THE DEVELOPMENT OF THE EARLY-ANDROGEN SYNDROME IN THE FEMALE RAT

PROEFSCHRIFT

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PROMOTOR : PROF. DR. J.J. VAN DER WERFF TEN BOSCH

CO-REFERENTEN : PROF. DR. H.J. VAN DER MOLEN
PROF. DR. J. MOLL

Dit onderzoek werd verricht in de afdeling Endocrinologie, Groei en Voortplanting van de Faculteit der Geneeskunde, Erasmus Universiteit Rotterdam.

Aan mijn ouders,
aan Marijoto

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Voorwoord

Het is slechts uit academische gewoonte dat één naam op de omslag is vermeld. Alleen in team-verband kan wetenschappelijk werk tot stand komen. Ik denk daarbij niet alleen aan uitwisseling van ideeën, maar ook aan hulp op het gebied van proefdierverzorging, histologie, administratie etc. Ik heb samenwerking steeds gezocht en ook bij velen zowel binnen de afdeling als daarbuiten ondervonden.

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De omslag is vervaardigd onder leiding van de heer Oudenalder en de figuren zijn verzorgd door de grafische afdeling van de audiovisuele dienst.

Abbreviations

AH-POA : anterior hypothalamic-preoptic area
ARC-VMN : arcuate-ventromedial nuclear complex
BSA : bovine serum albumin
b.w. : body weight
Ci : curie
DC : direct current
ES : electrical stimulation
ECS : electrochemical stimulation
FSH : follicle stimulating hormone
FSH-RH : follicle stimulating hormone-releasing hormone
HCG : human chorionic gonadotrophin
IU : international units
i.v. : intravenous(ly)
LH : luteinizing hormone
LH-RH : luteinizing hormone-releasing hormone
mc : millicoulomb
NIAMD : National Institute of Arthritis and Metabolic diseases
NIH : National Institute of Health
NRS : normal rabbit serum
OAAD : ovarian ascorbic acid depletion
OB : oestradiol benzoate
PBS : phosphate buffered saline
PMSG : pregnant mare serum gonadotrophin
PVC : persistent vaginal cornification
RIA : radioimmunoassay
RP : reference preparation
S.E.M. : standard error of the mean
S.D. : standard deviation
s.c. : subcutaneous(ly)
TP : testosterone propionate
TSH : thyroid stimulating hormone

General introduction

The gonadal functions of male and female individuals are mainly regulated by two gonadotrophic hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both secreted by the pituitary. In spontaneously ovulating mammals (e.g. rat and man) periodic discharges of these hormones, resulting in ovulation, are characteristic of the female, whereas similar periodic discharges have never been observed in the male. Every four or five days female rats show a peak in serum LH on the afternoon of the day preceding ovulation (the day of prooestrus), which coincides with an increase in serum FSH. The high FSH level is maintained till the afternoon of oestrus (Gay et al, 1970; Daane and Parlow, 1971). The serum LH peak normally results in ovulation of a number of follicles, which are transformed to corpora lutea. In rats these processes recur every four or five days.

It has been known for more than thirty years that androgens administered neonatally to female rats can prevent ovulation and corpus luteum formation in the adult animal (Bradbury, 1941). These findings are of particular interest in conjuction to those of Pfeiffer (1936). He observed that ovaries transplanted into adult male rats, castrated at birth, showed follicular growth and corpus luteum formation similar to those in female rats. On the other hand, implantation of testes into newborn female rats resulted in the absence of ovulations in the ovaries in situ. Furthermore, ovaries transplanted into male rats, castrated in adulthood, do not display ovulation or formation of corpora lutea (Goodman, 1934). On the basis of these observations it was concluded that in the rat the type of gonadotrophin regulation in adulthood depends on the presence or absence of

testicular hormones in neonatal life (Pfeiffer, 1936). Neonatal absence of androgenic hormones in females as well as in males permits the development of cyclic activity of implanted ovaries. The presence of androgenic hormones neonatally, following injection (in the female) or by testicular secretion (in the male) results in non-cyclic activity of implanted ovaries. Since it has been convincingly proven that gonadotrophin release is controlled by the brain (Harris and Campbell, 1966) the sex difference in gonadotrophin release implies a sex difference in the functioning of brain centres controlling gonadotrophin release. It is generally assumed that in the presence of androgenic hormones during a certain period of neural development - in the rat perinatally - the undifferentiated brain mechanism controlling the gonadotrophin release is organised so as to prevent cyclic gonadotrophin release (Gorski, 1966; Harris, 1970). Since androgen administration in neonatal female rats results in the development of a pattern of gonadotrophin secretion similar to that of the male, the sterilizing effect of early-androgen treatment has often been called "masculinization". However, this term tends to oversimplify the regulation of gonadal function in the male and may erroneously suggest that all sex differences between the male and female hypothalamo-hypophysial-gonadal axis depend on the neonatal presence of androgens. Therefore we prefer the term "androgen-sterilization" or "androgenization" to describe the effect of neonatal (early) androgen treatment.

This thesis will deal with the influence of neonatal androgen injections on the hypothalamo-hypophysial-ovarian system in the female rat. Sexual differentiation of the regulation of the gonads was the subject of an earlier thesis from this laboratory (van der Schoot, 1973).Furthermore, several excellent reviews on sexual differentiation of gonadotrophin regulation are available (Barraclough, 1966[a], 1968; Neumann et al, 1970; Harris, 1970; Gorski, 1971).

Androgen-sterilization has been obtained in several mamm-

alian species viz mice (Barraclough and Leathem, 1954; Lamond, 1969), hamsters (Alleva et al, 1969; Swanson, 1966) and guinea-pigs (Brown-Grant and Sherwood, 1971). It is conceivable that androgen-sterilization also occurs in primates, including man. At the adrenogenital syndrome high levels of androgenic hormones are present in female fetuses. (see Thomas, 1968). Until the present, studies on the effect of prenatal androgen in primates have failed to demonstrate an effect on fertility (Treloar et al, 1972; Goy and Resko, 1972). However, behavioral changes have been reported in such cases (Money and Ehrhardt, 1972; Goy and Resko, 1972).

Regulation of gonadotrophin release.

Release of the two gonadotrophic hormones, LH and FSH, from the pituitary is controlled by the releasing hormone(s) LH-RH (and FSH-RH) (McCann, 1970; Schally et al, 1973). It is thought that the releasing hormone is produced in neurons ending on capillary loops in the median eminence (Barry et al, 1973). The hormone reaches the pituitary via the portal vessels. (Harris, 1955). The releasing hormone producing elements are probably, located within the medio-basal part of the hypothalamus. Releasing hormone activity has been observed from the optic chiasm to the pituitary stalk (Watanabe and McCann, 1968; Crighton et al, 1970). The medio-basal part of the hypothalamus, by itself, does not produce a stimulus releasing sufficient LH-RH for an ovulatory surge of LH. Female rats with lesions in the anterior hypothalamus (Hillarp, 1949; Greer, 1953) or knife cuts between the anterior hypothalamus and the medial hypothalamus (Halász and Gorski, 1967) showed the condition of constant oestrus, which is characterized by follicular growth without ovulation. Such animals are capable of producing FSH and LH, although they do not produce an ovulatory surge of LH. From the experiments of Halász and Gorski (1967) its is concluded that the area rostral to the knife cuts is indispensable for the induction of the ovulatory LH surge. There is fairly good evidence that the anterior hypothalamic-

preoptic area (AH-POA) plays a major role in the regulation of the cyclic LH release. Knife cuts frontal to the AH-POA, leaving the neural connections of the AH-POA with the medial hypothalamus intact, may be followed by spontaneous ovulations (Köves and Halász, 1970; Kaasjager et al, 1971). This suggests that the neural mechanism required for triggering of the ovulatory LH surge lies within the preoptic area. Also, it has been reported that limbic structures (the amygdala and the hippocampus) are involved in the regulation of gonadotrophin release (for review see Zolovick, 1972; Wildschut, 1972). The relative importance of these extra-hypothalamic influences for cyclic LH release is still unclear.

Current theories of the regulation of gonadotrophin release involve a dual control mechanism. (1) The medial hypothalamus regulates the tonic release, the gonadotrophin concentration in the blood being regulated by the inhibitory feedback action of steroids on this area (Taleisnik et al, 1970). Also an influence of steroids on the sensitivity of the pituitary to releasing hormones is involved in the regulation of the tonic gonadotrophin release (Schally et al, 1973). (2) The AH-POA regulates the cyclic gonadotrophin release. Under adequate hormonal conditions, in which the stimulatory influence of a high oestrogen level plays a role (Brown-Grant, 1971), the AH-POA activates the RH-producing neurons in the medio-basal hypothalamus, and this results in an ovulatory discharge of LH from the pituitary.

Regulation of gonadotrophin release in androgen-sterilized rats.

Although ovulatory LH release is absent in androgen-sterilized rats, the presence of growing follicles in the ovaries and the signs of oestrogen secretion (near normal uterine weight and permanent vaginal cornification) indicate that some gonadotrophin release is maintained. Direct measurements of serum gonadotrophins with radioimmunoassay (RIA) in rats made anovulatory by 50 µg TP show serum LH

concentrations similar to those at dioestrus and oestrus in cyclic rats (Kawakami and Terasawa, 1972; Kawakami et al, 1973; Mallampati and Johnson, 1973). In rats made anovulatory by 1250 µg TP, Barraclough (1968) reported extremely high concentrations of serum LH as measured by bioassay, even higher than those observed in ovariectomized, untreated rats. However, data obtained by the more reliable RIA revealed serum LH concentrations in high dose TP-treated rats slightly higher than those at oestrus (Labsethwar, 1970, 3 x 100 µg TP; Uilenbroek, 1972, 1250 µg TP; Velasco and Rothchild, 1973, 1250 µg TP). Data on serum FSH concentrations in adult androgen-sterilized rats are limited. It has been assumed that the secretion of FSH is tonic, since 4- or 5-day cyclic changes in serum FSH have never been observed. FSH values not different from those at dioestrus in cyclic female rats were reported by Johnson (1972) (RIA and bioassay) and by Kawakami et al (1973) (RIA).

An inhibitory feedback action of steroids on gonadotrophin secretion which is normally present in intact rats is also operative in androgen-sterilized rats as indicated by the following findings. After ovariectomy serum gonadotrophins increase as was demonstrated in parabiosis experiments (Johnson and Witschi, 1963; Kurcz and Gerhardt, 1968; Moguilevsky et al, 1967). However, the increase in ovarian weight or accessory genital organs of the parabiotic partner of androgenized females was smaller than in the parabiotic partner of control females or even absent, if the animals were androgenized both with a high dose of TP and at an early age (500 µg TP at day 1) (Kurcz et al, 1969; Kurcz and Gerhardt, 1968). It has been suggested that in these cases the tonic gonadotrophin control was also affected. Direct measurements by bioassay or RIA of serum LH concentrations after ovariectomy in rats treated with 100 or 1250 µg TP on day 5 revealed either no differences (Barraclough, 1968; Schiavi, 1969; Taleisnik et al, 1969) or lower concentrations (Neill, 1972; Uilenbroek, 1972) as compared to ovariectomized control rats. Administration of oestrogen

to ovariectomized TP-treated rats can suppress or prevent the postcastration rise of serum LH (Barraclough, 1968; Uilenbroek, 1972).

Data indicative of the regulation of FSH secretion have been obtained with experiments in which compensatory ovarian hypertrophy was observed after unilateral ovariectomy in androgen-sterilized rats (Gorski and Barraclough, 1962; Swanson and van der Werff ten Bosch, 1964[a]; Dunlapp et al, 1972). This hypertrophy could be prevented by oestrogen administration (Petrusz and Nagy, 1967). Direct measurement of serum FSH by bioassay in spayed androgenized rats revealed no difference with those in spayed untreated rats (Schiavi, 1969).

The absence of cyclic gonadotrophin release and the presence of a tonic gonadotrophin release in TP-treated rats suggests an effect of neonatal TP at the AH-POA level. This might cause a different reaction to stimulation of the AH-POA in TP-treated rats as compared to normal rats. However, results obtained with stimulation of the AH-POA to induce ovulation are contradictory. Electrical stimulation of the AH-POA failed to induce ovulation in androgenized female rats (Barraclough and Gorski, 1961; Gorski and Barraclough, 1963), although this type of stimulation resulted in ovulation in nembutal blocked prooestrous rats (Critchlow, 1958; Everett, 1961). From these experiments it was concluded that following neonatal androgen treatment the neurons of the AH-POA had become refractory to electrical stimulation (Barraclough, 1966[a]). In contrast, results obtained with electrochemical stimulation of the AH-POA did not show a difference between normal and androgenized rats (Terasawa et al, 1969; Everett et al, 1970; Kawakami and Terasawa, 1972), which suggested that neonatal TP-treatment had no effect on the AH-POA. Arai (1971[b]) suggested that the limbic structures are affected by neonatal TP-treatment, because in contrast to normal rats ovulation could not be induced by electrochemical stimulation of the medial amygdaloid nucleus in androgenized rats.

Androgenization.

