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ADRENERGIC INFLUENCE ON PROGESTERONE METABOLISM AND CYCLICITY IN THE RAT OVARY: AUTOTRANSPLANTATION AND CHEMICAL SYMPATHECTOMY

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Summary—Elimination of adrenergic nerve endings by chemical sympathectomy with 6-hydroxydopamine of normally cycling rats produced no differences in the weights of body, uterus, ovaries or adrenals, but suppressed significantly proestrus/estrus stages. Unilateral fully denervated (autotransplanted) ovaries showed the following changes in [^{14}C]progesterone metabolism: the formation of 20α-hydroxy-4-pregnen-3-one increased, whereas 5α-pregnane-3α,20α- and 3β,20α-diol, 3α- and 3β-hydroxy-5α-pregnan-20-one, 20α-hydroxy-5α-pregnan-3-one, an unidentified metabolite Y and a group of hydrophobic metabolites decreased dramatically. Enzyme activities could not be restored with epinephrine. Sympathectomy changed the spectrum of [^{14}C] progesterone metabolites in the same direction, but only at diestrus and metestrus. Autotransplantation suppressed 5α-reductase, 3α- and 3β-hydroxysteroid dehydrogenase activities (-HSD) measured by the sum of all 5α-, 3α-, and 3β-metabolites, respectively. Sympathectomy suppressed significantly 5α-reductase and 3α-HSD at metestrus. 20α-HSD was not changed in any experiment. These studies provide evidence that 5α-reductase depends on adrenergic input in ovaries of rats at metestrus, a stage of nadir of gonadotropins. During the estrous cycle 5α-reductase may be a regulatory enzyme for progesterone metabolism and also influence estradiol biosynthesis.

INTRODUCTION

Though the intraovarian innervation was reported more than a century ago and investigations with modern methods leave no doubt of adrenergic innervation of mammalian ovary [recent reviews: 1–3] its physiological role is still hardly understood.

Most studies of the influence of ovarian adrenergic nerves or catecholamines (CA) concern their involvement in ovulation, compensatory hypertrophy [reviews: 3–6] and follicular development and dynamics [7–9], see also [10, 11]. The difficulties of studying CA effects result from their only permissive and mostly modulatory role. They are ineffective in stimulating basal secretion of steroids and effective in increasing hormone stimulated effects [12, 13].

The relation between CA and steroid metabolism concerns studies of secretion of progesterone, estradiol and androgens [12, 14–19]. Investigations of steroid metabolizing enzymes and their relation to adrenergic activity are rare: ovarian denervation decreased Δ5-3β-hydroxysteroid dehydrogenase (-HSD) in both interstitial gland cells and corpus luteum [20]. β_2-Agonists inhibited 20α-HSD and increased 3β-HSD in a granulosa cell model primed with follicle stimulating hormone (FSH) [17, 21].

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Abbreviations: **Steroids:** progesterone, 4-pregnene-3,20-dione; 20α-OH-P, 20α-hydroxy-4-pregnen-3-one; 3α- and 3β-diol, 5α-pregnane-3α,20α- and 3β,20α-diol; 3α- and 3β-20-one, 3α- and 3β-hydroxy-5α-pregnan-20-one; 20α-3-one, 20α-hydroxy-5α-pregnan-3-one; Zone I and IV, see Experimental, Y, unidentified metabolite; Σ5α-, Σ3α-, Σ3β-, Σ20α-, sum of all 5α, 3α, 3β, 20α-reduced metabolites, respectively. **Enzymes:** -HSD, hydroxysteroid dehydrogenase, hydroxysteroid: NAD^+($NADP^+$)-oxidoreductase; 3α-HSD (EC 1.1.1.50); 3β-HSD (EC 1.1.1.51) or (EC 1.1.1.145); 20α-HSD, 20α-hydroxysteroid: $NADP^+$-oxidoreductase (EC 1.1.1.149); 5α-reductase, 3-oxo-5α-steroid: $NADP^+$-Δ4-oxidoreductase (EC 1.3.1.4). **Others:** FSH, follicle stimulating hormone; LH, luteinizing hormone; PMSG, pregnant mare serum gonadotropin; CA, catecholamines; 6OHDA, 6-hydroxydopamine; EDTA, ethylenediaminetetra-acetate; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; TLC, thin-layer chromatography; SD, standard deviation. **Estrous stages:** Di, diestrus; Pro, proestrus; E, estrus; Met, metestrus.

