Effect of gonadal steroids and gamma-aminobutyric acid on LH release and dopamine expression and activity in the zona incerta in rats

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A dopaminergic system in the zona incerta stimulates LH release and may mediate the positive feedback effects of the gonadal steroids on LH release. In this study the mechanisms by which steroids might increase dopamine activity in the zona incerta were investigated. In addition, experiments were conducted to determine whether the inhibitory effects of gamma-aminobutyric acid (GABA) on LH release in the zona incerta are due to suppression of dopamine activity in this area or conversely whether the stimulatory effects of dopamine on LH release are due to suppression of a tonic inhibitory GABAergic system. Ovariectomized rats were treated s.c. with oil, 5 µg oestradiol benzoate or 5 µg oestradiol benzoate followed 48 h later by 0.5 mg progesterone, and killed 54 h after the oestradiol benzoate injection. At this time the LH concentrations were suppressed in the oestradiol benzoate group and increased in the group treated with oestradiol benzoate and progesterone. The ratio of tyrosine hydroxylase:β-actin mRNA in the zona incerta was significantly increased by the oestradiol benzoate treatment, but the addition of progesterone resulted in values similar to those in the control group. At the same time, the progesterone treatment increased tyrosine hydroxylase activity in the zona incerta as indicated by an increase in L-dihydroxyphenylalanine (L-DOPA) accumulation after 100 mg 3-hydroxybenzylhydrazine hydrochloric acid (NSD1015) kg⁻¹ and an increase in dopamine release as indicated by a increase in dihydroxyphenylacetic acid (DOPAC) concentrations (one of the major metabolites of dopamine). Ovariectomized rats treated with oestradiol benzoate plus progesterone were also injected i.p. with 75 mg gamma-acetylenic GABA kg⁻¹ (a GABA transaminase inhibitor) to increase GABA concentrations in the brain. This treatment had no effect on the ratio of tyrosine hydroxylase:β-actin mRNA but decreased L-DOPA accumulation and DOPAC concentrations in the zona incerta, indicating a post-translational inhibition of dopamine synthesis and release. Treatment of ovariectomized rats with oestradiol benzoate followed by 100 mg L-DOPA i.p. to increase dopamine concentrations in the whole brain had no effect on glutamic acid decarboxylase mRNA expression in the zona incerta, although it increased the glutamic acid decarboxylase:β-actin mRNA ratio in other hypothalamic areas (that is, the medial preoptic area, ventromedial nucleus and arcuate nucleus). In conclusion, the steroids act to increase dopamine activity in different ways: oestrogen increases tyrosine hydroxylase mRNA expression and progesterone acts after translation to increase tyrosine hydroxylase activity and dopamine release (as indicated by increases in DOPAC concentrations). This latter effect may be due to progesterone removing a tonic GABAergic inhibition from the dopaminergic system.

Introduction

The zona incerta situated in the dorsal hypothalamus is an important integrating centre, receiving and conveying neuronal tracts from the spinal cord, mid-brain, thalamus,

cortex and limbic system (Ricardo, 1981; Eaton *et al.*, 1994; Mihailoff, 1995; Nicolelis *et al.*, 1995; Moga and Moore, 1996; Shammagh-Lagnado *et al.*, 1996). The zona incerta is involved in a number of physiological processes such as sleep-waking cycles, ingestive behaviour, motor activity, visual perception, eye movement and nociceptive response (Kaelber and Smith, 1979; Legg, 1979; Dalton and Grossman, 1982; Kendrick and Baldwin, 1986; Tonelli and Chiaraviglio,

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1995; May *et al.*, 1997). Oestrogen receptors (of both the α and β type) are present in the zona incerta (Sar, 1984; Shrughrue *et al.*, 1997) as are steroid metabolizing enzymes (Haglund *et al.*, 1984), and this area regulates steroid-dependent functions such as gonadotrophin release (MacKenzie *et al.*, 1984; James *et al.*, 1987; Sanghera *et al.*, 1991a) and male and female sexual behaviour (Dornan *et al.*, 1991; Wilson *et al.*, 1991).

The zona incerta forms part of the dopaminergic incerto-hypothalamic tract that originates from three nuclear areas of dopamine cell bodies termed A11, A13 and A14 (Bjorklund and Nobin, 1973). The cell bodies of the A11 group are sited in the caudal thalamus and their terminals reach the medial-anterior part of the zona incerta. The A13 group are present in the zona incerta close to these nerve terminals and immunocytological techniques reveal that their axons lead to various hypothalamic nuclei, that is, the dorsal medial nucleus, paraventricular nucleus, lateral hypothalamus and anterior hypothalamus (Bjorklund and Nobin, 1973; Bjorklund *et al.*, 1975). There is controversy as to whether the preoptic area is innervated by dopamine neurones from the zona incerta (Bjorklund *et al.*, 1975; Day *et al.*, 1980), but if they are present, they do not impinge on GnRH neurones (Horvath *et al.*, 1993). The anterograde tracer technique shows that neurones in the zona incerta project ipsilaterally to the paraventricular nucleus, anterior hypothalamus, ventromedial nucleus, supraoptic nucleus, arcuate nucleus and medial preoptic area (Wagner *et al.*, 1995), but these are not necessarily dopaminergic and may belong to other neuronal systems originating in the zona incerta; examples include gamma-aminobutyric acid (GABA) (Oertel *et al.*, 1982), adrenaline (Ross *et al.*, 1984), aspartate (Moga and Moore, 1996), melanin concentrating hormone (MCH) (Bittencourt *et al.*, 1992) and orexin (Sakurai *et al.*, 1998).