In early experiments on the influence of neonatal androgen treatment on ovulation in adult female rats high doses of testosterone propionate (TP) were administered for several weeks (Shay et al, 1939; Bradbury, 1940, 1941; Wilson et al, 1941). In later experiments a single injection of TP given before day 10 of life also resulted in the absence of ovulations in all the injected animals (Barraclough and Leathem, 1954 and Barraclough, 1955 in mice; Barraclough, 1961 in rats). The period during which administration of androgen resulted in absence of ovulations has been called "the critical period". In the rat this period extends from a few days before birth (Swanson and van der Werff ten Bosch, 1965) till about the 10th day of life. It appeared that all the animals injected subcutaneously with a dose of 100 µg TP or more were anovulatory at 10-14 weeks of age (see review Neumann et al, 1970). With lower doses only a low percentage of the animals was anovulatory at 10-14 weeks of age (Gorski and Barraclough, 1963). However, by studying the effect of TP at two different ages Swanson and van der Werff ten Bosch (1964[a]) observed that with 5 or 10 µg TP, given on day 5, a number of animals which were ovulatory at the age of 10 weeks were anovulatory at the age of 21 weeks. These findings show that the early-androgen treatment does not exert an all- or none-effect, but show that the syndrome can develop at later ages. These findings have been confirmed by Kikuyama and Kawashima (1966), Gorski (1968) and Arai (1971[a]) and has been called "the delayed anovulation syndrome" (Gorski, 1968) or "the incomplete or delayed early-androgen syndrome" (van der Werff ten Bosch et al, 1971).

Scope of this thesis.

Up until now, most of the investigations concerning neonatal androgenization have been restricted to the later, adult function of the hypothalamo-hypophysial-ovarian axis. The aim of the present study was to clarify the development

of the early-androgen syndrome from the day of androgen injection onwards. Two types of TP-treated rats have been studied: rats treated with a single high dose of TP, which exhibited the anovulation syndrome regardless of when they were studied, and rats treated with a single low dose of TP, which exhibited the anovulation syndrome only after a variable period of ovarian cyclicity. These two types of androgenized rats have been compared during the prepuberal period and also in adulthood when the low dose TP-treated rats had finally reached the anovulatory state.
By establishing the earliest disturbances of sexual maturation following neonatal TP as well as the differences between high and low dose TP-treated rats, it was hoped to elucidate the development of the early-androgen syndrome.

Chapter 2 provides the materials and methods section in which particularly the radioimmunoassay methods for gonadotrophins are described.
In chapter 3 the effects of a single high and of a single low dose of TP given on day 5 on ovarian cyclicity in adulthood are described.
The effects of neonatal TP on ovarian and uterine weights, serum gonadotrophins and follicular development during the prepuberal period are reported in chapter 4.
In chapter 5 studies on the influence of neonatal TP on the capacity of the brain to induce ovulatory surges of LH are described.
Studies on the effect of neonatal TP-treatment on induction of ovulation by electrochemical stimulation of the brain are reported in chapter 6.
Chapter 7 provides a general discussion.

Chapter 2

Materials and methods

2-1 Animals and treatments.

Animals.

All animals used in these experiments were albino rats of the Wistar strain. Two substrains were used; an inbred substrain from the Department of Endocrinology and Diseases of Metabolism at Leiden University (here called "Endo" substrain) and an inbred substrain from the Netherlands Cancer Institute at Amsterdam (called R-Amsterdam substrain). Animals of both substrains were born in the laboratory and housed under conditions of constant temperature and humidity. Food, consisting of standard dry pellets (Hope Farms), and water were always available. The lighting schedule for both substrains was 14 hrs light and 10 hrs darkness. In the animal rooms of the "Endo" substrain lights were on from 08.00 hrs to 22.00 hrs, while in the rooms of the R-Amsterdam substrain lights were on from 05.00 hrs to 19.00 hrs. Employing the convention of Everett and Sawyer (1950), all subsequent references to time in the experiments refer to "colony times". Midday colony time is the midpoint of the light period. This was 15.00 hrs standard time for the "Endo" substrain and 12.00 hrs standard time for the R-Amsterdam substrain. The substrain used will be specified for every experiment.

On day 5 (day of birth is denoted as day 1) all females born on the same day were randomly distributed among lactating mothers. The group sizes with one mother were kept at eight, females being preferred.

Hormone treatments.

On day 5 all the females of a group received a single

injection of either 5 or 1250 µg testosterone propionate (TP, Neohombreol, Organon) in 0.05 ml sesame oil or 0.05 ml sesame oil alone. The injection was given subcutaneously in the neck area. Within a group all animals underwent identical treatments.

Other hormones used in the experiments are: pregnant mare serum gonadotrophin (PMSG, Gestyl, Organon), human chorionic gonadotrophin (HCC, Pregnyl, Organon), synthetic luteinizing hormone releasing hormone (LH-RH, Beckman), and progesterone (Organon). PMSG, HCG and LH-RH were diluted in a 0.9% NaCl solution and administered subcutaneously (s.c.) or intravenously (i.v.) in the vena jugularis in a volume of 0.1 ml. Progesterone was diluted in sesame oil and given s.c. in a volume of 0.1 ml.

Operations.

Operations were performed under ether anaesthesia. Hypophysectomy was carried out by the parapharyngeal approach; completeness was checked at autopsy by examination of the sella turcica under the dissecting microscope.

Blood for determination of gonadotrophic hormones was collected by puncture of the ophthalmic venous plexus with a broken glass capillary under light ether anaesthesia. Blood was allowed to clot in a refrigerator overnight prior to centrifugation. Serum was stored at -20°C.

Autopsy.

The animals were killed with chloroform. The ovaries and uteri were dissected free from adjacent fat and from the fallopian tube under a dissecting microscope. "Fluid" was removed from the uteri by pressing between filter paper. The organs were weighed on a Mettler-balance to the nearest 0.1 mg. Ovaries and uteri from animals younger than 15 days and pituitary glands (after removal of the posterior lobe) were weighed to the nearest 0.01 mg on a torsion-balance.

For detection of ovulation the fallopian tubes were dissected and opened with the aid of fine forceps. The ova

were counted under a microscope.

Histology.

Ovaries and brains were fixed in a 5% formalin solution, and sectioned at 10μ; every tenth section was mounted. For counting follicles 5μ sections were cut and serial sections were mounted. Sections of ovaries were stained with haematoxylin and eosin. Brain sections were stained with luxol-fast-blue (Romeis, 1968). Vaginal smears were stained with Giemsa's solution.

Statistical methods.

To test the probability of a difference between two samples with a normal distribution the data were subjected to Student's t-test. When the distribution of data was skewed, the probability of a difference was tested using a Mann-Whitney U test.(Siegel, 1956).

Nominal variables are presented in percentages, and to test the probability of a difference, the data were subjected to either the Fischer-test ($N<10$) or a χ^2-test ($N>10$) (Siegel, 1956).

A probability ≤ 0.05 (2-tailed) was accepted as the level of statistical significance.

2-2 Radioimmunoassay of gonadotrophins.

2-2-1 Introduction.

Until recently quantative estimation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were mainly performed with biological assays employing the ovarian ascorbic acid depletion test (OAAD, Parlow, 1961) for LH estimation and the HCG augmentation test (Steelman and Pohley, 1953) for FSH estimation. These bioassays are reliable for measuring gonadotrophins in pituitary extracts but are not sufficiently sensitive to permit reliable measurements in sera from individual rats. The development

of the radioimmunoassay (RIA) technique by Yalow and Berson (1969) as well as the method of labeling polypeptide hormones with radioactive iodine by Greenwood et al (1963) have permitted measurement of peptide hormones with a high degree of sensitivity. This RIA technigue has been applied to the measurement of rat LH by Monroe et al (1968). These authors used an antiserum against rat LH and purified rat LH for iodination. This homologous assay system has been designated as the "RR rat LH RIA", according to the convention of Niswender et al (1968[b]). The first capital letter refers to the species from which LH has been obtained for immunization while the second capital refers to the species from which the purified LH for radioiodination has been obtained. The sensitivity of this assay system is at least 200 times higher than that of the OAAD-test. However, this sensitivity is not sufficient to measure serum LH concentrations during oestrus and dioestrus of cyclic female rats. A more sensitive RIA for rat LH has been developed by Niswender et al (1968[a]) based on the crossreaction of rat LH with anti-ovine LH (OO rat LH RIA). This heterologous rat LH RIA has a sensitivity 20 times as high as that of the RR rat LH RIA and is at present the most sensitive RIA for rat LH.

An RIA method for rat FSH has been described by Daane and Parlow (1971). The sensitivity of this RR rat FSH RIA system permits reliable measurements of serum FSH concentrations during late prooestrus and early oestrus, but fails to give reliable data during the rest of the cycle (Daane and Parlow, 1971). A rat FSH RIA with an antiserum against ovine FSH (OR rat FSH RIA) has been developed (Uilenbroek and Dullaart unpublished results) but was not available at the time of the experiments to be reported here.

2-2-2 Assay systems and standards.

Two RIA systems were used for measuring rat LH in the experiments described in this thesis. They are:

1.RR rat LH RIA

The antiserum (NIAMD-anti-rat-LH S-2) and the purified rat LH (NIAMD-rat-LH I-1) were obtained from the National Institute of Arthritis and Metabolic Diseases (NIAMD). Daane and Parlow (1971) stated that this system is identical to that described by Monroe et al (1968).

2.OR rat LH RIA

The antiserum (anti-ovine-LH 610 V) was obtained by immunization of rabbits with NIH-LH-S-17. NIAMD-rat-LH I-1 was used for iodination.

Reliability data of these systems are given in 2-2-4.

For measuring rat FSH the RR rat FSH RIA was used (for details see 2-2-5). The antiserum (NIAMD-anti-rat-FSH S-1) and the purified rat FSH (NIAMD-rat-FSH I-1) were obtained from the NIAMD.

LH and FSH concentrations are expressed in the widely accepted NIAMD rat reference preparations: LH in ng NIAMD-rat-LH RP-1 (bioassay potency 0.03 NIH-LH-S-1), FSH in ng NIAMD-rat-FSH RP-1 (bioassay potency 2.1 NIH-FSH-S-1). These preparations were obtained from the NIAMD.

2-2-3 Procedure.

Radioiodination.

The purified hormones were iodinated according to the method of Greenwood et al (1963) with some minor modifications. Prior to the radioiodination procedure a Biogel P 60 column (Bio Rad Labs, Richmond Cal.) was prepared. The column (length about 15 cm, diameter 1 cm) was equilibrated with 0.01 M phosphate buffered saline (PBS) solution (PH 7.6). Prior to use 1 ml normal rabbit serum (NRS) or 0.01 M PBS containing 2% bovine serum albumin (BSA, Pentex fraction V) was passed through the column to reduce nonspecific binding of protein. Of the purified hormone 2.5 µg dissolved in 25 µl distilled water was mixed with 10 µl 0.5 M sodium

phosphate buffer (PH 7.6), 25 µg chloramine-T dissolved in 10 µl 0.01 M PBS and 1 millicurie (mCi) Na ^{125}I (Radiochemical Centre Amersham or Philips-Duphar). The reaction mixture was agitated on a Vortex mixer for exactly 60 sec. The reaction was stopped by addition of 62.5 µg sodium metabisulphite in 100 µl 0.01 M PBS. Carrier-solution (200 µl) containing 10 mg NaI/ml PBS was added and the content of the vial was transferred to the Biogel column with a pipette coated with 0.01 M PBS-2% BSA. The vial was rinsed with 300 µl carrier-solution, which was then also transferred to the column. The column was eluted with 0.01 M PBS. Ten drops of the column eluate were collected in tubes containing 100 µl 0.01 M PBS-2% BSA. The elution pattern shows two peaks of radioactivity: the first one (elution volume: 12 ml) of iodinated hormone, the second one (elution volume: 14 ml) of free ^{125}I. An aliquot of the protein peak with the highest amount of radioactivity was diluted with 0.01 M PBS-0.1% BSA to an activity of 10.000-15.000 cpm/100 µl and stored at -20°C. The specific activity of the labelled hormone is in the order of 50 to 200 µCi/µg; these are rough estimates since the amount of ^{125}I used and the amount of protein lost during the iodination procedure are not exactly known.

Radioimmunoassay.

Various amounts of reference preparation or aliquots of unknown samples were diluted to 500 µl with PBS-1% BSA. Two hundred µl antiserum, diluted to an appropriate concentration in 0.05 M EDTA-PBS containing 0.25% NRS, was added to each tube. After preincubation of this mixture for 24 hrs at 4°C, 100 µl iodinated hormone solution (10.000-15.000 cpm) was added. After an additional incubation for 72 hrs at 4°C the antibody bound iodinated hormone was separated from the free iodinated hormone by the double antibody technique: 200 µl of an appropriate dilution of anti-rabbit gamma globulin serum (donkey anti-Rgg, Wellcome) in PBS-1% BSA was added to precipitate the first antibody

together with the gamma globulin of the NRS. The tubes were incubated for another 48 hrs and the total radioactivity of the incubation mixture was counted in a Packard automatic gamma spectrometer. The tubes were then centrifuged at 3000 rpm for 20 min. The supernatant was aspirated, the precipitate washed with 1 ml PBS, centrifuged and the supernatant aspirated again. Finally the precipitate was counted.