The aim of this study was to investigate the influence of CA or adrenergic nerves on ovarian steroid metabolism by autotransplantation of ovaries as a fully denervated system, by chemical sympathectomy as an adrenergically denervated system and to try to restore lost enzyme activities by epinephrine.

The investigation of [^{14}C]progesterone metabolism allows indirect measurement of the relative activities of four enzymes (5α-reductase, 3α- and 3β- and 20α-HSD), in their environment.

Progesterone is not only an effective hormone by itself whose concentration must be regulated, but also the precursor of estradiol, the other effective hormone of the ovary. Studying the dependence of its metabolism on CA may increase our understanding of the complicated regulatory system of the ovary.

EXPERIMENTAL

Animals

Female Sprague Dawley rats were used, 76–84 days old on the day of experiment, supplied by Zentralinstitut für Versuchstierzucht Hannover, day–night cycle: 12 h [22].

Sympathectomy

Chemical sympathectomy was achieved by i.v. (tail vein) application of 6-hydroxydopamine (6OHDA) [23]. Fifty mg/kg 6OHDA HBr (Regis) was dissolved in 0.2 ml 0.001 N HCl (treated animals) or HCl only (control animals) and injected twice within 24 h. Two days later the same treatment was repeated. The experiment was carried out on day 8 after the first application of 6OHDA. The estrous cycle stage was monitored daily by vaginal smear: diestrus (Di), proestrus (Pro), estrus (E) and metestrus (Met). Twelve days before starting treatment with 6OHDA (see Table 2), 8 days starting with the first application of 6OHDA and from the 9th day of first injection (possible regeneration phase).

Autotransplantation

The animals were anaesthetized with ether, the right ovary was cut out, freed from adjacent tissue and replaced. Twenty four hours later both ovaries (intact and autotransplanted) were removed and separately homogenized as described below.

Incubation

After decapitation the organs were removed and weighed. Ovaries were homogenized with 0.25 M sucrose and 1.0 mM EDTA and incubated in Tris buffer, pH 7.35, with a NADPH regenerating system [22]. Sympathectomy experiment: 20 mg ovary tissue was incubated in a final volume of 1.02 ml. The incubation (30 min, 37°C) was started by the addition of 3.55 nmol [4-^{14}C]progesterone in 20 μl ethanol (sp. act.: 2.08 GBq/mol); number of control animals (=repeats) 5–8, number of treated animals was 5–10 with the exception of only one animal at the E stage, because E was suppressed by 6OHDA. Autotransplantation experiments: 10 mg ovarian tissue in a final volume of 0.540 ml was incubated: 0.25 ml homogenate plus 0.25 ml buffer, pH 7.35 (see above). The incubation (90 min, 37°C) was started by the addition of 20 μl epinephrine in 0.1% ascorbate, (final concentration 10^{-5} M), or 0.1% ascorbate only and [4-^{14}C]progesterone (see above) in 20 μl ethanol (6 repeats per group).

Metabolite isolation

The method has been described previously [22]. After extracting the steroids with dichloromethane, evaporating and dissolving in methanol, the metabolites were separated by a descending paper chromatography system for 5 h at 25°C (cyclohexane–methanol–water, 100:85:15, by vol) in 4 zones: Zone I (no further separation) represents the higher hydroxylated, hydrophilic metabolites. Zone II [further separation by a two-dimensional thin-layer chromatography (TLC) system: Al_2O_3, 1st dimension: ethanol–benzene, 1:24, v/v, developed twice; 2nd dimension: chloroform–acetone, 9:1, v/v, developed three times]. From this zone the following metabolites were isolated: 5α-pregnane-3α,20α- and 3β,20α-diol (3α- and 3β-diol), 20α-hydroxy-4-pregnen-3-one (20α-OH-P) and Y, an unidentified metabolite: the migration distance of Y in relation to 3α-diol and 3β-diol is 0.7 and 0.8 in the 1st dimension, respectively, and 0.9 to both in the 2nd dimension. Zone III (further separation by a one-dimensional TLC system: Al_2O_3, ethanol–benzene, 1:32.3, v/v, developed twice). From this zone the following metabolites were isolated: progesterone, 3α- and 3β-hydroxy-5α-pregnan-20-one (3α- and 3β-20-one) and 20α-hydroxy-5α-pregnan-3-one (20α-3-one). Zone IV (no further separation) represents a group of hydrophobic metabolites,