The dopamine system in the zona incerta exerts a stimulatory effect on the secretion of LH, since application of dopamine into the zona incerta stimulates LH release, whereas dopamine antagonists have the opposite effect (MacKenzie *et al.*, 1984; James *et al.*, 1987). This action is probably of physiological significance, since selective lesions of the dopaminergic nerve terminals in the zona incerta and lesions of all the cell bodies in this area prevent normal oestrous cyclicity (MacKenzie *et al.*, 1984, 1988) and electrolytic lesions prevent the pro-oestrous preovulatory LH surge (Sanghera *et al.*, 1991a) and decrease concentrations of GnRH in the median eminence (Wilkes *et al.*, 1979). Conversely, dopamine turnover in the zona incerta increases at the time of the preovulatory LH surge in intact female rats and the LH surge induced by ovarian steroids in ovariectomized rats (MacKenzie *et al.*, 1988; Wilson *et al.*, 1991).

GABA neurones in the zona incerta also regulate LH release. Administration of GABA agonists into the zona incerta results in inhibition of LH release, whereas antagonists have the opposite effect (Wilson *et al.*, 1990). Furthermore, ovarian steroid treatment reduces the expression of the rate-limiting synthetic enzyme glutamic acid decarboxylase (GAD) in the zona incerta at the time of the expected increase in LH secretion (Leigh *et al.*, 1990).

The observations that administration of oestradiol benzoate and progesterone to ovariectomized rats can alter

dopamine turnover and glutamic acid decarboxylase mRNA expression in the zona incerta at the same time as stimulating release of LH indicate that dopamine and GABA may mediate ovarian steroid regulation of the cyclical release of LH. The aim of the present study was to elucidate the mechanisms by which the steroids might alter dopaminergic activity in the zona incerta and to determine whether their effects are exerted on mRNA expression or post-translationally or on other events along the pathway. In addition, the possible interaction between the dopaminergic and GABAergic systems in the zona incerta was investigated. Whole brain GABA concentrations were increased and any changes in dopamine activity in the zona incerta were recorded. Conversely, whole brain dopamine concentrations were increased and changes in glutamic acid decarboxylase mRNA expression were measured. The modulation of these possible interactions by ovarian steroids was considered.

Materials and Methods

Animals

Female Wistar rats (180–220 g) bred at St George's Hospital Medical School were ovariectomized under halothane (Fluothane, Zeneca Ltd, Macclesfield) and nitrous oxide anaesthesia and allowed to recover for at least 2 weeks before experimentation. The rats were housed in groups of five in a fixed lighting system (lights on 07:00 h to 19:00 h) and given food and water *ad libitum*.

General procedures and treatments

There were three treatment groups in each experiment and treatment was initiated at 12:00 h. Rats were treated s.c. with either 0.1 ml corn oil, 5 μ g oestradiol benzoate in 0.1 ml oil, or 5 μ g oestradiol benzoate followed 48 h later by either 0.1 ml oil or 0.5 mg progesterone in 0.1 ml oil. At 6 h after progesterone or 54 h after the first injection of oil or oestradiol benzoate, the animals were decapitated. Blood was collected from the cervical cut and the brain was removed, placed immediately in solid CO₂ and stored at -80°C or frozen in liquid nitrogen-cooled isopentane for subsequent mRNA extraction. The blood samples were centrifuged at 400 g at 4°C for 15 min and the plasma was stored at -20°C until assayed for LH.

In one experiment, groups of ovariectomized rats were treated with oestradiol benzoate alone and killed at 0, 6, 24, 30, 48, 51 or 54 h after the oestradiol benzoate injection (that is, at 12:00 or 18:00 h on day 1, 12:00 or 18:00 h on day 2 and 12:00, 15:00 or 18:00 h on day 3). Groups of oil-treated rats were killed at 12:00, 15:00 and 18:00 h to serve as controls for a possible circadian rhythm.

