% inhibition of [^{3}H]DHA binding being achieved at approximately 5×10^{-7} moles/l *l*-propranolol and at 5×10^{-5} moles/l *d*-propranolol.

The data from radioligand ([^{3}H]DHA) saturation studies were analysed by means of Scatchard plots [29]. An example of a Scatchard plot for Merino pineal β-receptors is shown in Fig. 1. From similar plots, the apparent dissociation constant (K_D) for the ligand–receptor complex and the maximum density of β-receptor sites (β_{max}) were determined. Figure 1 also shows the mean K_D and β_{max} values and standard errors of these means obtained at 37° for β-adrenoceptors in pineal homogenates from Merinos killed at approximately 10:30 a.m. under natural daylight conditions.

For all experiments in the presence or absence of steroids, Hill plots yielded straight lines with gradients (Hill coefficients) not significantly different from unity, indicating an absence of co-operative effects and demonstrating the presence, under these experimental conditions, of a homogenous population of β-receptors in the pineal gland.

Preliminary displacement experiments indicate that catecholamines displace specific [^{3}H] binding from pineal binding sites with the order of potency

Table 1. Effects of altered assay conditions on pineal β-receptor parameters

Assay conditions	N	K_D* (nmoles/l)	β_{max}† (pmoles/mg)
Whole homogenate, 25°	14	13.2 ± 1.1	3.5 ± 0.3
Whole homogenate, 37°	18	17.2 ± 2.6	1.6 ± 0.3‡
Washed membrane fraction, 25°	6	5.8 ± 0.8‡	4.4 ± 0.5

* *KD* = dissociation constant.
† β_{max} = β-receptor density.
‡ $P \leq 0.01$, cf. whole homogenate at 25°, unpaired *t*-test.

Table 2. Effect of gonadal steroid implants on Merino pineal β-adrenoceptors and jugular venous catecholamine levels*

Treatment	β-Receptor parameters K_D (nM)	β_{max} (pmoles/mg)	Plasma catecholamines (pmoles/ml) NA	A	DA
Controls (7)	14.6 ± 3.8	0.9 ± 0.1	8.3 ± 1.8	2.1 ± 0.7	0.5 ± 0.4
17β-Estradiol (7)	9.5 ± 2.3	1.0 ± 0.2	3.8 ± 1.1†	1.1 ± 0.5‡	0.7 ± 0.6
Progesterone (7)	12.6 ± 3.3	1.4 ± 0.3	2.7 ± 0.9†	1.2 ± 0.4†	1.0 ± 0.8

* Abbreviations: K_D = dissociation constant; β_{max} = β-receptor density; NA = noradrenaline; A = adrenaline; and DA = dopamine. The numbers in parentheses equal the number of sheep.
† $P < 0.02$, cf. untreated controls; Student's paired *t*-test.
‡ $P < 0.001$, cf. untreated controls; Student's paired *t*-test.

isoprenaline > adrenaline > noradrenaline, indicating that the pineal binding sites may correspond to physiological β_2-adrenoceptors [30].

The effect of incubation temperature on specific DHA binding was also investigated (Table 1). At 25° the dissociation constant of the DHA from the receptors was not significantly different from that seen at 37° ($P < 0.4$, $N = 32$, unpaired *t*-test); however, the apparent β_{max} was higher than that seen at 37° ($P < 0.01$, $N = 32$, unpaired *t*-test).

β-Receptor binding parameters were also determined using washed pineal membranes assayed at 25°. When compared to whole homogenates assayed under the same conditions (Table 1), there was a significant increase in affinity ($P = 0.01$), while the non-significant ($P < 0.3$) trend towards increased receptor density on a protein weight basis may reflect the removal of non-receptor protein during the wash procedure.

For convenience, in the following studies with steroids, whole pineal homogenates and incubation temperatures of 37° were routinely used. The higher incubation temperature is in keeping with physiological conditions, mean deep body temperature in sheep being in excess of 38°.