such as 5α-pregnane-3,20-dione, which, however, could not be identified.

The 4 zones were monitored by radiochromatography scanning, whereas the metabolites were monitored by autoradiography. The zones and the spots were extracted with methanol followed by evaporating, dissolving and scintillation counting and presented as percent of total recovered radioactivity which was set 100%. The recovery rate was 90–95%. Final identification was reached by rechromatography in different systems, by derivative formation [22] and by recrystallization of the isolated radioactive metabolites with nonradioactive carrier to constant specific activity.

Statistics

Differences of the metabolite concentrations of treated and control animals were tested by Duncan's new multiple range test at least at a level of $\alpha = 0.05$. The differences of the distribution of cycle stages were tested by chi square (χ^2)-test.

RESULTS

The influence of autotransplantation of the ovary on [^{14}C]progesterone metabolism

Autotransplantation of the ovaries did not change the weight of the ovary: 33.3 ± 3.7 and 33.8 ± 9.9 mg of intact vs autotransplanted ovaries. After incubation of homogenates of these ovaries with [^{14}C]progesterone besides the substrate the following ^{14}C-metabolites were isolated, identified and quantified: 3α- and 3β-diol, 20α-OH-P [Fig. 1(a)], 3β-20-one, 20α-3-one, 3α-20-one [Fig. 1(b)]. A group of hydrophylic (Zone I) and hydrophobic (Zone IV) metabolites were quantified as a whole (not shown). Zone I includes estradiol and estrone.

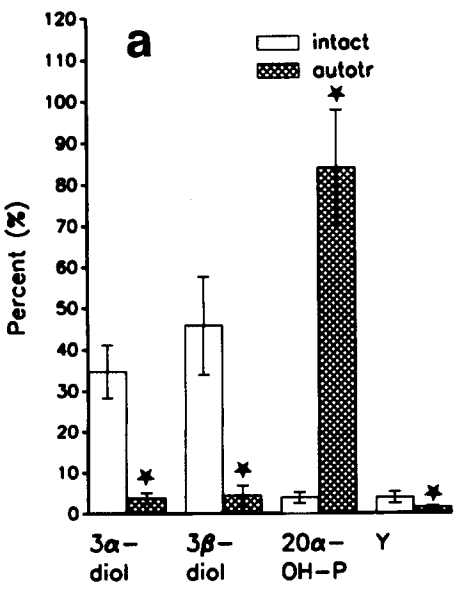
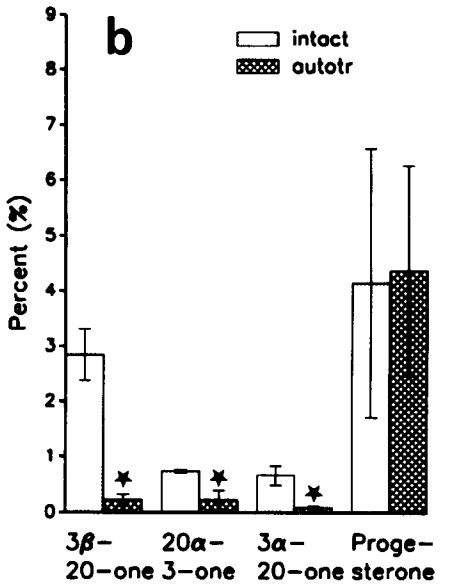