Other treatments included 3-hydroxybenzylhydrazine hydrochloric acid (NSD1015, 100 mg kg⁻¹ i.p.; Sigma Chemical Co. Ltd, Poole), an aromatic-acid decarboxylase inhibitor, administered 20 min before the animals were killed. This time was chosen after determination of the rate of

accumulation of L-3,4-dihydroxyphenylalanine (L-DOPA) in the zona incerta during 10–60 min after NSD1015, which showed that accumulation reached a plateau only after 30 min. The GABA transaminase inhibitor, gamma-acetylenic GABA (GAG; Merrell Dow Labs, Strasbourg) was administered (75 mg kg⁻¹ i.p.) 1 h after progesterone, that is 5 h before the rats were killed. The dopamine precursor L-DOPA (Sigma Chemical Co. Ltd, Poole) was administered (100 mg kg⁻¹ i.p.) to the oestradiol benzoate-treated group at 60 min before the rats were killed, to raise whole brain concentrations of dopamine.

Preparation of brain samples for analysis

Microdissection of the zona incerta was carried out according to the method of Palkovits (1973) using a flat-tipped needle of 0.7 mm diameter as the punch. Other areas were dissected at the same time, including the preoptic area, ventromedial nucleus and arcuate nucleus; all sites were identified according to Paxinos and Watson (1982). Samples were stored at -80°C until analysed for amines within 4 weeks. In the case of mRNA assessment, RNA extraction was carried out immediately after the microdissection.

HPLC and electrochemical detection

Dopamine and dihydroxyphenylacetic acid (DOPAC). The micropunch samples were sonicated in cold 0.1 mol perchloric acid l⁻¹ (HPLC grade; Merck, Lutterworth) containing 0.01 µg isoprenaline as the internal standard. After centrifuging at 11 000 g for 15 min, 50 µl supernatant was injected via a Rheodyne 7125 injection valve into a Beckman ultrasphere ion-pair 5 µm column (15 cm × 4.6 mm) protected by a guard column (Uptight Pre-column Kit-C-135β; Jones Chromatograph Ltd, Hengoed). The dopamine and DOPAC were detected using an LC4C amperometric detector (BAS Technicol Ltd, Congleton) set in oxidative mode with a potential of +0.7 V versus an Ag-AgCl reference electrode (Model TL5A; BAS Technicol). The mobile phase consisted of filtered and de-gassed 0.1 mol citric acetate l⁻¹, 25 mmol NaOH l⁻¹ and 1 mmol EDTA-Na l⁻¹ (pH 3.55) containing 0.5 mmol octane sulphonate l⁻¹ as the ion-pair reagent and 6% (v/v) acetonitrile.

Chromatograms were analysed by a Shimadzu CR3A Chromatopac integrator (Shimadzu UK, Milton Keynes) and the sensitivity was 10–15 pg for dopamine and DOPAC dependent upon laboratory conditions. Protein content was estimated on the remaining pellet after extraction with perchloric acid, using the modified Coomassie blue method of Bradford (1976).

L-DOPA. All details were as described above except the mobile phase consisted of 0.1 mol citric acetate buffer l⁻¹ at pH 4.85 containing 1.1 mmol heptane sulphonate l⁻¹ and 3% methanol. The internal standard was dihydroxyphenylglycol (Sigma Chemical Co. Ltd, Poole). The L-DOPA was detected by the Coulochem electrochemical detection (ESA Analytical, St Ives, Huntingdon) and the chromatograms

were analysed by a Spectra-Physics SP4270 integrator (Thermo Separation Products, Stone). The sensitivity for L-DOPA was 25 pg.

RNA extraction and slot-blot analysis of tyrosine hydroxylase, glutamic acid decarboxylase and β-actin mRNAs

Total RNA was extracted from individual micropunch samples using RNazol B (Biogenics Ltd, Bournemouth) according to the manufacturer's protocol. The quality of the extracted RNA was assessed by standard northern blotting procedures using glyoxal to denature the RNA and [³²P]d-CTP-labelled (Amersham International, Amersham) tyrosine hydroxylase cDNA (Tufts University, Boston, USA), glutamic acid decarboxylase 67 cDNA (UCLA, USA) or β-actin (Galton Laboratories, University College London; used as a 'house-keeping' gene) as the probes. The signal obtained from autoradiography was discrete, indicating undegraded RNA, and the size of the mRNA molecule hybridized with the relevant probe corresponded to published values (Leigh *et al.*, 1990; Lewis *et al.*, 1983).

The RNA derived from each micropunch sample was applied in 100 µl sterile water to a nylon membrane (Zetaprobe; Bio-Rad, Hercules, CA) using a manifold slot-blot apparatus (Schleicher & Schuell, Dassel). The filters were baked at 80°C for 1 h to fix the RNA, prehybridized and then hybridized overnight at 42°C with either [³²P]d-CTP-labelled tyrosine hydroxylase cDNA or glutamic acid decarboxylase 67 cDNA. For rehybridization, the probes were stripped off the filter by washing in boiling 0.1 × SSC (saline sodium citrate)-0.1% (w/v) SDS and cooling gradually to room temperature. The filter was subjected to autoradiography to ensure that all the probe had been removed. After prehybridization, the filter was rehybridized with [³²P]d-CTP-labelled β-actin.