Effects of steroids on pineal β-adrenoceptors in vivo

Gonadal steroids. Pineal β-receptor parameters in 17β-estradiol- or progesterone-implanted castrated male Merino sheep 14 days post-implantation and in untreated paired controls are shown in Table 2. Neither steroid alone caused a significant change in β-receptor density or in binding affinity. Interpretation of some apparent trends in these parameters is rendered difficult by a concurrent depression in jugular venous plasma noradrenaline and adrenaline levels (Table 2). The possible involvement of autoregulation of the β-receptors by the altered plasma catecholamine levels may obscure any direct effects of the steroids on the measured β-receptor parameters.

The effects of the two steroid implants in combination on pineal β-receptor parameters remain to be investigated in this species.

Dexamethasone. In marked contrast to the apparent lack of significant effects of the gonadal steroid implants on pineal β-receptor parameters, injection of the synthetic glucocorticoid Dex resulted in significant decreases in both K_D and β_{max} with time after injection (Table 3). Figure 2a shows the plasma levels of Dex up to 8 days post-treatment. Jugular venous noradrenaline levels were not affected by Dex treatment (Fig. 2b); similar curves (not shown) were also obtained for adrenaline and dopamine.

The Dex dosage used was sufficient to decrease depilatory force in these sheep from a mean pretreatment control value of 13.9 Newton/kiloTex (S.E.M. = 0.2, N = 6) to 9.4 Newton/kiloTex (S.E.M. = 0.9, N = 6, $P < 0.001$, paired *t*-test) 8 days after treatment. By contrast, vehicle-treated controls showed no such decrease (20.1 Newton/kiloTex, S.E.M. = 0.9, N = 6, cf. 19.3 Newton/kiloTex, S.E.M. = 1.3, N = 6, NS).

DISCUSSION

The current results elucidate some properties of [^{3}H]DHA binding sites in pineal glands harvested

Table 3. Time course of dexamethasone effects on Merino pineal β-adrenoceptors

Time post-injection (days)	Vehicle control K_D (nM)	β_{max} (pmoles/mg)	Dexamethasone (400 mg) K_D (nM)	β_{max} (pmoles/mg)
1	14.4 ± 5.1 (3)*	1.4 ± 0.5 (3)	7.4 ± 1.1 (3)	0.9 ± 0.2 (3)
2	14.2 ± 5.1 (3)	1.7 ± 0.4 (3)	5.6 ± 2.1 (3)	0.7 ± 0.3†(3)
8	10.0 ± 0.7 (5)	1.2 ± 0.1 (5)	6.6 ± 0.7†(5)	0.9 ± 0.2†(5)

* The numbers in parentheses equal the number of observations.
† $P < 0.05$, cf. vehicle control; Student's paired *t*-test.

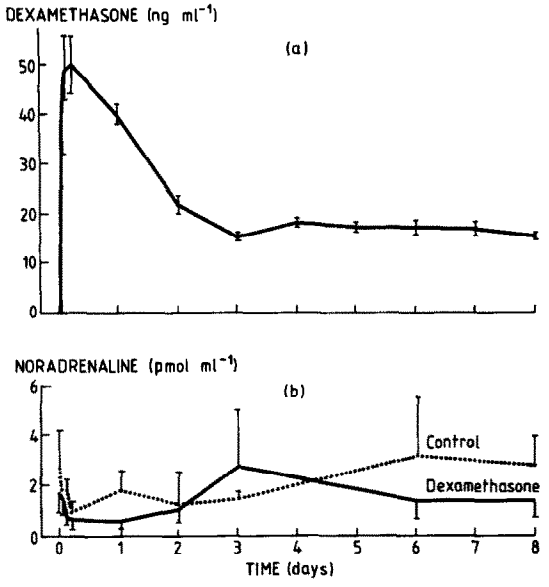

Fig. 2. (a) Jugular venous plasma concentration of dexamethasone up to 8 days post-dexamethasone treatment. Each point is the mean of five determinations, and the vertical bars represent the standard errors of these means. (b) Jugular venous plasma noradrenaline concentrations in vehicle (. . . .)- and dexamethasone (——)-treated sheep up to 8 days post-dexamethasone treatment. Plasma adrenaline and dopamine levels (not shown) similarly exhibited no significant differences between control and dexamethasone-treated sheep.