Fig. 1—*continued.*



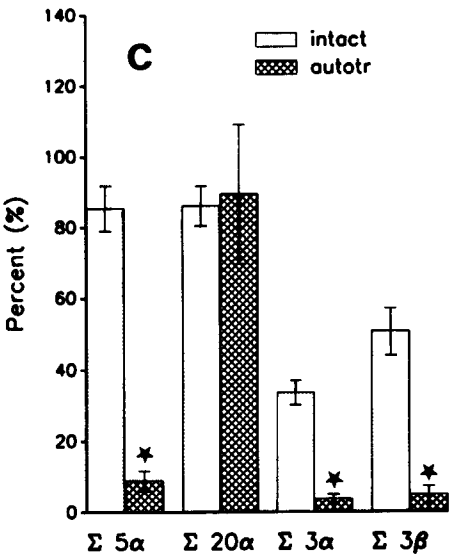


Fig. 1. Metabolites of [^{14}C]progesterone in intact and autotransplanted (autotr) ovaries. 10 mg of ovary tissue (of intact and autotransplanted ovaries) was incubated with [^{14}C]progesterone. Additional conditions are described under Experimental. Means are given as percent of recovered radioactivity (set 100%; recovery rate was 90–95%) ± SD, $n = 6$. Metabolites of Zone I and IV (not shown, see Experimental) account for the difference between the combined percentages of metabolites shown and 100%. Y (a) is an unidentified metabolite of Zone II, see Experimental. All 5α-, 20α-, 3α- or 3β-reduced metabolites from (a) and (b) were combined and presented in (c): Σ5α, Σ20α, Σ3α and Σ3β, respectively. Significant differences between intact and autotransplanted ovaries are marked by ★.

Table 1. The influence of chemical sympathectomy on organ weights

	Diestrus	Proestrus	Estrus	Metestrus
		Body weight (g)		
Control	226 ± 14[a]	222 ± 14	228 ± 8	219 ± 22
6OHDA	204 ± 14	203 ± 19	193	212 ± 11
		Right adrenal gland (mg)		
Control	24 ± 1	26 ± 2	28 ± 2	26 ± 1
6OHDA	26 ± 3	30 ± 5	25	31 ± 7
		Ovaries (mg)		
Control	76 ± 14	84 ± 5	90 ± 6	76 ± 5
6OHDA	70 ± 9	79 ± 7	83	70 ± 9
		Uterus (mg)		
Control	298 ± 30	389 ± 75	396 ± 22	318 ± 39
6OHDA	248 ± 47	391 ± 73	389	294 ± 76

[a]Means ± SD

5α-Pregnane-3,20-dione, which would run in Zone IV could not be detected. Y [Fig. 1(a)] is a metabolite which could be separated from the diols in a two-dimensional TLC system (see Experimental).

Quantitatively most important are the 3α- and 3β-diols in the intact ovaries [Fig. 1(a) intact] and only 20α-OH-P in the autotransplanted ovaries [Fig. 1(a), autotr]. Other metabolites represent a few percent. More than 95% of progesterone was metabolized during 90 min of incubation to the same extent in intact and autotransplanted animals [Fig. 1(b)]. The sum of all 5α-, 3α-, 3β- or 20α-reduced metabolites showed no change in 20α-metabolites while 5α-, 3α- and 3β-metabolites dramatically decreased [Fig. 1(c), autotr].

The attempt to restore adrenergic influence with epinephrine revealed only very small effects: the transformation of the substrate progesterone is enhanced and the production of 3β-20-one and 20α-3-one is increased but only in intact ovaries (not shown).

The influence of chemical sympathectomy on organ weights, cyclicity and metabolism of [^{14}C]progesterone during the estrous cycle

Chemical sympathectomy (treatment of the animals with 6OHDA) did not have an effect on the weights of body, adrenals, ovaries and uterus (Table 1), but suppressed Pro/E stages (Table 2).

In the ovary homogenates of control and 6OHDA treated animals 3α- and 3β-diols and 20α-OH-P appeared as the major metabolites of [^{14}C]progesterone during the 4 estrous stages: Di, Pro, E and Met. Other metabolites represent only a few percent. Progesterone was metabolized to >95% during 30 min of incubation. Control animals showed no significant changes of [^{14}C]progesterone metabolite spectrum during the estrous cycle [Fig. 2(a) and (c)]. 6OHDA caused significant changes in comparison with control animals at Di and Met: decrease of 3α-diol at Met, 3β-diol at Di [Fig. 2(b)], 3α-20-one at Di and Met [Fig. 2(d)], Zone I-metabolites at Met (not shown) and increase of 20α-OH-P at Di and Met [Fig. 2(b)].