The filters were washed with solutions containing SSC and SDS. The stringency of washing was increased gradually by decreasing the SSC concentration and increasing the temperature; the final wash was carried out using 0.1 × SSC-0.1% (w/v) SDS at 60°C for 5 min. The filters were subjected to autoradiography with X-omat-AR X-ray film for periods of up to 2 weeks. The film was developed using Kodak developer and fixer. The intensity of the bands obtained after autoradiography was measured using a scanning densitometer (Chromoscan 3; Joyce Loebl, Gateshead). Results were expressed as a ratio of peak intensity obtained after hybridization with tyrosine hydroxylase cDNA probe:β-actin cDNA probe or glutamic acid decarboxylase 67 cDNA probe:β-actin cDNA probe.

Radioimmunoassay for plasma LH

The assay was conducted according to Kendle *et al.* (1978). Plasma samples (10 µl) were assayed in duplicate using reagents supplied by the National Hormone and Pituitary Program (Baltimore, MD). The standard was NIADDK-rLH-RP3 and the antibody NIADDK-anti-rLH S10. The inter- and

intra-assay coefficients of variation were 8.0 and 9.5%, respectively, and the sensitivity was 10 pg per tube (1 ng ml⁻¹).

Statistical analysis

The significance of difference between two groups was assessed by the Student's *t* test together with their 95% confidence limits. Comparisons among more than two groups were carried out by ANOVA followed by Gabriel's test for comparison of unequal groups.

Results

Effect of steroids on dopamine activity in the zona incerta

Expression of tyrosine hydroxylase mRNA in the zona incerta. Ovariectomized rats were treated with either 5 µg oestradiol benzoate, which reduced LH release as noted 54 h later, or 5 µg oestradiol benzoate followed at 48 h by 0.5 mg progesterone, which stimulated LH release (Table 1). At this time, slot-blot analysis of micropunch samples of the zona incerta showed that the expression of mRNA for tyrosine hydroxylase, the rate-limiting enzyme for catecholamines,

was significantly increased by treatment with oestradiol benzoate alone. This was indicated by the ratio of tyrosine hydroxylase:β-actin mRNA compared with that in the oil-treated animals. Administration of progesterone to the oestradiol benzoate-treated animals decreased the ratio to a value similar to that in the controls.

The tyrosine hydroxylase:β-actin mRNA ratio at several intervals after the oestradiol benzoate treatment is shown (Table 2); only the value at 54 h after injection was significantly higher in the oestradiol benzoate-treated animals than in the controls.

Effect on tyrosine hydroxylase activity. Ovariectomized rats primed with either oil, oestradiol benzoate alone or oestradiol benzoate plus progesterone were all treated i.p. with 100 mg NSD1015 kg⁻¹ at 20 min before the animals were killed. NSD1015 is an aromatic-acid decarboxylase inhibitor and prevents the conversion of L-DOPA to dopamine. Thus the accumulation of L-DOPA in the 20 min after the NSD1015 injection is an indication of tyrosine hydroxylase activity (that is, in converting tyrosine to L-DOPA). Treatment with oestradiol benzoate alone had no effect on L-DOPA accumulation, but oestradiol benzoate plus progesterone at the time of increased LH release significantly increased L-DOPA concentrations, indicating an increase in tyrosine hydroxylase activity (Table 3).

Table 1. Effect of gonadal steroids and increased gamma-aminobutyric acid (GABA) concentrations on the ratio of tyrosine hydroxylase:β-actin mRNA in the zona incerta in female ovariectomized rats

	Steroid treatment			
	Oil	Oestradiol benzoate + oil	Oestradiol benzoate + progesterone + saline	Oestradiol benzoate + progesterone + GAG
Tyrosine hydroxylase:β-actin mRNA	0.87 ± 0.16 (7)	2.72 ± 0.48** (6)	1.00 ± 0.17 (7)	1.63 ± 0.09 (6)
Plasma LH (ng ml ⁻¹)	7.50 ± 0.43 (7)	3.43 ± 1.05* (6)	42.64 ± 6.39*** (6)	14.90 ± 0.45† (12)

Ovariectomized rats were injected s.c. with 0.1 ml oil, 5 µg oestradiol benzoate or 5 µg oestradiol benzoate followed 48 h later by 0.1 ml oil or 0.5 mg progesterone. The progesterone-treated animals were also injected i.p. with 1 ml saline kg⁻¹ or 75 mg gamma-acetylenic GABA kg⁻¹ (GAG; gamma-acetylenic GABA; GABA T inhibitor) at 1 h after the progesterone treatment. All animals were killed 54 h after oestradiol benzoate, that is, 6 h after progesterone and 5 h after GAG. Values are mean ± SE. Numbers in parentheses represent number of micropunch samples or plasma samples in each group.

P* < 0.01, *P* < 0.001 compared with oil-treated controls; †*P* < 0.001 compared with groups treated with oestradiol benzoate alone; ‡*P* < 0.001 compared with oestradiol benzoate + progesterone group (ANOVA and Gabriel's test for multiple comparison).