The relative percentage bound was calculated as follows:

$$\text{relative \% bound} = \frac{B - B_{bl}}{B_0 - B_{bl}} \times 100\%$$

B = percentage bound (counts precipitate per total counts added)

B_0 = percentage bound obtained in tubes containing no reference preparation or sample

B_{bl} = percentage precipitated counts in tubes containing no reference preparation or sample and no antiserum

The amount of LH or FSH in unknown samples was estimated by comparing the relative percentage bound of the unknown sample with the amount of reference preparation at the same relative percentage binding.

Criteria.

The specificity of the RIA system was studied by comparing the dose response curves of a number of rat pituitary preparations with varying biological potencies of LH, FSH and TSH. An RIA system was considered as specific e.g. for LH, if the ratios of the amounts of these preparations required to achieve 50% relative binding was similar to the ratios of their biological LH activities and if hormone-free serum gave no displacement.

The sensitivity of the RIA systems was defined as the amount of hormone required to achieve 90% relative binding.

2-2-4 Radioimmunoassay of rat LH.

OR rat LH RIA.

An antiserum with a high binding capacity for rat LH was obtained by immunizing rabbits with ovine LH. For this purpose NIH-LH-S-17 was used which was available in sufficient quantity. Since an RIA system is generally more reliable when the hormones competing for the antibody are of the same species, an OR rat LH RIA was developed with NIAMD-rat-LH I-1 as the labelled hormone.

Six rabbits were immunized by intracutaneous injections of 150-200 µg NIH-LH-S-17 per animal. This amount was dissolved in 0.3 ml 0.9% NaCl solution, emulsified in 0.7 ml complete Freund's adjuvant and injected at different sites on the back. The injections were given 3 times at 3-weeks intervals. Ninety days after the last injection a booster injection of 1 mg NIH-LH-S-17 dissolved in 0.5 ml 0.9% NaCl solution with 0.5 ml complete Freund's adjuvant was given intracutaneously. Ten days later blood was collected from the ear vein for estimation of antibody titer. Thereafter booster injections were given every month. A serum with a high antibody titer (anti-ovine-LH 610 V) was selected for further study. The antibody was used in an initial

Table 2-1 **Radioimmunoassay of rat LH.**
Influence of FSH and TSH on rat LH determination in an RIA system (OR rat LH RIA) with NIAMD-rat-LH I-1 ^{125}I as tracer and anti-ovine LH (610 V, 1/70000) as antiserum. FSH/LH and TSH/LH reflect the ratios of biologically assayed FSH (expressed as mg NIH-FSH-S1), respectively TSH (expressed as USP TSH) to LH (expressed as mg NIH-LH-S1).

preparation tested	bioassay					immunoassay
	O.A.A.D. NIH-LH-S1	HCG-AUGM. NIH-FSH-S1	McKenzie USP TSH	FSH/LH	TSH/LH	immuno potency relative to NIAMD-rat-LH I-1
NIAMD-rat-LH RP-1	0.03	0.54	0.22	18	7.3	0.02
NIAMD-rat-LH I-1	1.0	<0.04	0.4	<0.04	0.4	=1
NIAMD-rat-FSH RP-1	0.02	2.1	0.3	105	15	0.01
NIAMD-rat-FSH I-1	<0.002	± 100	-	>50000	-	<0.003
NIAMD-rat-TSH I-1	0.02	<0.1	35	<5	1750	0.1

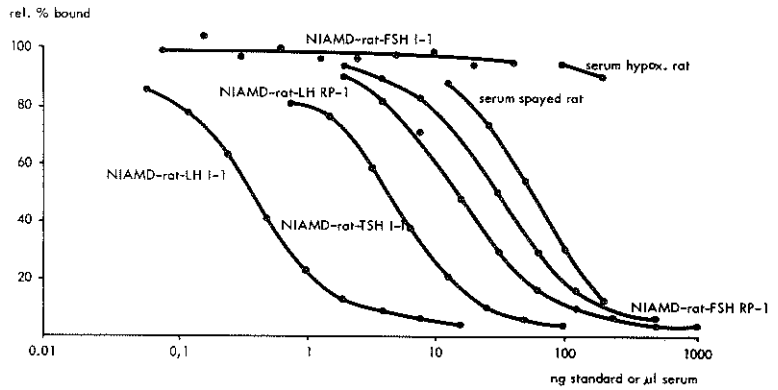


Fig. 2-1 Dose response curves of rat pituitary preparations and serum in an RIA system (OR rat LH RIA) with NIAMD-rat-LH I-1 ^{125}I as tracer and anti-ovine LH (610 V, 1/70000) as antiserum.

concentration of 1 to 70.000 resulting in a binding of approximately 25%.

Data on the specificity of this antibody for rat LH are given in Table 2-1. The dose response curves of a number of rat pituitary preparations with widely varying ratios of LH, FSH and TSH as known from bioassay data provided by the NIAMD run parallel except for that of NIAMD-rat-FSH I-1 (Fig. 2-1). The immunopotencies of these preparations were calculated relative to NIAMD-rat-LH I-1. Notwithstanding the extreme FSH/LH and TSH/LH ratios, the immunopotencies of these preparations were close to the LH bioassay data. Only in the preparation with a very high amount of TSH (NIAMD-rat-TSH I-1) was a five-fold higher LH immunopotency observed. Since such high TSH concentrations are only present in purified pituitary preparations it has been assumed that within the physiological range, interference by FSH and TSH is negligible.

The influence of serum was studied by comparing dose response curves of various sera with the rat pituitary standard. Although the dose response curve of the serum of a spayed rat seemed to run parallel to the rat standard

Table 2-2 Comparison between different RIA systems for rat LH.

assay system	sensitivity** mean ± S.D.	N	LH* estimated in standard serum (10-S) 100 µl mean ± S.D.	coeff. of variation***	N	200 µl mean ± S.D.	coeff. of variation	N
OO rat LH RIA	0.79 ± 0.34	3	40 ± 7.5	18.7%	3	37.2 ± 5.1	14.7%	3
RR rat LH RIA	16.6 ± 2.5	7	N.D.			95.0 ± 22.7	24 %	7
OR rat LH RIA	2.65 ± 0.34	14	31 ± 3.6	11.5%	14	31.7 ± 4.5	14.3%	14

* expressed in ng NIAMD-rat-LH RP-1/ml serum
** ng NIAMD-rat-LH RP-1 calculated at 90% relative binding
*** coefficient of variation = $\frac{\text{S.D.}}{\text{mean}} \times 100\%$

curve, repeated assays of this serum showed a significantly higher LH concentration at 100 µl (295 ± 5.1 ng rat-LH RP-1/ml) than at 50 µl (275 ± 4.5 ng rat-LH RP-1/ml). Also serum of a hypophysectomized animal showed slight displacement of iodinated rat LH from the antibody. This indicates that a serum component cross reacted with LH for the anti-ovine LH serum.

The sensitivity of this assay system is 2.65 ng rat-LH RP-1 (90% relative binding), which is six times higher than that of the RR rat LH RIA (Table 2-2).

In a standard serum (10-S) an LH concentration equivalent to 31 ng/ml (coefficient of variation 11.5%) and 31.7 ng/ml (coefficient of variation 14.3%) was estimated when assayed at dose levels of 100 and 200 µl respectively. These results agree with the data obtained with the very sensitive OO rat LH RIA of Niswender et al (1968[a]) (Table 2-2).

RR rat LH RIA.

This system made available by the NIAMD has been described by Monroe et al (1968). It was used for the serum LH determinations in the PMSG experiments (chapter 5). As already mentioned above the sensitivity of this system is low (16.6 ng NIAMD-rat-LH RP-1 at 90% relative binding). The LH in the standard serum (10-S) could only be detected at a dose level of 200 µl and resulted in an LH concentration of 95 ng/ml

(coefficient of variation 24%). This estimated concentration of LH is two to three times higher than that obtained with the OR and OO rat LH RIA (Table 2-2). This is in contrast with the observations of Niswender et al (1968[a]) and Monroe et al (1969), who observed that estimates of LH potency obtained with the OO and RR rat LH RIA systems were in good agreement. Hence, in our hands the RR rat LH RIA appears to overestimate the true LH values.

2-2-5 Radioimmunoassay of rat FSH.

This system has been described by Daane and Parlow (1971). Since its distribution by the NIAMD, it has been employed for measuring serum FSH in rats by many investigators.

Data on the specificity of this assay system for rat FSH are given in Fig. 2-2 and Table 2-3. The dose response curves of three rat pituitary preparations (rat-FSH RP-1, rat-LH RP-1 and the highly purified rat-FSH I-1) run parallel and the immunopotencies, as calculated from these curves, relative to NIAMD-rat-FSH RP-1 were close to the FSH bioassay data of these preparations. However, in the purified rat pituitary preparations with high ratios of LH/FSH (rat-LH

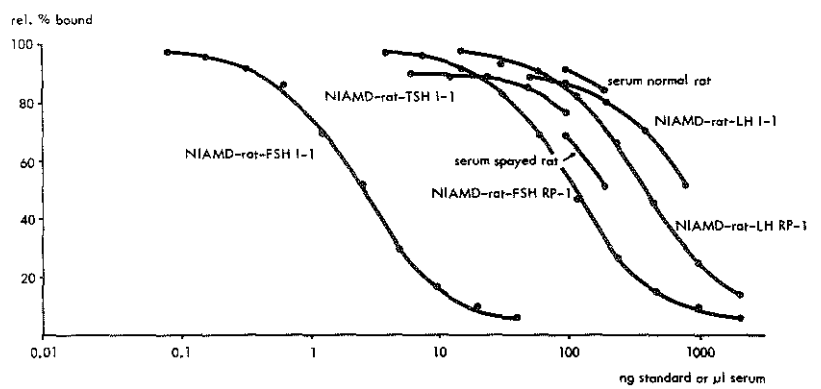


Fig. 2-2 Dose response curves of rat pituitary preparations and serum in an RIA system (RR rat FSH RIA) with NIAMD-rat-FSH I-1 ^{125}I as tracer and NIAMD-anti-rat-FSH S1 (1/875) as antiserum.

Table 2-3 Radioimmunoassay of rat FSH.
Influence of LH and TSH on rat FSH determination in an RIA system (RR rat FSH RIA) with NIAMD-rat-FSH I-1 ^{125}I as tracer and NIAMD-anti-rat-FSH S1 (1/875) as antiserum. LH/FSH and TSH/FSH reflect the ratios of biologically assayed LH (expressed as mg NIH-LH-S1), respectively TSH (expressed as USP TSH) to FSH (expressed as mg NIH-FSH-S1).

	bioassay					immunoassay
preparation tested	O.A.A.D. NIH-LH-S1	HCG-AUGM. NIH-FSH-S1	McKenzie USP TSH	LH/FSH	TSH/FSH	immuno potency relative to NIAMD-rat-FSH RP-1
NIAMD-rat-LH RP-1	0.03	0.54	0.22	0.06	0.4	0.60
NIAMD-rat-LH I-1	1.0	<0.04	0.4	>25	>10	0.27
NIAMD-rat-FSH RP-1	0.02	2.1	0.3	0.01	0.14	= 2.1
NIAMD-rat-FSH I-1	<0.002	± 100	-	<0.00002	-	93
NIAMD-rat-TSH I-1	0.02	<0.1	35	>0.2	>350	± 0.8

I-1) or TSH/FSH (rat-TSH I-1) cross reactions with LH and TSH were found. These results are in agreement with those of Seki et al (1971), who demonstrated good correlations between the FSH potency of pituitary extracts estimated by bioassay and by this RIA system.

An influence of serum has been reported by Swerdloff et al (1971) and Seki et al (1971). They demonstrated that the binding of the labelled hormone to the antibody was significantly inhibited when serum from hypophysectomized rats was added to the FSH assay. We also observed non-specific interference of serum. When sera with high FSH concentrations were measured at two dose levels lower FSH concentrations were found at 200 µl than at 100 µl, suggesting a partial inhibition of the displacement reaction in the presence of more serum. For this reason all serum samples were assayed at a constant serum volume of 100 µl.

The sensitivity of the FSH assay was 26.3 ng NIAMD-rat-FSH RP-1 (90% relative binding). This means that FSH concentrations below 200 ng FSH RP-1/ml are undetectable when 100 µl samples are used. For this reason FSH could only be detected in the standard serum (10-S) at a dose level of 200 µl (FSH concentration 174 ng NIAMD-rat-FSH RP-1/ml, coefficient of variation 18.4%).

Chapter 3

Ovarian activity in adulthood after neonatal administration of a high or a low dose of testosterone propionate

3-1 Introduction.

A single injection of TP in neonatal female rats may prevent ovarian cyclicity in adulthood (Barraclough, 1961; Gorski and Barraclough, 1963). In these studies the effectiveness of neonatal androgen treatment was established at one age only (usually 10 or 20 weeks) using the occurrence of prolonged vaginal cornification and the absence of corpora lutea in the ovaries as criteria. The dosage used by Barraclough (1961), 1250 µg TP on day 5, was effective in all the animals injected. When lower doses were used (5 or 10 µg TP on day 5) it was found that the incidence of anovulatory ovaries depended on the age at which the ovaries were examined. The proportion of anovulatory rats rose between the ages of 10 and 21-24 weeks (Swanson and van der Werff ten Bosch, 1964[a]). These observations indicated that with a small dose of TP some animals showed ovulatory cycles for some time after puberty, but stopped cycling at a later age while with a large dose of TP the animals became anovulatory earlier.