The sum of all metabolites with 5α, 3α-, 3β- or 20α-reduced group can be taken to reflect the relative activities of 5α-reductase, 3α-, 3β-, and 20α-HSD, respectively (Fig. 3). Significant changes in these activities were observed at Met for 5α-reductase and 3α-HSD. The decrease of 3β-HSD was not significant though that of 3β-diol (Di) was [Fig. 2(b)]. The decrease of 3α- and 3β-metabolites may be a secondary effect because 5α-reductase action provides substrates for the two HSDs. 20α-Metabolites and thus the

Table 2. The influence of chemical sympathectomy on cyclicity

	Before treatment		Number of diagnoses of stages from first injection (8 days)		From 9th day of first injection	
	Pro/E	Met/Di	Pro/E	Met/Di	Pro/E	Met/Di
Control	173	328	144	209	61	64
6OHDA	196	311	85*	241	44	56

*Significant by χ^2-test.

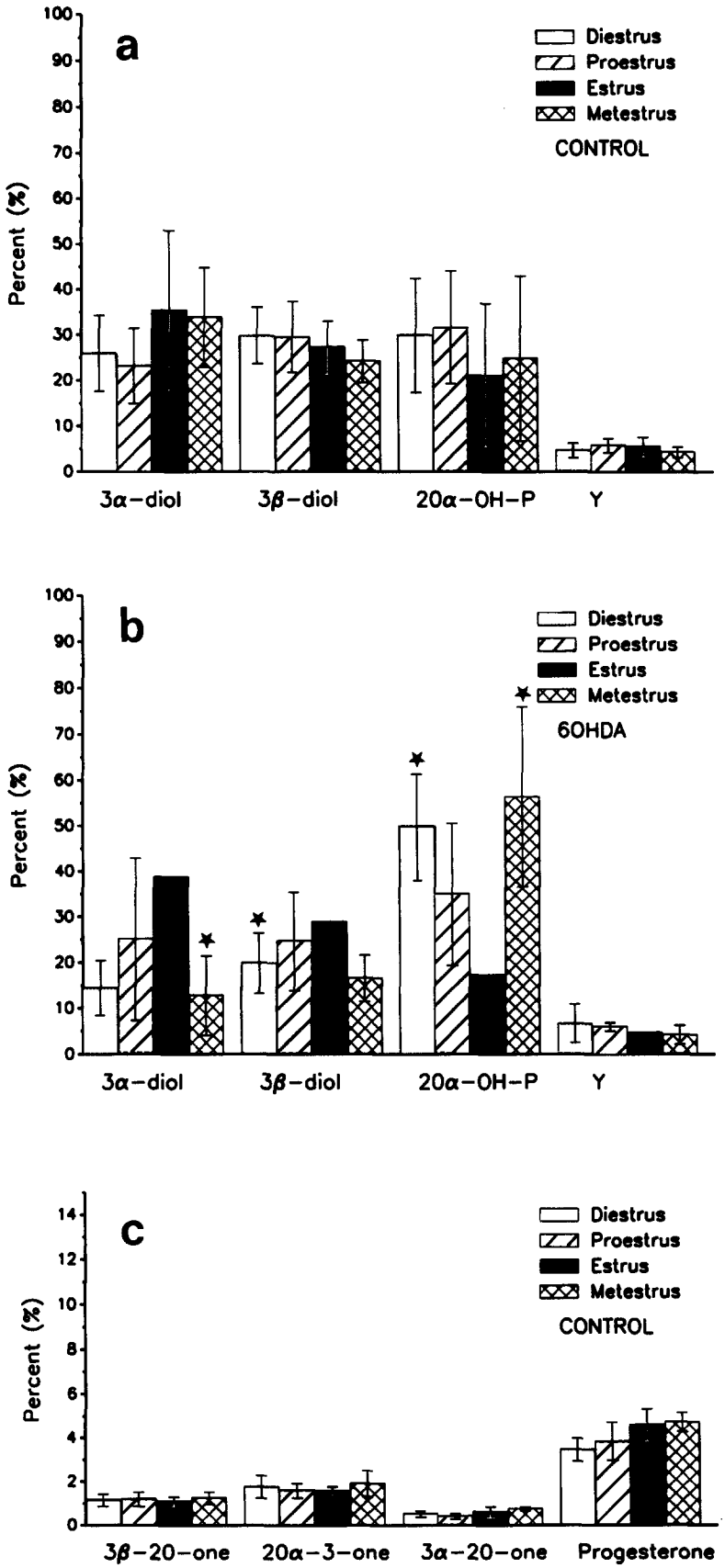


Fig. 2—*legend overleaf.*

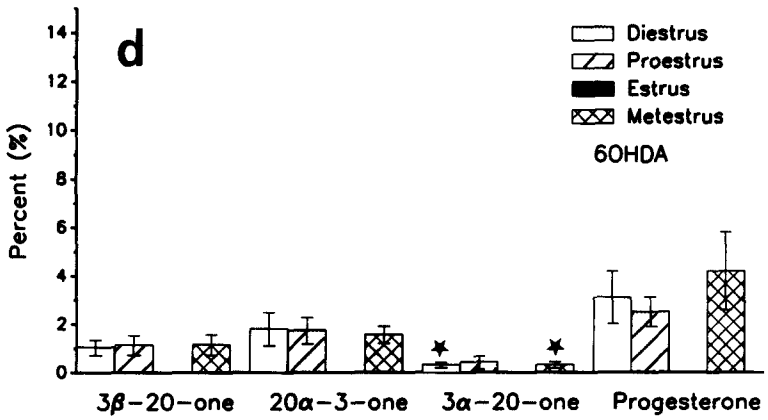


Fig. 2. Metabolites of [^{14}C]progesterone during the estrous cycle in ovaries of control (a, c) and 6OHDA treated animals (b, d). 20 mg of ovary tissue was incubated. Additional experimental conditions are described under Experimental. Means are given as percent of recovered radioactivity (set 100%; recovery rate was 90–95%) ± SD, control: $n = 5$–8; 6OHDA: $n = 5$–10, with exception of E, because 6OHDA suppressed E (see Table 2). Metabolites of Zone I and IV (not shown, see Experimental) account for the difference between the combined percentages of metabolites shown and 100%. Y (a, b) is an unidentified metabolite of Zone II, see Experimental. Significant differences between treated and untreated animals are marked by ★.

20α-HSD is not influenced by 6OHDA treatment (Fig. 3).

DISCUSSION

Cyclicity

The basis for the cyclicity changes, seen as suppression of Pro/E stages (Table 2), is a complicated regulatory system of the ovary–pituitary–hypothalamus axis. In these sympathectomized animals the peripheral adrenergic nerves with the exception of adrenal medulla [23] are destroyed. 6OHDA does not penetrate the blood–brain barrier [24]. Thus it is unlikely that peripherally applied 6OHDA acts directly on the brain. Intracerebral lesions with 6OHDA have no effect on the cyclicity of rats and on the regulatory mechanism of gonadotropin secretion in spite of norepinephrine depletion [25], but the authors recorded the reiniation of consecutive 4 day estrous cycles on day 9 after surgery, a time of return to normal cyclicity (see Table 2, from 9th day of first injection). Local application of 6OHDA to both ovaries delayed the first E cycle in 53% of golden hamsters [9].