Table 2. Changes in the ratio of tyrosine hydroxylase:β-actin mRNA in the zona incerta after administration of 5 µg oestradiol benzoate in female ovariectomized rats

Treatment	Time after oestradiol benzoate administration						
	0 h (Day 1: 12:00 h)	6 h (Day 1: 18:00 h)	24 h (Day 2: 12:00 h)	30 h (Day 2: 18:00 h)	48 h (Day 3: 12:00 h)	51 h (Day 3: 15:00 h)	54 h (Day 3: 18:00 h)
Ratio tyrosine hydroxylase:β-actin mRNA (oil)	0.64 ± 0.07 (6)	0.74 ± 0.09 (15)	0.64 ± 0.07 (6)	0.74 ± 0.09 (15)	0.64 ± 0.07 (6)	0.74 ± 0.20 (3)	0.74 ± 0.09 (15)
Ratio tyrosine hydroxylase:β-actin mRNA (oestradiol benzoate)	0.65 ± 0.08 (5)	0.97 ± 0.27 (5)	0.92 ± 0.12 (4)	0.48 ± 0.09 (6)	0.48 ± 0.11 (7)	0.87 ± 0.25 (4)	1.65 ± 0.23** (10)
Plasma LH (ng ml ⁻¹) (oestradiol benzoate)	13.51 ± 2.37† (5)	2.53 ± 0.66 (8)	3.57 ± 0.41 (5)	2.89 ± 0.67 (5)	4.33 ± 0.90 (6)	3.62 ± 0.41 (4)	4.78 ± 0.71 (13)

Values are mean ± SE. Numbers in parentheses represent number of micropunch samples or plasma samples in each group.

**P* < 0.01 compared with oil-treated controls at the same time (Student's *t* test); †*P* < 0.005, ‡*P* < 0.001 compared with all other time intervals (ANOVA and Gabriel's test for multiple comparisons).

Table 3. Effect of gonadal steroids and increased gamma-aminobutyric acid (GABA) concentrations on L-dihydroxyphenylalanine (L-DOPA) accumulation in the zona incerta in female ovariectomized rats

	Steroid treatment			
	Oil	Oestradiol benzoate + oil	Oestradiol benzoate + progesterone + saline	Oestradiol benzoate + progesterone + GAG
at-DOPA (pg μg^{-1} protein)	2.68 $\pm$ 0.16 (7)	3.87 $\pm$ 0.81 (6)	5.30 $\pm$ 0.32* [†] (7)	3.49 $\pm$ 0.45* [†] (6)
Plasma LH (ng ml ⁻¹)	10.66 $\pm$ 0.76 (7)	3.70 $\pm$ 0.41** (7)	20.41 $\pm$ 5.44** [†] (4)	1.97 $\pm$ 0.28** (6)

Treatments were as for Table 1.

at-DOPA: accumulation assessed 20 min after administration of 100 mg 3-hydroxybenzylhydrazine hydrochloric acid (NSD1015) kg⁻¹ i.p.

Values are mean $\pm$ SE. Numbers in parentheses represent number of micropunch samples in each group.

* $P < 0.01$, ** $P < 0.001$ compared with oil-treated controls; [†] $P < 0.001$ compared with group treated with oestradiol benzoate alone; ^{††} $P < 0.01$, ^{†††} $P < 0.001$ compared with oestradiol benzoate + progesterone + saline group (ANOVA and Gabriel's test for multiple comparison).

**Effect on dopamine and DOPAC concentrations.* The concentrations of dopamine and one of its major metabolites DOPAC were measured in ovariectomized animals treated with oil, oestradiol benzoate alone or oestradiol benzoate plus progesterone. Oestradiol benzoate had no effect on the concentration of either dopamine or DOPAC, whereas in the animals treated with oestradiol benzoate plus progesterone, there was a significant increase in DOPAC concentrations at the time of the increased release of LH; dopamine concentrations were not affected by the oestradiol benzoate plus progesterone treatment (Table 4).

Effect of increasing whole brain GABA concentrations on dopaminergic activity in the zona incerta

Ovariectomized animals primed with oestradiol benzoate plus progesterone were treated i.p. with either 0.1 ml saline kg⁻¹ or 75 mg GAG kg⁻¹ at 1 h after progesterone treatment, that is 5 h before the animals were killed. GAG is a GABA transaminase inhibitor and so increases endogenous GABA concentrations by preventing its degradation. Groups treated with oestradiol benzoate plus progesterone showed high concentrations of LH, and GAG significantly reduced these

Fig. 1. The possible relationships between dopamine (DA) and gamma-aminobutyric acid (GABA) in the zona incerta and GnRH secretion. Other systems linking neuronal activity in the zona incerta with the GnRH neurones are probably present at the level depicted by the dotted line. +, indicates stimulatory effect; -, indicates inhibitory effect.