In order to study the development of the early-androgen syndrome in rats treated with a high dose of TP and in rats treated with a low dose of TP, it was necessary to study the extent to which a low dose of 1250 µg TP given on day 5 or 5 µg TP given on day 5 would affect ovarian cyclicity in our strain of rats (the R-Amsterdam substrain).

3-2 Experiment and results.

Female rats of the R-Amsterdam substrain were injected

on day 5 either with oil or with 5 or 1250 µg TP. Vaginal smears were made daily from day 50 till day 120. Thereafter some animals were killed for inspection of the ovaries.

All female rats treated with 1250 µg TP were anovulatory during the entire period studied as judged from the vaginal smears. These smears always contained cornified cells, sometimes mixed with non-cornified epithelial cells or leucocytes but cyclic variations, which in normal rats are indications of ovulation and corpus luteum formation, were lacking. At autopsy it appeared that the ovaries contained follicles and interstitial tissue, but no corpora lutea. In addition to follicles capable of ovulating with exogenous gonadotrophins (see chapter 6), many atretic follicles were present. Vaginal smears of the majority of the low dose TP-treated animals showed regular oestrous cycles from day 50 till day 60. No differences between low dose TP-treated

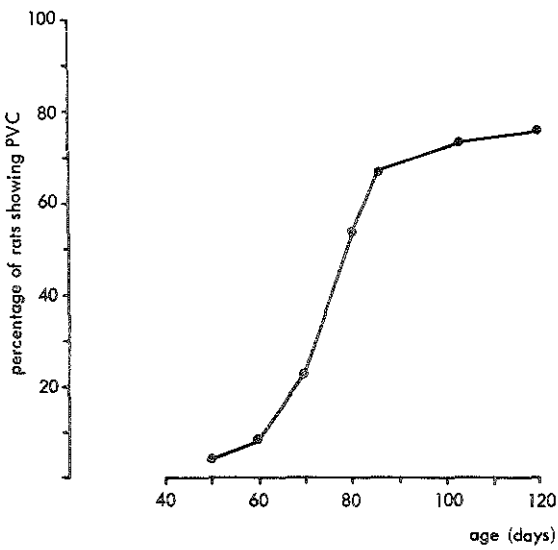


Fig. 3-1 **Percentage of rats showing persistent vaginal cornification (PVC) as a function of age in a group of 74 rats treated with 5 µg testosterone propionate on day 5 . PVC has been defined as the beginning of a period of at least one sequence of 10 consecutive days with cornified smears.**

rats and oil-treated rats were found in this period. However, with increasing age the cycles became irregular, sometimes with prolonged oestrous periods eventually resulting in persistent vaginal cornification (PVC) in nearly all animals. In Fig. 3-1 the percentage of rats showing PVC is given from day 50 till day 120. The ovaries of animals killed while vaginal cycles were normal contained follicles and corpora lutea. The ovaries of animals killed during the period of PVC contained exclusively follicles. No fresh corpora lutea were found.

In conclusion it can be said that high dose TP-treated rats were anovulatory at any time studied while most of the low dose TP-treated rats became anovulatory after a period of spontaneous ovulatory cycles. These findings are similar to those described earlier (Swanson and van der Werff ten Bosch, 1964[a,b]). In the R-Amsterdam substrain the majority of the 5 µg TP-treated rats became anovulatory between 70 and 90 days of age.

Chapter 4

Serum gonadotrophins and follicular development during the prepuberal period and the onset of puberty in female rats after neonatal administration of testosterone propionate

4-1 Introduction.

During the prepuberal period of the female rat several maturational processes take place in the brain, the hypophysis and the ovaries resulting in ovulation and oestrous cycles (for reviews see Donovan and van der Werff ten Bosch, 1965; Critchlow and Bar-Sela, 1967; Meijs-Roelofs, 1973).

In the hypophysis LH and FSH are present at birth. The LH content reaches maximal values in the period between days 30 and 40 (Lisk, 1968; Suzuki et al, 1971), while the FSH content reaches maximal values around day 25 (Corbin and Daniels, 1967; Kragt and Ganong, 1968; Watanabe and McCann, 1969; Suzuki et al, 1971). LH and FSH are present in the blood long before puberty occurs. The serum FSH concentrations reach a peak value on day 15 and decline after day 21 (Kragt and Dahlgren, 1972; Ojeda and Ramirez, 1972; Meijs-Roelofs et al, 1973[b]).

In the brain hypothalamic mechanisms regulating gonadotrophin release develop. An inhibitory ovarian feedback on gonadotrophin release is operative shortly after birth (Goldman and Gorski, 1971; Caligaris et al, 1972; Meijs-Roelofs et al, 1973[a]). The presence of a stimulatory feedback action of ovarian steroids on gonadotrophin release can be demonstrated from day 20 onwards (Caligaris et al, 1972, 1973).

In the ovaries a number of follicles start to grow between birth and day 10. Thereafter growth continues and small antral follicles appear. The follicles increase in volume to large antral follicles after day 20 (for review see

Critchlow and Bar-Sela, 1967).

The question arises as to how these maturational processes develop after neonatal administration of TP. Data on the influence of neonatal androgen on the various maturational processes during the prepuberal period are limited. Compared to normal rats ovarian weights of androgenized rats were lower 10 days after a single injection of 1500 µg TP on day 5 (Jacobsohn, 1964) and after an injection of 100 µg TP (Matsuyama et al, 1966). In mice, Peters et al (1970) observed a reduced number of small oocytes in the ovaries two days after injection of 1 mg TP on day 5 and a reduced number of growing follicles at 28 days of age. Pituitary LH content was lower than in normal rats on days 15, 21 and 28 (100 µg TP on day 5; Matsuyama et al, 1966) and day 30 (1250 µg TP on day 5; Barraclough, 1966[b]). Serum LH concentrations lower than those in untreated rats have been found by Weisz and Ferin (1970) on days 7 to 21 (100 µg TP on day 5) and lower serum FSH concentrations by Johnson (1971) on day 30 and thereafter (50 µg TP on day 5).

In androgen-treated rats as compared to normal rats the first cornification of the vagina occurs earlier (Jacobsohn, 1964) and uterine weights before "puberty" are higher (Johnson, 1972). Therefore, it has been assumed that early-androgen administration accelerates sexual maturation (van der Werff ten Bosch et al, 1971).

In order to study the influence of neonatal administration of TP on sexual maturation prepuberal serum levels of FSH and LH, follicular development and the age at which vaginal opening, first oestrous smear and first ovulation occurs are studied in rats treated with either a low or a high dose of TP.

4-2 Experiments and results.

4-2-1 Effect of neonatal administration of TP on serum gonadotrophins and follicular development during the prepuberal period.

Female rats of the R-Amsterdam substrain were injected on day 5 with oil, 5 µg TP or 1250 µg TP between 09.00 and 10.00 hrs. Six litters of each treatment group were formed, each consisting of 8 animals. Starting on day 5 and continuing on every fifth day till day 35, one animal of each litter was killed at 15.00 hrs. Blood was collected for LH and FSH determination. Body weight, ovarian weights and vaginal opening were recorded at autopsy. Ovaries were fixed in Bouin's fluid, sectioned at 5 µ and serial sections were mounted for counting follicles.

Follicular volumes were determined by the method of Boling et al (1941), modified in two ways: (1) two diameters were measured in the section in which the nucleolus of the ovum was found. (2) the third diameter was substituted by the mean of the two other diameters. Only follicles with a volume exceeding 100 x 10^5 μm^3 were counted in order to compare the results with those obtained in a previous study in untreated prepuberal rats (Meijs-Roelofs et al, 1973[b]). These follicles are all antral follicles, and their incidence varies during the oestrous cycle in adult rats (Mandl and Zuckerman, 1952; Welschen, 1973). Three categories were distinguished: (1) 100 - 249 x 10^5 μm^3, (2) 250 - 499 x 10^5 μm^3, (3) $\geq$ 500 x 10^5 μm^3. In the oestrous cycle follicles of the last category will ovulate after HCG treatment (Welschen and Rutte, 1971). To establish the numbers of follicles, rats with ovaries containing corpora lutea have been excluded.

Both FSH and LH were measured in peripheral blood of individual animals except for animals younger than 10 days of age, in which case blood from 2 to 10 animals was pooled. An additional group of similarly treated animals was used for blood collection till day 10. Serum LH was determined by the OR rat LH RIA, serum FSH by the RR rat FSH RIA. Serum samples were assayed in duplicate in an aliquot of 100 µl. LH concentrations are expressed in ng NIAMD-rat-LH RP-1/ml, FSH concentrations in ng NIAMD-rat-FSH RP-1/ml.

Data on body weight, ovarian weights and uterine weight

Table 4-1 Body weight, ovarian and uterine weights of female rats treated on day 5 with oil, 5 or 1250 µg testosterone propionate (mean ± S.E.M.).

treatment on day 5	age (days)	vaginal opening*	no. of rats	body weight (g)	ovarian weights (mg)	uterine weight (mg)
oil	5	-	6	9.5 ± 0.2	0.47 ± 0.03	2.22 ± 0.16
	10	-	5	17.2 ± 1.0	1.32 ± 0.18	6.27 ± 0.99
	15	-	6	27.8 ± 1.2	3.37 ± 0.30	16.6 ± 1.2
	20	-	6	38.2 ± 1.5	7.49 ± 0.17	19.1 ± 0.9
	25	-	6	46.9 ± 1.3	14.1 ± 0.2	20.0 ± 0.8
	30	-	6	64.4 ± 2.2	17.7 ± 0.5	32.4 ± 1.6
	35	-	6	84.4 ± 2.3	22.0 ± 0.5	54.0 ± 5.5
5 µg TP	5	-	6	8.5 ± 0.2	0.38 ± 0.03	1.96 ± 0.09
	10	-	7	15.7 ± 0.8	1.05 ± 0.07	5.33 ± 0.34
	15	-	6	26.4 ± 0.5	3.17 ± 0.18	15.1 ± 0.7
	20	-	6	40.2 ± 1.5	8.06 ± 0.94	21.9 ± 1.0
	25	-	6	46.8 ± 2.7	14.1 ± 0.7	20.1 ± 1.2
	30	-	6	63.8 ± 2.1	18.3 ± 0.7	32.8 ± 1.6
	35	-	2	85.3 ± 2.2	21.4 ± 0.3	69.5 ± 30.5
		±	2	86.8 ± 1.7	22.0 ± 0.8	96.0 ± 0.8
		+	2	85.0 ± 2.5	26.5 ± 4.8	129.7 ± 18.0
1250 µg TP	5	-	8	10.0 ± 0.2	0.49 ± 0.05	2.55 ± 0.12
	10	-	8	16.9 ± 0.6	0.98 ± 0.04	5.43 ± 0.34
	15	-	6	27.5 ± 1.0	**1.72 ± 0.17**	**10.4 ± 0.5**
	20	-	7	38.1 ± 1.1	**4.45 ± 2.26**	17.5 ± 0.9
	25	-	6	49.0 ± 1.4	**12.4 ± 0.2**	19.6 ± 0.9
	30	-	3	63.7 ± 1.9	15.7 ± 0.4	34.2 ± 4.4
		pinhole	3	70.5 ± 2.8	16.5 ± 0.2	39.5 ± 0.8
	35	pinhole	3	86.5 ± 3.3	19.1 ± 2.7	79.2 ± 13.4
		+	3	99.3 ± 8.6	24.5 ± 3.0	154.7 ± 10.1

* vaginal opening is indicated by: - (closed vagina), ± (partial vaginal opening), + (open vagina) and "pinhole" (small vaginal opening in a flat vaginal membrane)
bold face means significantly different from the corresponding oil-treated group ($P<0.05$ Student's t-test)
statistics were not performed in groups smaller than 5 animals

are given in Table 4-1.

On day 35 the condition of the vagina appeared different in the three treatment groups. Since in the normal female rat differences in vaginal development reflect differences in maturational stage, the data of rats killed at day 35 have been subdivided according to the state of vaginal development. Oil-treated animals had a closed vagina on day 35. Two of the six 5 µg TP-treated rats had a closed vagina,

two rats showed an indication of vaginal opening such as is usually seen in normal rats on the day before first ovulation and two rats showed a well developed, open vagina. In the rats treated with 1250 µg TP vaginal development was abnormal. As early as day 30, 3 out of 6 animals showed a "pinhole" in a flat vaginal membrane, while on day 35, 3 out of 6 animals had an open vagina and the remaining three the "pinhole" type of vaginal opening.

Body weights of the three treatment groups were not different during the period studied.

Ovarian weights of oil- and 5 µg TP-treated animals were not different. On days 15,20, and 25 ovarian weights of the 1250 µg TP-treated groups were significantly lower than those of both control and 5 µg TP-treated animals.