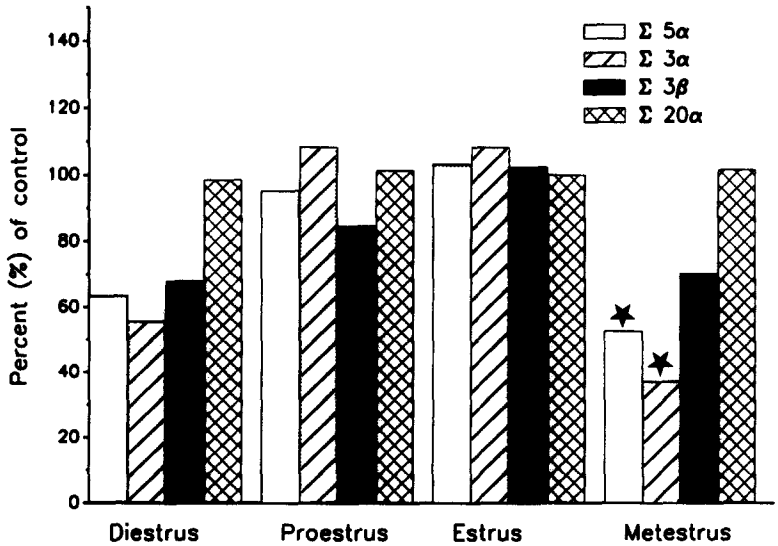


Fig. 3. Metabolites of [^{14}C]progesterone during the estrous cycle in ovaries of control and 6OHDA treated animals. All 5α-, 3α-, 3β- or 20α-reduced metabolites (Fig. 2) were combined and data presented as percent of 6OHDA treated groups to control groups: Σ5α, Σ3α, Σ3β and Σ20α, respectively. Significant differences are marked by ★.

Signalling from peripheral nerves to brain were discovered by Cardinali *et al.* [26]: E cycle is delayed and gonadotropin and prolactin release is inhibited during sympathetic nerve degeneration after superior cervical ganglionectomy of rats. That means peripheral nerves gave signals directly to the brain and changed gonadotropin release. Compensatory hypertrophy of the remaining ovary with unilaterally ovarectomized rats was reported [20, 27]. Intact adrenergic, afferent and efferent neural elements of the ovary are required for the development of compensatory ovarian hypertrophy [28].

An efferent neural system from brain to ovaries has been suggested to work supplementary to the brain–pituitary–ovarian hormonal mechanisms in the regulation of ovarian steroid secretion and the system may be required for adjustment [29]. Although far from being understood, peripheral nerves seem to play an important role in the regulation of ovary cycle.

[^{14}C]progesterone metabolism

Autotransplantation interrupts blood flow and fully denervates the organ. Autotransplanted ovaries of the rat are revascularized after 48 h and reinnervated after 28 days, but even after 4 days 40% of the antral follicles had escaped necrosis [30]. Our experiment was done 24 h after autotransplantation at a time of a minimum of additional effects. In contrast to autotransplantation as a fully denervated system, sympathectomy destroys specifically adrenergic nerve endings. Both treatments result in changes of progesterone metabolism in the same direction: increasing 20α-OH-P and decreasing significantly the sum of 5α-reduced metabolites: in autotransplanted ovaries the effects are more pronounced in that all metabolite concentrations with the exception of 20α-OH-P [Fig. 1(a)] and Zone I (not shown) decrease dramatically. The sum of all 3α-, 3β-, 5α- or 20α-reduced metabolites shows a dramatic decrease of 5α-reductase activity and of 3α- and 3β-HSD activities after autotransplantation in comparison with the nontransplanted ovary [Fig. 1(c)]. After sympathectomy a significant decrease of 5α-reductase and 3α-HSD activity is observed at Met (Fig. 3). The change in 3α- and 3β-HSD could be a secondary effect, because the substrate of these enzymes are 3-oxo-5α-reduced metabolites. No change was seen in 20α-HSD-activity in any experiment.

These two experiments complement each other in that the gradual denervation results in a gradual response of the measured system. This supports the view that the effect is more dependent on denervation than on interruption or change of blood flow, though some influence of sympathectomy on blood flow is not fully excluded. The dependence of 5α-reductase on adrenergic input at Met is particularly interesting, because Met is a stage where the concentrations of gonadotropins, FSH and LH, are very low. In the other stages the gonadotropins may cover the effect of CA or adrenergic input. Thus, the regulatory effect of CA can only be seen in the stage with the lowest gonadotropin concentration. An interplay between CA and hormones of hypophysis is shown by the depletion effect of ovarian CA after gonadotropin surge in normally cycling rats [31, 32]. Epinephrine receptors can be desensitized by hCG [33]. CA responsiveness depends on FSH in granulosa cells of the rat [15] and changed during the estrous cycle: β-adrenergic receptors have the highest concentration at Pro and the lowest at E [34].