Table 4. Effect of gonadal steroids and increased gamma-aminobutyric acid (GABA) concentrations on dopamine and dihydroxyphenylacetic acid (DOPAC) concentrations in the zona incerta in female ovariectomized rats

	Steroid treatment			
	Oil	Oestradiol benzoate + oil	Oestradiol benzoate + progesterone + saline	Oestradiol benzoate + progesterone + GAG
Dopamine (pg μg^{-1} protein)	4.50 $\pm$ 0.52 (12)	4.19 $\pm$ 0.86 (14)	4.50 $\pm$ 0.56 (12)	4.41 $\pm$ 0.64 (9)
DOPAC (pg μg^{-1} protein)	1.52 $\pm$ 0.24 (7)	1.18 $\pm$ 0.12 (12)	2.40 $\pm$ 0.39** (6)	1.63 $\pm$ 0.09† (6)
Plasma LH (ng ml ⁻¹)	6.78 $\pm$ 1.04 (12)	3.67 $\pm$ 0.47* (16)	29.62 $\pm$ 4.87*** (9)	1.30 $\pm$ 0.15†† (5)

Treatments were as for Table 1.

Values are mean $\pm$ SE. Numbers in parentheses represent number in each group.

* $P < 0.01$, ** $P < 0.001$ compared with oil-treated controls; † $P < 0.001$ compared with group treated with oestradiol benzoate alone; †† $P < 0.001$ compared with oestradiol benzoate + progesterone + saline group (ANOVA and Gabriel's test for multiple comparison).

concentrations. GAG had no effect on the ratio of tyrosine hydroxylase: β -actin mRNA expression (Table 1), whereas it significantly reduced L-DOPA accumulation and DOPAC concentrations in the zona incerta (Tables 3 and 4). The effect on DOPAC was specific to the zona incerta and did not occur in the other areas analysed (preoptic area, ventromedial nucleus and arcuate nucleus) (see Table 5). In addition, GAG treatment had no effect on L-DOPA accumulation in the arcuate nucleus and ventromedial nucleus, but did induce a decrease in L-DOPA in the preoptic area (Table 5).

Effect of increasing whole brain dopamine concentrations on GAD mRNA in hypothalamic areas

Ovariectomized rats primed with 5 μg oestradiol benzoate were injected i.p. with 100 mg L-DOPA kg^{-1} at 1 h before the animals were killed. This treatment increased dopamine concentrations approximately threefold in the zona incerta, preoptic area, ventromedial nucleus and arcuate nucleus (for

example, in the zona incerta, dopamine concentrations (pg μg^{-1} protein $\pm$ SE (n)): oestradiol benzoate + saline, 4.19 $\pm$ 0.86 (14); oestradiol benzoate + L-DOPA, 12.08 $\pm$ 3.28 (8); $P < 0.001$), but did not significantly alter LH concentrations (LH concentrations (ng ml⁻¹ $\pm$ SE (n)): oestradiol benzoate + saline, 3.7 $\pm$ 0.47 (16); oestradiol benzoate + L-DOPA, 3.8 $\pm$ 1.06 (8)). The L-DOPA treatment increased GAD: β -actin ratios to a similar extent in all areas examined, that is, there was an increase of between 1.52- and 2.07-fold in the four areas, which are all within the 95% confidence limits of each group. In comparison with the relevant saline groups, the increase was significant in all areas, except the zona incerta (Table 6).

Discussion

Other studies showed that administration of dopamine into the zona incerta stimulates LH release, whereas dopamine antagonists have the opposite effect (MacKenzie *et al.*, 1984;

Table 5. Effect of gonadal steroids and increased gamma-aminobutyric acid (GABA) concentrations on dopamine and dihydroxyphenylacetic acid (DOPAC) concentrations and L-dihydroxyphenylalanine (L-DOPA) accumulation in the preoptic area, ventromedial nucleus and arcuate nucleus in female ovariectomized rats

Area of brain		Oestradiol benzoate + progesterone + saline	Oestradiol benzoate + progesterone + GAG
Preoptic area	L-DOPA	5.52 $\pm$ 0.51 (6)	3.75 $\pm$ 0.29* (7)
	Dopamine	5.38 $\pm$ 0.65 (14)	7.99 $\pm$ 1.28 (8)
	DOPAC	2.98 $\pm$ 0.36 (16)	2.53 $\pm$ 0.16 (8)
Arcuate nucleus	L-DOPA	3.83 $\pm$ 0.62 (7)	4.37 $\pm$ 0.49 (6)
	Dopamine	9.02 $\pm$ 1.97 (13)	12.70 $\pm$ 3.25 (8)
	DOPAC	2.53 $\pm$ 0.43 (5)	2.68 $\pm$ 0.39 (9)
Ventromedial nucleus	L-DOPA	4.42 $\pm$ 0.25 (7)	3.59 $\pm$ 0.34 (7)
	Dopamine	5.14 $\pm$ 0.70 (16)	5.72 $\pm$ 0.85 (9)
	DOPAC	2.20 $\pm$ 0.41 (17)	1.73 $\pm$ 0.13 (9)

Ovariectomized rats were injected s.c. with 5 μg oestradiol benzoate followed 48 h later with 0.5 mg progesterone. At 1 h after progesterone the rats were injected i.p. with either 1 ml saline kg^{-1} or 75 mg gamma-acetylenic GABA (GAG) kg^{-1} . All animals were killed 54 h after oestradiol benzoate, that is, 6 h after progesterone and 5 h after GAG. Some animals also received 100 mg 3-hydroxybenzylhydrazine hydrochloric acid (NSD1015) kg^{-1} i.p. 20 min before they were killed to assess L-DOPA accumulation.