Uterine weights of all rats increased till day 20, then remained constant till day 25 and increased further with a sharp rise in weight at vaginal opening. No differences were found between the different treatment groups, except on day 15, when in 1250 µg TP-treated rats a lower uterine weight was recorded and on day 35, when uterine weights of TP-treated rats were higher than in control rats.

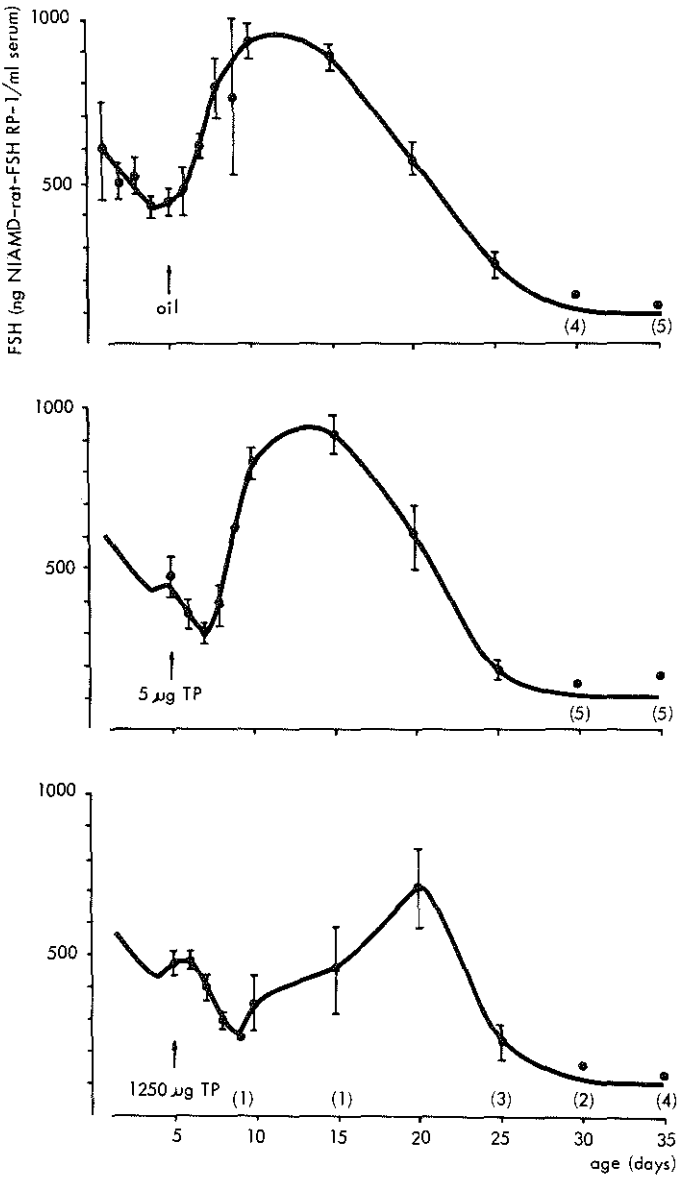
The results of follicular measurements are given in Table 4-2. In oil-treated rats the first follicles of the category 100 - 249 x 10^5 μm^3 appeared on day 20. On day 25 their number had increased and a maximum was reached on day 30. Follicles of the second volume class (250 - 499 x 10^5 μm^3) appeared on day 25 and remained constant in number till day 35. Large follicles ($\geq$ 500 x 10^5 μm^3) were only occasionally found. In the 5 µg TP-treated rats the pattern was essentially the same. Only the numbers of small follicles (100 - 249 x 10^5 μm^3) were slightly smaller during the period studied. In the 1250 µg TP-treated rats the pattern was different. Significantly smaller numbers of the small follicles were present on days 20 and 30, while on day 35 follicles $\geq$500 x 10^5 μm^3 were present in a significantly higher number than in the two other treatment groups.

Table 4-2 Numbers of follicles of various volume classes in one ovary of rats treated on day 5 with oil, 5 or 1250 µg testosterone propionate. Numbers in parentheses are numbers of animals.

Follicular volume	treatment on day 5	mean numbers of follicles in 1 ovary (± S.E.M.)				
		day 15	day 20	day 25	day 30	day 35
100 - 249 $\times 10^5$ µm^3	oil	-	2.5 ± 0.8 (6)	11.6 ± 1.9 (5)	16.2 ± 1.2 (6)	10.8 ± 2.9 (5)
	5 µg TP	-	2.0 ± 0.5 (6)	9.2 ± 1.1 (6)	15.5 ± 2.5 (6)	8.8 ± 2.6 (4)
	1250 µg TP	-	**0.2 ± 0.2** (6)	9.3 ± 1.4 (6)	**9.2 ± 1.6** (5)	8.2 ± 1.2 (5)
250 - 499 $\times 10^5$ µm^3	oil	-	0.2 ± 0.2	6.6 ± 1.3	5.5 ± 1.6	5.4 ± 1.6
	5 µg TP	-	-	8.0 ± 1.3	6.8 ± 1.7	7.7 ± 0.6
	1250 µg TP	-	-	7.0 ± 1.1	7.4 ± 1.5	5.8 ± 1.5
≥ 500 $\times 10^5$ µm^3	oil	-	-	1.0 ± 0.4	-	1.0 ± 0.5
	5 µg TP	-	-	1.0 ± 0.8	-	1.3 ± 0.5
	1250 µg TP	-	-	1.5 ± 0.6	0.8 ± 0.4	**5.2 ± 1.3**

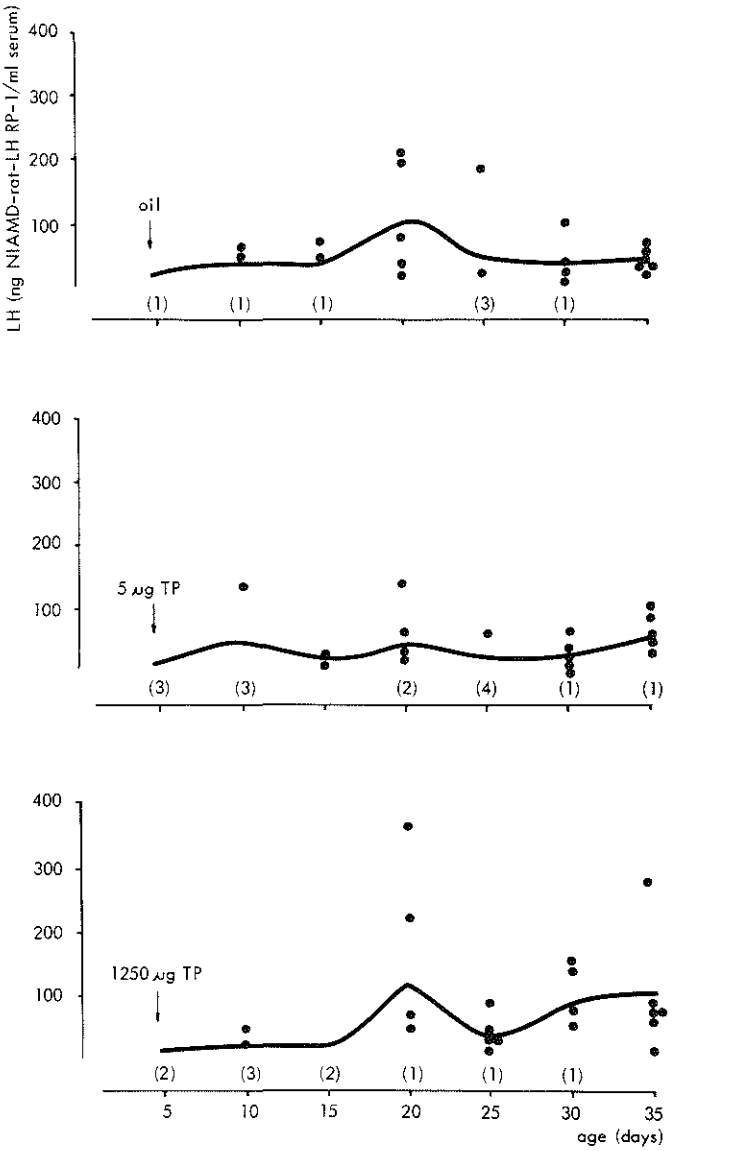
bold face mean significantly different from oil-treated group ($P<0.05$ Student's t-test)

Serum FSH concentrations measured in these animals, supplemented by data obtained on similarly treated rats killed at other ages, are presented in Fig. 4-1. In control animals serum FSH concentrations were lower on day 5 (500 ng/ml) than on days 10-15 (1000 ng/ml). Thereafter, values decreased gradually to almost undetectable values on days 30 and 35 (< 100 ng/ml). Note that serum FSH concentrations measured with the same method during the oestrous cycle varied from 100 ng/ml at dioestrus to 500 ng/ml during late prooestrus and early oestrus. Following the day of injection (day 5) 5 µg TP-treated rats showed lower serum FSH concentrations on day 7 than control rats (290 ng/ml vs 597 ng/ml). From day 10 onwards the FSH concentrations were not different from those of control rats. Injection of 1250 µg TP on day 5 resulted in lower FSH concentrations than in oil-treated control rats for a much longer period than in 5 µg TP-treated rats. The lowest concentration was found on day 9 (238 ng/ml). On day 20 the FSH concentrations were not different from those of the control rats. From this day on the FSH pattern was similar to that of oil- or 5 µg TP-treated rats.



() numbers of samples with undetectable levels (<100 ng/ml)

Fig. 4-1 Serum FSH concentrations in prepuberal female rats treated on day 5 with oil, 5 or 1250 µg testosterone propionate (TP). Each point before day 10 represents the mean (± S.E.M.) of 2-4 pools of blood (2-10 animals). From day 10 onwards each point represents the mean (± S.E.M.) of individual samples of 5-7 animals.



() numbers of pools or individual samples with undetectable levels (<20 ng/ml)

Fig. 4-2 Serum LH concentrations in prepuberal female rats treated on day 5 with oil, 5 or 1250 µg testosterone propionate (TP). Each point represents one determination. Before day 10 pools were used of 2-10 animals. On day 10 and thereafter samples of individual animals were used. The curve represents the mean levels.

Serum LH concentrations are given in Fig. 4-2. Control rats showed, in contrast to the regular FSH pattern with high levels before day 25, LH levels with much variation. The levels were generally low (< 100 ng/ml).
Only on day 20 a few rats showed high LH concentrations. Note that serum LH concentrations during the oestrous cycle, measured with the same method, varied from <50 ng/ml in dioestrus to >1000 ng/ml during late prooestrus. In 5 and 1250 µg TP-treated rats serum LH concentrations were also low. An influence of neonatal TP on serum LH could not be detected.

Body weights, ovarian weights, uterine weights and serum gonadotrophin levels of oil-treated animals are in agreement with the data obtained in a previous study in untreated rats (Meijs-Roelofs et al, 1973[b]). Also the measurements of follicular development are essentially the same.

4-2-2 Effect of neonatal administration of TP on the onset of puberty.

This experiment was carried out to investigate more systematically the time of puberty in TP-treated rats. 9 Animals of each treatment group were inspected daily to see whether the vagina was open. From the day of vaginal opening vaginal smears were taken daily for about 10 days. Then the animals were killed at oestrus and the number of tubal ova counted.

Table 4-3 Effect of neonatal administration of testosterone propionate on vaginal opening and ovarian function (mean ± S.E.M.).

treatment on day 5	number of animals	"pinhole" vagina at age (days)	"pinhole" vagina at body weight (g)	vaginal opening and first oestrous smear at age (days)	vaginal opening and first oestrous smear at b.w. (g)	rats with oestrous cycles days 38-48	number of ova at oestrus
oil	9	-	-	38.2 ± 0.5	89.9 ± 3.1	100 %	10.5 ± 0.4 (6)
5 µg TP	9	-	-	37.7 ± 0.4	89.6 ± 3.6	100 %	10.0 ± 0.2 (6)
1250 µg TP	9	31.3 ± 0.2	63.1 ± 1.0	38.2 ± 0.4	93.3 ± 1.8	0 %	-

The results are given in Table 4-3. In oil-treated animals vaginal opening occurred at 38.2 days of age at a mean body weight of 89.9 g. All these animals showed a cornified vaginal smear on the day of vaginal opening. In 5 µg TP-treated rats vaginal opening and first observed vaginal cornification occurred at an age (37.7 days) and body weight (89.6 g) not different from those of oil-treated rats. Note that in the previous experiment the vaginal development of the 5 µg TP-treated rats was slightly accelerated. All 5 µg TP-treated animals showed regular 5-day cycles between days 38 and 48. The number of ova of these animals, killed at oestrus, were also similar to those of oil-treated rats. In 1250 µg TP-treated rats an abnormal vaginal development was observed. As in the previous experiment, a small hole was seen in a flat vaginal membrane (a "pinhole" vagina) at an early age (31.3 days) and at a low body weight (63.1 g). The vaginal smears taken daily from this "pinhole" vagina showed only a few epithelial cells and a few leucocytes without daily changes till, at an age of 38.2 days and a body weight of 93.3 g, not different from those of oil- and 5 µg TP-treated rats, the vaginal opening increased to normal size. From this day on the vaginal smears were continuously cornified. At autopsy 10 days later the ovaries contained no corpora lutea.