The inability to see an effect of epinephrine in the intact ovary or in the autotransplanted ovary may be a problem of concentration and receptor specificity and may be influenced by narcosis and surgery. The possible influence of narcosis and surgery is supported by the comparison of the controls of autotransplanted and sympathectomized rats (Figs 1 and 2, 20α-OH-P vs all other metabolites). 5α-, 3α- and 3β-reduced metabolites are augmented in controls of autotransplanted ovaries in comparison with controls of sympathectomized rats of all estrous stages (5α- and 3β-metabolites) or during Di and Pro (3α-metabolites). Autotransplanted animals are stressed by narcosis and surgery 24 h before whereas in the sympathectomy experiment the animals are only stressed by injection and this 5 days before, at that time the metabolism should be returned to normal. These results confirm the conception that 5α-reductase and perhaps 3α- and 3β-HSD (see Discussion above) are dependent on adrenergic input here induced by stress. In follicles from untreated animals ^{14}C-5α- and 3β-reduced metabolites of [^{14}C]testosterone are significantly increased after incubation with the α-adrenergic agonist phenylephrine, whereas in follicles of anaesthetized animals no change in ^{14}C-metabolites of pregnenolone was found [35].

These *in vitro* findings are consistent with the present study and indicate that 5α-reductase is

dependent on adrenergic input. The physiological role of this enzyme may be the indirect control of aromatase system by elimination of the substrate testosterone or 4-androstenedione on the one hand and the inhibition of aromatase system by reduced androgens on the other hand [11].

20α-HSD

I did not find any change of 20α-HSD during the estrous cycle, during autotransplantation, sympathectomy or during prolactin deprivation with CB154 [36]. 20α-HSD-activity in preovulatory follicles of immature PMSG primed rats was found to be independent of the LH-surge [37].

Previously reported changes of 20α-HSD are related to pregnancy, corpus luteum or *in vitro* systems which simulate corpus luteum, like granulosa cells from immature hypophysectomized animals treated with estradiol and FSH. In these cells β-adrenergic agents inhibit 20α-HSD and act as luteotrophic agents [17, 21]. Inhibition of 20α-HSD was found in such granulosa cell models with FSH, testosterone or cAMP [38–40]. 20α-HSD was increased by neurectomy of pelvic nerves in preparturient and parturient periods of rats [41], and also after vagotomy of pregnant rats on day 8 of pregnancy while 3β-HSD in corpus lutea and interstitial glands decreased [42].

20α-HSD vs 5α-reductase

The physiological role of 5α-reductase in prepubertal rat ovary is well established [43–48]. In several models of prepubertal rats 5α-reductase was influenced by hormones of hypophysis [44, 46–48]. In immature PMSG treated animals 5α-reductase declined to a residual level upon first ovulation, whereas 20α-HSD was induced at the same time [46]. But in normally cycling rats 5α-pregnanes are produced in large quantities [49–51]. In small antral follicles from immature rats stripped of their theca layers the steroid metabolic pattern of progesterone is very similar to that obtained in interstitial cells with a high ratio of 5α-reductase/20α-HSD activity. Treatment with FSH suppressed only 5α-reductase in these follicles so that 20α-OH-P was the only steroid metabolite of progesterone [48]. In untreated animals, 29, 45, 66 and 929 days old, this ratio changed from 3, 1, 0.7 to 1.5, respectively [22]. It was 0.7 in large sized, intact follicles of adult, untreated animals with pregnenolone as a substrate [35] and was drastically reduced by autotransplantation, by sympathectomy at Met and by prolactin deprivation with CB154 at Pro and Met [36] caused exclusively by the suppression of 5α-reductase.

20α-HSD plays an important role in the regulation of progesterone metabolism in corpus luteum and during pregnancy, whereas in normally cycling rats 5α-reductase may regulate progesterone metabolism and also indirectly effect estradiol biosynthesis.

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