Values are mean $\pm$ SE pg μg^{-1} protein. Number of micropunch samples in each group is given in parentheses. LH concentrations for the groups assessed for L-DOPA accumulation are shown in Table 3 and for groups assessed for dopamine and DOPAC in Table 4.

* $P < 0.01$ compared with group treated with oestradiol benzoate + progesterone + saline.

Table 6. Effect of increased dopamine concentrations on ratio of glutamic acid decarboxylase (GAD): β -actin mRNA in hypothalamic areas in female ovariectomized rats

Hypothalamic area	Ratio of GAD: β -actin mRNA:		Ratio of L-DOPA: saline results	95% confidence limits
	Oestradiol benzoate + saline	Oestradiol benzoate + L-DOPA		
Zona incerta	1.37 $\pm$ 0.46 (5)	2.04 $\pm$ 0.45 (5)	1.59	0.63–3.97
Preoptic area	1.24 $\pm$ 0.18 (6)	1.82 $\pm$ 0.20* (9)	1.52	1.03–2.23
Ventromedial nucleus	1.26 $\pm$ 0.28 (7)	2.46 $\pm$ 0.37* (8)	2.07	1.22–3.50
Arcuate nucleus	1.19 $\pm$ 0.17 (7)	1.97 $\pm$ 0.21* (8)	1.71	1.05–2.78

Ovariectomized rats were injected s.c. with 5 μ g oestradiol benzoate and either 1ml saline kg⁻¹ or 100 mg L-DOPA kg⁻¹ (precursor of dopamine) was administered i.p. 1 h before the animals were killed at 54 h after oestradiol benzoate.

Values are mean $\pm$ SE. Numbers in parentheses represent number of micropunch samples in each group.

* $P < 0.05$ compared with group treated with oestradiol + saline (Student's t test after log transformation for each area).

James *et al.*, 1987). Furthermore, endogenous dopamine activity in the zona incerta is increased at the time of increased LH release as shown by a rise in turnover rate (assessed after inhibition of tyrosine hydroxylase by α -methyl tyrosine), an increase in the ratio of DOPAC:dopamine and an increase in DOPAC concentrations (MacKenzie *et al.*, 1988; Wilson *et al.*, 1991). The concentration of DOPAC has been used as a marker of dopamine release, as it is the main metabolite of dopamine after dopamine is released from nerve terminals (Tian *et al.*, 1991). These changes have been observed at the time of the preovulatory LH surge in intact females and also at the time of the LH surge induced in ovariectomized rats treated with oestradiol benzoate plus progesterone (MacKenzie *et al.*, 1988; Wilson *et al.*, 1991). Oestradiol benzoate alone, whether exerting a negative or positive feedback effect, has no effect on dopamine activity as assessed by the above parameters (Shoemaker and Schlumpf, 1977; Gunnet *et al.*, 1986; Wilson *et al.*, 1991). However, in long-term ovariectomized rats, chronic oestrogen treatment, either alone or with progesterone, restores the reduced immunoreactive tyrosine hydroxylase concentrations in dopamine neurones in the zona incerta to normal values (Sanghera *et al.*, 1991b).

The aim of the present study was to determine the possible mechanisms by which the ovarian steroids might influence dopamine activity, that is, by affecting the expression or activity of the rate-limiting catecholamine synthetic enzyme tyrosine hydroxylase or by acting on the release of dopamine. The results showed that oestradiol benzoate alone increased expression of tyrosine hydroxylase mRNA 54 h after treatment, as indicated by the significant increase in the ratio of tyrosine hydroxylase: β -actin mRNA, whereas the oestradiol benzoate plus progesterone treatment reversed this increase. These results are in conflict with those of Morrell *et al.* (1989) who reported that steroid treatments had no influence on tyrosine hydroxylase mRNA (assessed by *in situ* hybridization) in the zona incerta. Although the steroid regimen was different from that used in the present study and no LH measurements were made when the animals were killed, Morrell *et al.* (1989) expected to see low LH concentrations in the group treated with oestradiol benzoate alone and an LH surge in the animals treated with oestradiol benzoate plus progesterone. The animals were

killed at the mid-point of the dark phase in a 12 h light:12 h dark regimen, whereas in the present study, animals were killed 1 h before the start of the dark period; this may explain the observed differences and emphasises the sensitivity of the kinetics of the system. In the present study, the LH surge was induced to occur at a time similar to the physiological timing of the natural LH surge (between 18:00 and 19:00 h, just before the transition from light to dark).