4-3 Discussion

Following TP administration on day 5 a decrease in serum FSH concentration has been seen. Goldman and Gorski (1971) found decreased serum FSH concentrations 8-9 hrs after a single dose of TP on day 6 or after a single injection of OB on day 5 or 7. The present data show that the fall is temporary and presumably due to an inhibitory action of the injected TP. The existence in female rats of an inhibitory steroidal feedback before day 20 has been demonstrated for oestrogen in a previous report (Meijs-Roelofs et al, 1973[a]). In that study it was shown that ovariectomy on day 13

resulted in an increased FSH level two days later as compared to the control value. This increase was not observed when ovariectomy was followed by daily injections of 0.1 µg OB/100 g body weight.

The high FSH concentrations found in normal rats before day 20 may be of importance for follicular growth. This view is in agreement with findings of Eshkol et al (1970) who observed in mice a decreased follicular cell proliferation and organization at 7 and 14 days of age after daily injections of anti-gonadotrophic serum given from the day of birth. When FSH was given together with anti-gonadotrophic serum the follicular cell proliferation appeared normal. Fig. 4-3, which depicts the data on serum FSH concentrations and on numbers of follicles, shows for control rats a serum FSH peak on days 10-15 and a peak in the number of antral follicles in the volume class of 100 - 249 x 10^5 μm^3 on day 30. In the mouse the development of primordial follicles to antral follicles takes about 12-16 days (Pedersen, 1969). If the rat ovary undergoes changes parallel to the ovary of the mouse, the high FSH concentration around day 12 might stimulate massive growth of primordial follicles. The absence of the FSH peak in the 1250 µg TP-treated rats might then result in the reduction of the number of antral follicles in the volume class of 100 - 249 x 10^5 μm^3 noted on day 30. Another explanation is given by Peters et al (1970). These authors found after injection of 1000 µg TP in day 5 old mice a reduction in the number of growing follicles on day 28. They suggested that this effect was due to an accelerated reduction of the number of small oocytes observed two days after TP administration.

The reduction of the number of small follicles (100 - 249 x 10^5 μm^3) may be reflected in the lower ovarian weights on day 20 and thereafter following injection of 1250 µg TP. The lower ovarian weights before day 20 are probably caused by a reduction in the number of follicles < 100 x 10^5 μm^3.

The low uterine weight in the 1250 µg TP-treated rats on day 15 suggests a low oestrogen concentration in the blood

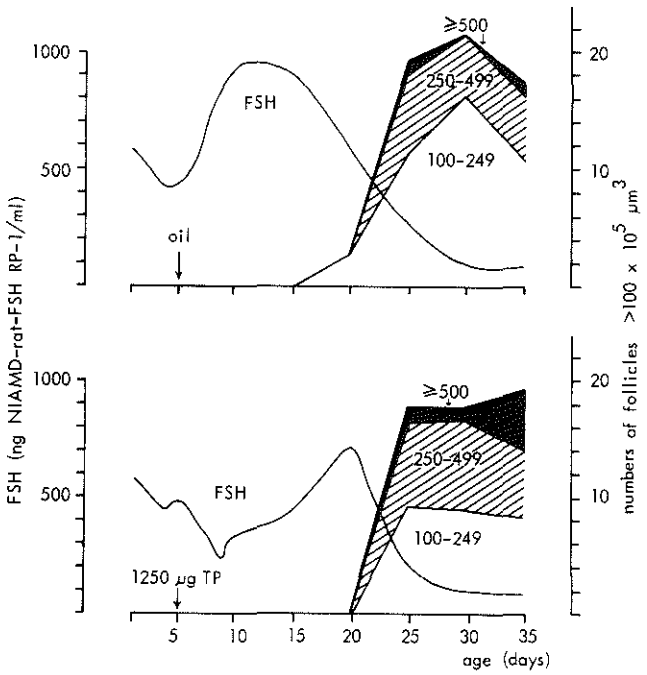


Fig. 4-3 Serum FSH concentrations and numbers of follicles of various volume classes in one ovary in prepuberal female rats treated on day 5 with oil or 1250 µg testosterone propionate.

In these rats. Such a low oestrogen concentration together with the observed decreased FSH concentration might also explain the retarded follicular growth. Goldenberg et al (1972) demonstrated that oestrogen increases both granulosa proliferation and incorporation of FSH in the follicles and in this way stimulates follicular growth. In addition Reiter et al (1972) found a significant decrease in follicular growth in immature rats treated with specific anti-oestradiol serum for periods of 4 days.

On day 35 the ovarian development in the rats treated with a high dose of TP seemed to be accelerated. Follicles in the largest volume class ($\geq$ 500 x 10^5 μm^3) were present in a significantly higher number in the 1250 µg TP-treated rats than in the 5 µg TP- or oil-treated rats. Also uterine weights were

higher and the vaginal development had been accelerated. An earlier vaginal opening after neonatal TP-treatment has been described previously (Segal and Johnson, 1959; Tramezzani et al, 1963; Jacobsohn, 1964; Forsberg et al, 1966). Although this suggests an earlier sexual maturation, vaginal opening is not a useful index of sexual maturation in these rats, since it has been demonstrated that TP itself causes the breakdown of the vaginal orifice (Jacobsohn 1964, Jacobsohn and Norgren 1965). A "pinhole" vaginal opening was seen in our experiments at a very early age and at low body weight in rats treated with a high dose of TP. However, the vaginal opening increased to normal size and the first cornified smears were obtained at an age and body weight not different from that of oil- and 5 µg TP-treated rats. This latter finding is in contrast to that of other investigators, who observed cornified smears a few days earlier in TP-treated rats than in control rats (Jacobsohn, 1964: 1500 µg TP on day 5; Sheridan et al, 1973[a]: 1 µg TP daily for the first 10 days of life). The presence of a higher number of large follicles on day 35 in the high dose TP-treated rats favours the concept of an accelerated sexual maturation. However, ovulation, which is in normal female rats, the culmination of sexual maturation cannot be achieved in high dose TP-treated rats. Therefore the present finding indicates that an accelerated sexual maturation is limited to an accelerated follicular growth.

Chapter 5

Induction of ovulation by PMSG in androgen-treated prepuberal rats

5-1 Introduction.

One of the maturational processes taking place in the female rat brain during the prepuberal period is the maturation of a mechanism for induction of ovulatory surges of LH. Such a mechanism is already present before puberty. Ovulation and corpus luteum formation can be induced by a single injection of OB (Hohlweg, 1934; Dörner and Döcke, 1964; Ying and Greep, 1971[a]) or PMSG (Cole, 1936; Zarrow and Quinn, 1963). The ovulation inducing effect of these agents is mediated by an endogenous ovulatory LH surge on the second day after administration of OB or PMSG. This was demonstrated by well-timed pentobarbital administration (Ying and Greep, 1971[b], in OB-treated rats; Strauss and Meyer, 1962; McCormack and Meyer, 1962, in PMSG-treated rats) and by direct measurements of serum LH (Ying et al, 1971 in OB-treated rats; Ying and Meyer, 1972; Sorrentino et al, 1972 in PMSG-treated rats). The presence of the mechanism inducing ovulatory surges of LH can be demonstrated from day 20 onwards. From that age on, ovulation can be induced by OB (Ying and Greep, 1971[b]) or PMSG (Zarrow and Quinn, 1963; McCormack and Meyer, 1964) and a rise in serum LH can be induced by administration of progesterone to OB-primed rats (Caligaris et al, 1972).

It is not known whether the development of this brain mechanism is disrupted by neonatal treatment with androgen. In the previous chapters it was shown that early administration of a single large dose of TP causes the anovulatory state from the time of vaginal opening, while a single small dose (5 µg TP on day 5) causes the anovulatory state after an initial period of ovarian cyclicity. Brown-Grant et al (1964)

demonstrated that no ovulation could be induced with PMSG in 30 day old rats, which had received 1250 µg TP on day 4. Also, Dörner and Döcke (1964) could not obtain corpus luteum formation after an injection of OB in rats treated with 1250 µg TP on day 3.

In this chapter the effects of PMSG administration to TP-treated prepuberal rats were studied to answer the following questions.

1) Can ovulation be induced with PMSG in animals treated neonatally with a low dose of TP?
2) Is the response to PMSG comparable to that in control rats?
3) If not, is the difference from normal animals due to changes in the mechanism controlling the release of LH or to a change in ovarian responsiveness?
4) Furthermore, is there a shift in the response to different doses of PMSG or in the age at which maximal response occurred that could be attributed to an acceleration of sexual maturation?

A part of this study has been published in the Journal of Endocrinology (Uilenbroek and van der Werff ten Bosch, 1972).

5-2 Experiments.

5-2-1 Effects of PMSG.

Female rats of the R-Amsterdam substrain were injected on day 5 with oil, 5 or 1250 µg TP. On day 30 all animals received 30 IU PMSG between 09.00 and 11.00 hrs. Body weights ranged from 65 to 70 g. Three groups of animals (A, B and C) were formed each consisting of oil, 5 or 1250 µg TP-treated rats. Group A and B were killed on day 30, 31, 32 or day 33 between 11.00 and 13.00 hrs.

From group A body weight, ovarian weights and uterine weight were recorded.

From Group B the adenohyphophysis was dissected, weighed on a torsion balance, quickly frozen and stored at -20°C for

LH determination.

From Group C blood was collected for serum LH determination on day 32 (two days after PMSG injection) at either 14.00 hrs, 15.00 hrs, 16.00 hrs or 18.00 hrs. The animals were bled only once in order to keep any possible disturbance of the ovulatory response as small as possible. On day 33 ova in the fallopian tubes were counted.

Prior to determination of the LH content the hypophyses were thawed and homogenized in 10 ml 0.01 M PBS (pH 7.6). The homogenates were then centrifuged for 15 min and the supernatants stored at $-20^{o}C$ until assayed. LH concentrations were estimated by the OR rat LH RIA (see chapter 2) at three dose levels in appropriate dilutions. The amounts of LH are expressed in µg NIAMD-rat-LH RP-1 per gland.

Serum LH concentrations were estimated by the RR rat LH RIA (see chapter 2). This relatively insensitive assay system was chosen because in a pilot experiment it had appeared that the antiserum used in the RR rat LH RIA did not crossreact with PMSG. In contrast, the OR rat LH RIA was not able to discriminate rat LH from the injected PMSG. This is of particular importance because the half-life time of PMSG is rather long (Parlow and Ward, 1961). Serum LH concentrations are expressed in ng NIAMD-rat-LH RP-1 per ml serum. Duplicate volumes were assayed at two different dose levels (100 and 200 µl).

Data on body weight, ovarian weights, uterine weight, adenohypophysial weight, pituitary LH content and ovulation response obtained in the three groups A,B and C are combined in Table 5-1.

In control rats (oil-treated neonatally) the dose of 30 IU PMSG uniformly resulted in ovulation with an average number of 10.1 ova per animal. In rats treated with 5 µg TP 33% of the animals responded to 30 IU PMSG, whilst the average number of ova per ovulating rat was 2.9. Animals treated with 1250 µg TP did not ovulate in response to 30 IU PMSG on day 30.

Ovarian weights increased rapidly in all three treatment groups (oil, 5 and 1250 µg TP) following PMSG administration.

Table 5-1 Body weight, ovarian weights, uterine weight, adenohypophysial weight, pituitary LH content and ovulation response after administration of 30 IU PMSG on day 30 in rats treated on day 5 with oil, 5 or 1250 µg testosterone propionate (mean ± S.E.M.). Data combined from 3 different experiments. Numbers in parentheses are numbers of animals.

treatment on day 5	age (days)	body weight (g)	ovarian weights (mg)	uterine weight (mg)	adenohyp. weight (mg)	adenohyp. LH content** (µg)	rats that ovulated number	%	ova per ovulating rat
oil	30	61.3 ± 2.1 (7)	15.5 ± 0.5 (7)	26.9 ± 2.1 (7)	2.49 ± 0.17 (5)	122 ± 21 (5)	-	-	-
	31	64.7 ± 2.7 (6)	30.2 ± 0.9 (6)	55.9 ± 5.3 (6)	2.83 ± 0.15 (3)	113 ± 17 (5)	-	-	-
	32	65.2 ± 1.4 (9)	61.3 ± 1.6 (9)	92.4 ± 2.3 (9)	3.31 ± 0.06 (5)	134 ± 17 (5)	-	-	-
	33	63.3 ± 1.1 (6)	100.6 ± 8.0 (6)	102.7 ± 0.9 (6)	3.42 ± 0.06 (5)	95 ± 15 (5)	27/27	100	10.1 ± 1.3
5 µg TP	30	55.5 ± 1.3 (4)	17.4 ± 0.6* (4)	26.4 ± 1.9 (4)	2.89 ± 0.14 (5)	140 ± 3 (5)	-	-	-
	31	60.5 ± 2.7 (6)	26.6 ± 1.2* (6)	67.1 ± 3.6 (6)	3.22 ± 0.10 (5)	143 ± 11 (5)	-	-	-
	32	64.7 ± 2.5 (6)	49.9 ± 1.4* (6)	89.8 ± 3.4 (6)	3.35 ± 0.18 (5)	139 ± 21 (5)	-	-	-
	33	-	92.5 ± 5.2 (7)	111.5 ± 5.2 (7)	3.62 ± 0.07 (5)	137 ± 6* (5)	9/27	33	2.9 ± 0.6
1250 µg TP	30	63.7 ± 1.8 (6)	12.7 ± 0.3* (6)	24.1 ± 1.3 (6)	2.64 ± 0.12 (5)	66 ± 11* (4)	-	-	-
	31	64.0 ± 1.5 (6)	24.9 ± 1.1* (6)	55.9 ± 5.9 (6)	3.07 ± 0.16 (5)	90 ± 15 (5)	-	-	-
	32	68.7 ± 1.8 (7)	52.9 ± 1.8* (7)	100.3 ± 2.5*(7)	3.14 ± 0.10 (4)	108 ± 25 (5)	-	-	-
	33	69.7 ± 2.0 (6)	76.4 ± 4.8* (6)	106.4 ± 2.5 (6)	3.26 ± 0.12 (4)	53 ± 10* (5)	0/29	0	-

* significantly different from the corresponding oil-treated group ($P \leq 0.05$ Student's t-test)
** expressed in µg NIAMD-rat-LH RP-1

However, ovarian weights of the two TP-treated groups were subnormal on days 31 and 32. The subnormal ovarian weights on day 33 in the group treated with 1250 µg TP probably reflects the absence of corpora lutea in these rats.