from Merino sheep during daytime under natural light conditions. The [^{3}H]DHA binding sites in this study may reasonably be considered to correspond to Merino pineal β-receptors in view of their saturability with increasing ligand concentration, stereospecificity or preferential displacement of labelled ligand by *l*-propranolol over that by the *d*-isomer, and the observed similar extents of displacement by a specific β-adrenoceptor antagonist (propranolol) and agonist (isoprenaline) (cf. Ref. 31). The rapid rate of association of the ligand to the receptor is also characteristic of neurotransmitters and their receptors.

The characteristics of these receptors have been determined, and a relatively low K_D (*ca.* 17 nmoles/l) corresponding to a high affinity of the receptors for [^{3}H]DHA has been observed at 37° in whole pineal homogenates. This relatively high affinity is in keeping with results obtained for pineals in other species [23]. The relatively higher receptor density is in good agreement with the observations of Romero *et al.* [14] in another species. The apparent decrease in β-receptor density with increased incubation temperature is atypical and may reflect increased protease action at the higher temperature.

The greater affinity of DHA to the receptors in washed pineal membranes compared to whole pineal homogenates may be indicative of the removal, during the purification step, of some endogenous cytoplasmic ligand(s); further experiments, currently in progress, support this possibility.

The results obtained for pineal β-receptor characteristics in sheep with gonadal steroid implants are inconclusive. Certainly the current results have failed to confirm in castrated male sheep the suggestions of Cardinali and Vacas [15] concerning a feedback regulation by gonadal steroids on the pineal glands. Possible reasons for this may include species differences or steroid concentration effects. For example, evidence for pineal regulation of hypothalamic releasing factors is strong in some species but absent in female sheep [32], indicating possible major differences between sheep and other species in central neuroendocrine mechanisms. In addition, in the case of 17β-estradiol, the implants resulted in a very low circulating level of steroid which, while in the physiological range, may not be optimal to elicit pineal β-receptor responses (cf. the biphasic dose-dependent effect of estradiol on other pineal parameters in rats [15]).

The results of *in vivo* dexamethasone treatment in the current study are of interest. In contrast to cultured human astrocytoma cells where dexamethasone has been reported to increase β-receptor density by a cyclohexamide-sensitive mechanism [18], the glucocorticoid decreased daytime β-receptor density in Merino pineals. Opposing effects of adrenal steroids on β-adrenoceptors have also been reported for other tissues; for example, adrenalectomy induces an increase in β_{max} in rat liver and a fall in rat lung [33]. The difference between these reports and the current study in sheep pineal does not appear to reside in the ability of the conscious animal to autoregulate the receptors via alterations in circulating plasma catecholamine levels, since no significant changes were noted in these levels between dexamethasone-treated and control animals, or in the same sheep before and after treatment.

The current results further indicate that the effect of the steroid on the β-receptors may be direct, since a general inhibitory effect of dexamethasone on protein synthesis does not explain the observed increased binding affinity in the pineal gland.

No relationship can be established as yet between the effects of this steroid on the pineal and on the strength of attachment of wool fibres. The similarities in time course of onset of both effects may reflect changes in a common underlying mechanism, such as DNA synthesis or replication, but the possibility of corticosteroids directly affecting wool growth in part by some pineal-related action cannot be excluded on the basis of the present results.

Further studies involving glucocorticoid effects on pineal receptors and other pineal parameters including the melatonin synthetic enzymes and adenyl cyclase will help to elucidate putative interactions between corticosteroids, the pineal, and wool growth in seasonally shedding and non-shedding sheep.

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