The results of the present study also showed that although progesterone appeared to reduce tyrosine hydroxylase mRNA in oestradiol benzoate-treated animals, at the same time it increased synthesis, as shown by the increase in L-DOPA accumulation, which is a marker of increased tyrosine hydroxylase activity. Progesterone also increased DOPAC concentrations, which could be interpreted as an increase in dopamine release (Tian *et al.*, 1991) or an increase in degradative enzyme activity. Either explanation, when taken together with the increase in synthesis, indicates an increase in dopamine turnover and supports previous findings on dopamine turnover using α -methyl tyrosine (MacKenzie *et al.*, 1988; Wilson *et al.*, 1991). The effect of oestradiol benzoate on tyrosine hydroxylase mRNA was observed at the time of reduced LH release, whereas the effect of progesterone on dopamine turnover was observed when LH was increased. The timing of the two effects might be different; the effect on tyrosine hydroxylase mRNA may precede translational events and any other events that occur further along the transmitter pathway. A time course of the effect of oestradiol benzoate on tyrosine hydroxylase mRNA showed that the increase does not occur earlier than 54 h after treatment and is not associated with any negative feedback effect on LH, which can be observed as early as 6 h after treatment. Thus, the stimulatory effect on expression of tyrosine hydroxylase mRNA may be in preparation for the potential positive feedback effect on LH release that occurs at a later stage and after any negative feedback effect, in the presence of sufficiently high concentrations of ovarian steroids. The post-translational effect of progesterone on tyrosine hydroxylase activity may have a feedback action to terminate further increased expression of tyrosine hydroxylase mRNA.

Oestradiol benzoate receptors are present in the nuclei of neurones in the zona incerta, whereas progesterone receptors have not been observed at that site (Sar, 1984; Fox *et al.*, 1990).

Thus it is possible that oestradiol benzoate acts directly on dopaminergic cell bodies to affect tyrosine hydroxylase mRNA expression, whereas the effect of progesterone on the release of dopamine from nerve terminals (as indicated by the increase in DOPAC) may occur indirectly via another system. The focus of the present study was the possibility that progesterone induces this effect via a GABAergic system.

Progesterone treatment reduces GAD mRNA expression at the time of increased LH release in several hypothalamic areas, including the preoptic areas and zona incerta (Leigh *et al.*, 1990; Unda *et al.*, 1995). Wilson *et al.* (1990) reported that GABA injected into the zona incerta acts on GABA B receptors to inhibit LH release. In the present study, the hypothesis that this inhibitory effect in the zona incerta is due to inhibition of the stimulatory dopamine system was investigated. It was found that increasing endogenous GABA activity in the whole brain by means of systemic administration of a GABA transaminase inhibitor reduced LH release and at the same time decreased L-DOPA accumulation and DOPAC concentrations in the zona incerta. These effects in the zona incerta were to some extent specific, as there was no decrease in L-DOPA concentrations in the preoptic area, ventromedial nucleus and arcuate nucleus, or in L-DOPA accumulation in the ventromedial nucleus and arcuate nucleus. The manipulation of GABA activity did not affect expression of tyrosine hydroxylase mRNA in the zona incerta, and thus, similar to progesterone, its effects appear to occur after translation rather than at the level of mRNA.

Another possible dopamine–GABA interaction might be the converse of that discussed above. When progesterone increases LH release, it also stimulates dopamine activity in the zona incerta (MacKenzie *et al.*, 1988; Wilson *et al.*, 1991; this study), which might act to remove the tonic inhibitory effects of GABA from a GnRH system. However, increasing dopamine concentrations in the whole brain increased, rather than decreased, GAD mRNA expression, indicating this interaction is unlikely to occur at transcription. This increase was seen in all the areas examined and indicates that there is an interaction between the dopaminergic and GABAergic systems that is not specific to a particular area of the hypothalamus.

Therefore, the positive feedback effect of progesterone is probably not mediated via an ovarian steroid-induced increase in dopamine activity leading to removal of the inhibitory effects of a GABAergic system, but rather is a progesterone-induced reduction in GABA activity (Fig. 1). This proposal is supported by the finding that progesterone reduces GAD mRNA expression in the zona incerta (Leigh *et al.*, 1990). This effect of progesterone removes the inhibitory GABAergic influence on the dopamine system in the zona incerta, which is exerted at post-translational sites.

In summary, the results of the present study confirm that steroids stimulate dopamine activity in the zona incerta and show that oestrogen increases tyrosine hydroxylase mRNA expression, whereas progesterone stimulates tyrosine hydroxylase activity and dopamine release (as indicated by an increase in DOPAC concentrations). In addition, the inhibitory effect of GABA on LH release in the zona incerta may be exerted by decreasing tyrosine hydroxylase activity

and dopamine release in this area, and progesterone may act to remove this inhibitory effect of GABA.

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