Uterine weights also increased following PMSG administration. No systematic differences appeared between the three treatment groups.

Adenohypophysial weights increased in all three groups during the four days studied. Whether this is due to the PMSG injection is not known, since no saline injected animals were included.

Hypophysial LH content remained constant in the control and 5 µg TP-treated groups. Between days 30 and 33 the LH content in the 1250 µg TP-treated rats tended to be lower than in oil- and 5 µg TP-treated rats. However, only on days 30 and 33 the difference was significant.

Serum LH concentrations in control animals were below the detectable level on days 30 and 31. On day 32, the day preceding ovulation, control animals showed an increase of

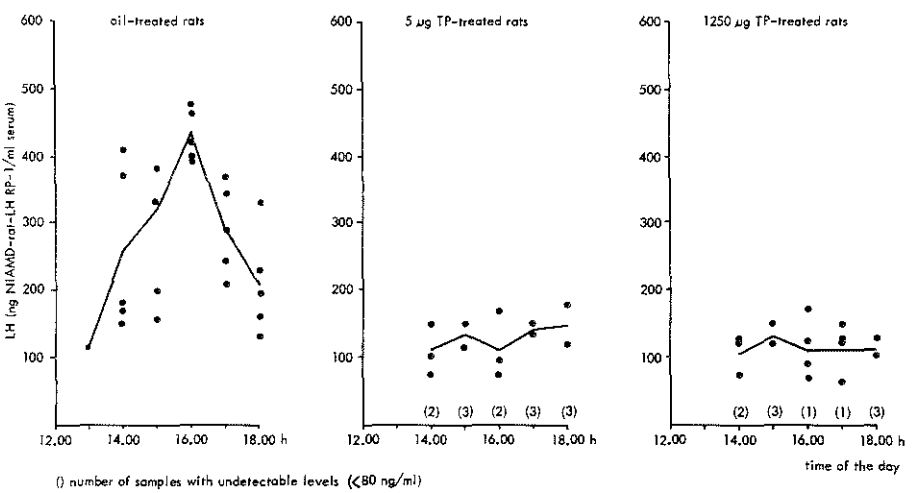


Fig. 5-1 Serum LH concentrations two days after administration of 30 IU PMSG on day 30 in rats treated on day 5 with oil, 5 or 1250 µg TP. Each point represents the LH concentration of one individual animal.

LH during the afternoon with a mean maximal value of 431 ng/ml at 16.00 hrs (Fig. 5-1). The 5 and 1250 µg TP-treated groups did not show an increase of LH on day 32 between 14.00 hrs and 18.00 hrs.

It is concluded that the lowered ovulation response seen in the TP-treated rats on day 33 after PMSG administration on day 30 is due to a lowered release of LH on day 32.

5-2-2 Effect of progesterone on PMSG induced ovulation.

Progesterone injected two days after PMSG administration has been reported to result in an increase of the ovulatory response (McCormack and Meyer, 1963, 1964). Zarrow and Hurlbut (1967) obtained maximal effect of progesterone with a single injection of 0.5-1 mg 48-56 hrs after administration of PMSG. It has been suggested that progesterone acts on the neural machanism controlling ovulatory LH release (Kaasjager et al, 1971; Taleisnik et al, 1970). Since this mechanism may be affected by neonatal TP-treatment it seemed important to establish whether progesterone could also increase the PMSG-induced ovulation response in TP-treated rats.

In this and the following experiments of this chapter female rats of the "Endo" substrain were used. Animals of the three treatment groups (oil, 5 and 1250 µg TP) were given 30 IU PMSG on day 28 between 08.00 and 09.00 hrs. The body weight of the animals ranged from 50-70 g. On day 30, 52 hrs after PMSG administration, animals of each treatment group received a single s.c. injection of 0.5 mg progesterone. The animals were killed the following morning and the number of ova was counted.

The results are given in Table 5-2. A comparison is first made of the data obtained with PMSG alone in the substrain used in this experiment and those obtained in the substrain used in the previous experiment (Table 5-1 and Table 5-2).

Table 5-2 **Effect of progesterone on induction of ovulation by 30 IU PMSG on day 28 (52 h before progesterone) in rats treated on day 5 with oil or testosterone propionate (TP).**

treatment on day 5	progesterone (0.5 mg)	rats that ovulated number	%	ova per ovulating rat (mean ± S.E.M.)
oil	-	18/18	100	40.9 ± 3.0
5 µg TP	-	5/14	36	2.6 ± 1.0
1250 µg TP	-	8/15	53	2.5 ± 0.3
oil	+	21/21	100	34.6 ± 3.0
5 µg TP	+	21/23	91	14.5 ± 3.1*
1250 µg TP	+	3/16	19	2.7 ± 1.2

* significantly different from 5 µg TP-treated rats without progesterone ($P<0.01$ Mann-Whitney U test)

With both substrains all control animals ovulated in response to a dose of 30 IU PMSG given on day 28. The number of ova released per rat however, was considerably higher in the "Endo" substrain (40.9 vs 10.1). The response to PMSG obtained in 5 µg TP-treated rats was low (36% ovulation, 2.6 ova/ovulating rat) and not different from that obtained in the animals of the previous experiment (33% ovulation, 2.9 ova/ovulating rat). Rats treated with 1250 µg TP now also responded, although the average number of ova per ovulating animal was small (53% ovulation, 2.5 ova/ovulating rat). In contrast to the results obtained with R-Amsterdam substrain, there was no difference between the two TP-treated groups.

If PMSG was followed by progesterone, a different picture emerged. Although progesterone had no significant influence on the response of control rats, there was a marked effect upon the rats treated with 5 µg TP. Nearly all (91%) of these animals now ovulated and the number of ova reached an average of 14.5 per ovulating rat. Progesterone had no significant effect on the group treated with 1250 µg TP. Here the incidence of ovulation and the number of ova per ovulating rat remained very small (19% ovulation, 2.7 ova/ovulating rat).

It is concluded that progesterone facilitated PMSG induced ovulation only in the 5 µg TP-treated rats. In the 1250 µg TP-treated group and the control group no increased ovulatory response could be obtained: in the latter probably because a maximum response had already been reached. In comparison with the R-Amsterdam substrain, the "Endo" substrain responded with a much higher number of ova. However, after neonatal TP-treatment the response to PMSG was very low in both substrains.

5-2-3 Ovarian sensitivity.

It was demonstrated in the previous chapter that in rats treated with a high dose of TP the numbers of small antral follicles were lower than in oil-treated rats. Two experiments were performed to determine whether in addition to the absence of an ovulatory LH surge, the sensitivity of the ovaries of the androgen-treated rats to gonadotrophic stimuli might also be affected.

In one experiment animals treated with oil, 5 or 1250 µg TP were given 30 IU PMSG on day 22. At that age the incidence of ovulation caused by PMSG alone is almost zero, probably because insufficient LH is released. There is no difference between the three neonatal treatment groups in this respect. A single s.c. injection of HCG was given 52 hrs after the PMSG administration in a dose ranging from 1 to 20 IU.

Fig. 5-2 shows that with additional HCG treatment all animals of the three treatment groups could be made ovulating. All TP-treated rats showed a lack of response to doses of 5 IU HCG and below. With higher doses of HCG the response of rats treated with 5 µg TP resembled that of controls, while the animals treated with 1250 µg TP were less responsive.

In a second experiment animals treated with 5 or 1250 µg TP were given a single injection of 30 IU PMSG on day 28 followed 52 hrs later by HCG in a single dose of 2 to 20 IU.

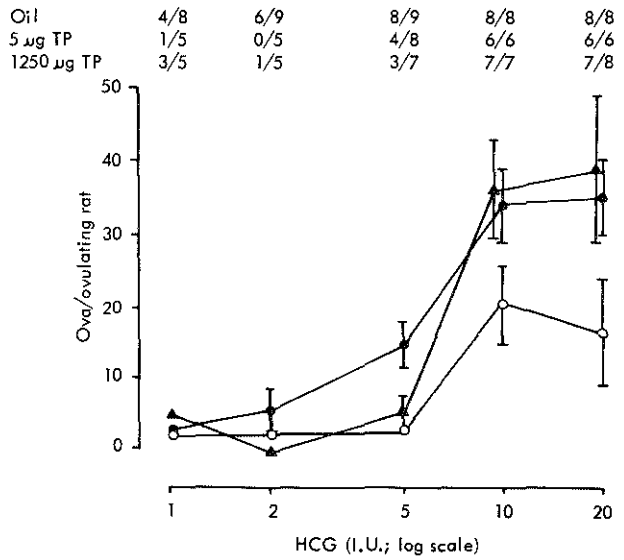


Fig. 5-2 Effects on 30 IU pregnant mare serum gonadotrophin given on day 22, followed by different doses of human chorionic gonadotrophin (HCG) 52 h later, on the ovaries of rats treated on day 5 with oil (●-●) or with 5 µg (▲-▲) or 1250 µg (O-O) testosterone propionate (TP). Vertical lines indicate ± S.E.M. Tabulated data = no. of ovulating rats/total no. of rats.

A group of animals which had undergone no treatment previously was given PMSG on day 28, hypophysectomized on day 29 and given HCG on day 30.

When 30 IU PMSG was given on day 28 numerous ovulations occurred in all oil-treated animals but in none of the neonatally TP-treated ones (Table 5-2). With the addition of 10 IU HCG (Fig. 5-3) a normal quota of eggs was now also shed in rats treated with 5 µg TP, but not in those treated with 1250 µg TP. Further doubling of the dose of HCG also normalized the response of the latter group of animals. Hypophysectomized animals showed the same response as the rats treated with 5 µg TP.

It is concluded that the ovaries of the TP-treated rats

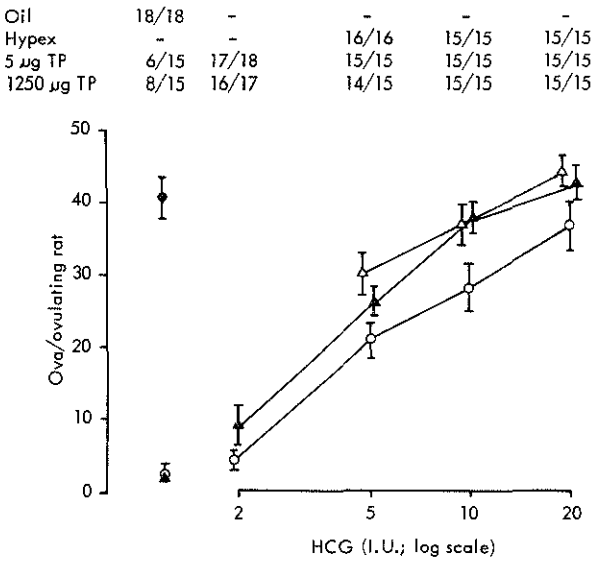


Fig. 5-3 Effects of 30 IU pregnant mare serum gonadotrophin (PMSG) given on day 28, followed by different doses of human chorionic gonadotrophin (HCG) 52 h later, on the ovaries of rats treated on day 5 with 5 µg (▲-▲) or 1250 µg (O-O) testosterone propionate (TP). Control animals were hypophysectomized (hypex) 1 day after PMSG injection (△-△). On the left : oil (●) and neonatally TP-treated groups without HCG treatment. Vertical lines indicate ± S.E.M. Tabulated data = no. of ovulating rats/total no. of rats.

are responsive to the combined gonadotrophic stimulation of PMSG and HCG. The ovarian sensitivity of the rats treated with 5 µg TP grossly resembled that of the oil-injected controls. However, the ovaries of rats treated with 1250 µg TP were less responsive.

5-2-4 Response to different doses of PMSG.

An experiment was carried out to test whether in TP-treated rats there occurred a shift in response to different doses of PMSG.

Animals of the three neonatal treatment groups were given a single dose of PMSG (varying between different animals

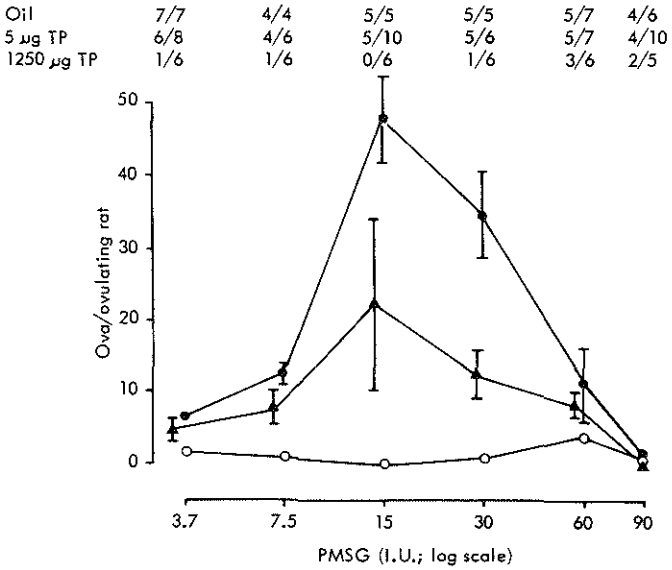


Fig. 5-4 Effects of different doses of pregnant mare serum gonadotrophin (PMSG) given on day 28, followed by 0.5 mg progesterone 52 h later, on the ovaries of rats treated on day 5 with oil (●-●) or with 5 µg (▲-▲) or 1250 µg (O-O) testosterone propionate (TP). Vertical lines indicate ± S.E.M. Tabulated data = no. of ovulating rats/total no. of rats.

from 3.7 to 90 IU) on day 28 and 0.5 mg progesterone 52 hrs later.

The results are given in Fig. 5-4. With doses of PMSG of 3.7 to 30 IU all control animals shed eggs. The average number of ova varied with a maximum (of 47.8 per animal) after a dose of 15 IU. Very few of the animals treated with 1250 µg TP ovulated and the average number of ova per ovulating rat remained very low. The most effective dose was 60 IU PMSG (50% ovulation, 4 ova/ovulating rat). The rats treated with 5 µg TP occupied an intermediate position between the previous two groups, but resembled the controls much more than the 1250 µg TP-treated animals. At nearly all dose levels of PMSG, ovulation occurred in the majority of animals and the average numbers of ova shed showed a pattern

similar to that of controls.

5-2-5 Effect of age on response to PMSG.

A possible shift with age in the responsiveness to PMSG was also tested in the TP-treated rats.

Animals of the three treatment groups were given 30 IU PMSG on one of the following days: day 22 (body weight range 30-45 g), day 24 (35-50 g), day 26 (40-55 g), day 28 (50-65 g) or day 30 (60-75 g). All animals received progesterone (0.5 mg) 52 hrs after the injection of PMSG.

The results are given in Fig. 5-5. Nearly all control animals ovulated at every age tested. However, as tests were carried out at later ages more ova were shed. In rats treated with 1250 µg TP the average numbers of ova remained uniformly

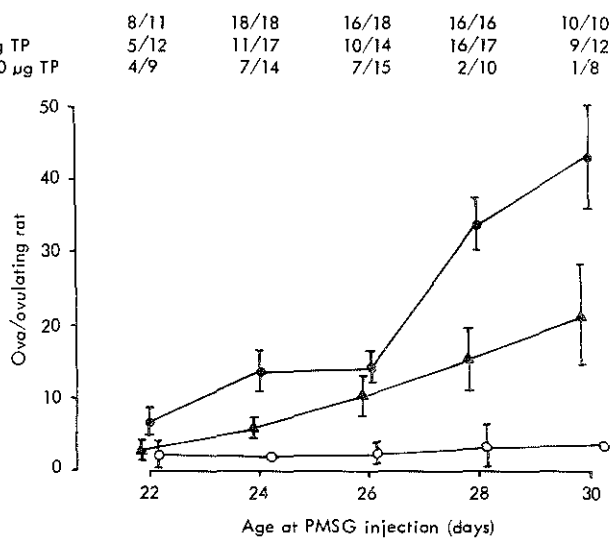


Fig. 5-5 Effect of age on the induction of ovulation by 30 IU pregnant mare serum gonadotrophin (PMSG), followed by 0.5 mg progesterone 52 h later, in rats treated on day 5 with oil (●-●) or with 5 µg (▲-▲) or 1250 µg (O-O) testosterone propionate (TP). Vertical lines indicate ± S.E.M. Tabulated data = no. of ovulating rats/total no. of rats.

very low at every age tested. The rats treated with 5 µg TP resembled the controls. The average numbers of ova rose gradually with increasing age in these animals.

It is concluded from the experiments in 5-2-4 and 5-2-5 that 5 µg TP-treated rats did not differ from control rats with respect to the dose of PMSG administered, at which maximal ovulatory responses could be obtained and to the age of administration, at which maximal effect was observed.

5-3 Discussion.

An ovulatory surge of LH in neonatally untreated rats following PMSG administration has been reported previously (Ying and Meyer, 1972; Sorrentino et al, 1972; Endersby et al , 1973). The present experiments confirm these findings and show that ovulation induced by a single dose of PMSG follows the release of LH on the afternoon of the second day after PMSG administration. The release of LH was not accompanied by a significant decrease in pituitary LH content between the second and third day after PMSG administration. This finding contrasts with the observations of other investigators, who found a fall in pituitary LH content either between 14.00 and 18.00 hrs on the second day after PMSG administration (Klausing and Meyer, 1968; Zarrow and Dinius, 1971) or between the second and third day after PMSG administration (Rennels and O'Steen, 1967; McCormack and Meyer, 1968; Ying and Meyer, 1972). There are at least two possible explanations for this discrepancy. The absence of a demonstrable fall in LH content in the experiment may be due to high individual variation. Another possibility is that the amount of LH released was too small in comparison with the amount present in the pituitaries, since in the R-Amsterdam substrain the LH surge two days after PMSG administration was rather low. A four-fold higher serum LH concentration at 15.00 hrs on day 32 was observed in F_1 hybrids of this substrain (R x U) after the

same PMSG treatment. This high LH concentration correlates well with a higher ovulation response in these F_1 hybrid animals. The large difference in the number of ova released, between the R-Amsterdam substrain and the "Endo" substrain indicates that great strain differences exist in the response to PMSG.

Observations in TP-treated rats warrant the following considerations. In the previous chapter it was demonstrated that female rats treated neonatally with a high dose of TP (1250 µg) will be anovulatory from the time of vaginal opening. In these animals a single dose of PMSG at the age of 30 days failed to induce ovulation. This corresponds with earlier findings that ovulation or corpus luteum formation in prepuberal rats receiving 1250 µg TP did not occur following a single injection of PMSG (Schuetz and Meyer, 1963; Brown-Grant et al, 1964) or OB (Dörner and Döcke, 1964). The absence of an LH surge on day 32 in the present experiments indicates that such animals do not ovulate for lack of an LH stimulus.

Female rats treated neonatally with 5 µg TP, showing ovarian cycles for some time after puberty, responded to PMSG with ovulation in a limited number of cases and with a very low number of ova. Also in these rats, no rise in serum LH could be found two days after PMSG administration. It is remarkable that although a third of the 5 µg TP-treated rats ovulated, the serum LH concentrations of these animals on day 32 were not different from those of non-ovulating 5 or 1250 µg TP-treated rats. It may be argued that the LH peak present in the 5 µg TP-treated rats, that did ovulate, occurred at a time different from that of oil-treated rats. Although this possibility cannot be excluded, it seems more likely that the LH assay lacks the sensitivity required to detect the minimal increase in serum LH which is sufficient to release a small number of ova.

The presence in low dose TP-treated rats of postpuberal ovulatory cycles and the subnormal ovulatory response to PMSG administered prepuberally indicate that while after

puberty these animals seem to be normal, before puberty the mechanism inducing ovulatory surges of LH is already diminished. In the light of these finding one could expect differences in the magnitude of the spontaneous ovulatory LH surge between cyclic 5 µg TP-treated rats and normal rats. To date no such differences have been described.

The finding of a lower pituitary LH content in 1250 µg TP-treated rats agrees well with previous observations (Matsuyama et al, 1966; Barraclough, 1966[b]). The absence of an ovulatory LH surge in these rats is probably not due to the low pituitary LH content, since both types of TP-treated rats showed an absence of an ovulatory LH surge, while a low pituitary LH content could be demonstrated only in rats treated with the high dose of TP.

It is of interest that an injection of progesterone two days after PMSG treatment increased the ovulation response in the 5 µg TP-treated rats. Others have reported that progesterone will not induce ovulation in adult TP-treated rats (Barraclough et al, 1964), although the ovulatory response to electrical stimulation of the brain increased if progesterone had previously been given (Barraclough and Gorski, 1961). The present data indicate that prepuberal 5 µg TP-treated rats are still sensitive to the facilitating action of progesterone.

The ovaries of both groups of TP-treated rats were responsive to the combined PMSG and HCG treatment. A clearly decreased sensitivity of the ovaries was found in rats treated with the high dose of TP. On day 22 as well as on day 28, the response to PMSG followed by HCG was lower in the high dose TP-treated rats than that of oil-treated or hypophysectomized rats. In this respect it is of interest that a lower number of small follicles was seen on days 20 and 30 in 1250 µg TP-treated rats than in control rats (see chapter 4). The decreased sensitivity of the ovaries of these animals may be due to a relative lack of small follicles.

The concept of an acceleration of sexual maturation in

TP-treated rats as proposed by van der Werff ten Bosch et al (1971) was tested in the PMSG experiments. The earlier presence of large follicles on day 35 in the 1250 µg TP-treated rats as compared with normal rats suggests that follicular growth is accelerated (see chapter 4). On the base of this concept it seemed possible, therefore, that PMSG-induced ovulation might occur at earlier ages in high dose TP-treated rats than in normal rats. This explanation is not supported by the data obtained. The ovulation response to PMSG in the high dose TP-treated rats was very low at all ages and with all doses of PMSG. The maximal response in control animals and 5 µg TP-treated animals occurred at the same dose level of 15 IU PMSG and at the same age.

Chapter 6

Induction of ovulation by electrochemical stimulation of the brain in androgen-sterilized rats

6-1 Introduction.

In the previous chapter it was pointed out that even before puberty the ovulatory LH release mechanism is affected by administration of a high as well as by a low dose of TP neonatally. All the same, low dose TP-treated rats show ovulatory cycles after puberty. The question arose whether after attainment of the anovulatory state, a difference in the potential of the brain to induce an ovulatory release of LH would persist between high and low dose TP-treated rats. Barraclough and Gorski found a difference between high and low dose TP-sterilized rats with regard to the ovulatory response after electrical stimulation of the brain (Barraclough, 1966[a]). Electrical stimulation (ES) of the anterior hypothalamic-preoptic area (AH-POA) was found to be ineffective in inducing ovulation in rats sterilized by 1250 µg TP (Barraclough and Gorski, 1961) but effective in 75% of the rats sterilized by 10 µg TP, although an injection of progesterone prior to ES was necessary (Gorski and Barraclough, 1963). ES of the arcuate-ventromedial nuclear complex (ARC-VMN) also revealed a difference between high and low dose TP-treated rats. While ES of the ARC-VMN failed to induce ovulation in 1250 µg TP-treated rats (Barraclough and Gorski, 1961) such a stimulation was effective in 10 µg TP-treated rats (Gorski and Barraclough,1963). However, after pre-treatment with progesterone the difference disappeared. Both types of TP-treated rats were now equally responsive to ES of the ARC-VMN. In normal nembutal blocked prooestrous rats ES of the AH-POA as well as of the ARC-VMN resulted in complete ovulation Critchlow, 1958; Everett, 1961), Therefore, Barraclough and

Gorski concluded that neonatal TP treatment exerts its effect on the AH-POA resulting in the absence of the cyclic LH release and that in high dose TP-treated rats the area of the brain involved in the regulation of the tonic gonadotrophin release (ARC-VMN) was also affected. In contrast to ES, electrochemical stimulation (ECS) of the AH-POA and of the ARC-VMN were equally effective in normal and androgen-sterilized rats (Terasawa et al, 1969; Everett et al, 1970; Kawakami and Terasawa, 1972). These results suggest that either no difference in exitability exist between cyclic and anovulatory animals at the level of the AH-POA and ARC-VMN or that this method is inadequate to detect such a difference between cyclic and anovulatory animals.

Since Barraclough and Gorski have drawn for reaching conclusions from their experiments with ES, the present experiments were carried out to examine if after ECS of the two different brain areas high and low dose TP-sterilized rats would react with a different ovulatory response.

6-2 Materials and methods.

Female rats of the Wistar substrain, R-Amsterdam, were injected on day 5 with either 5 µg or 1250 µg TP. For reasons mentioned in the discussion no oil-treated rats were used. At the age of 5 to 6 months vaginal smears were taken daily for at least 10 days. Only animals which showed a cornified smear during the entire period were used.

In one experiment HCG or LH-RH was used to induce ovulation. Both preparations were injected intravenously between 12.00 and 14.00 hrs on the day before autopsy.

ECS was performed under ether anaesthesia. The head of the animal was fixed with ear bars in a Kopf-stereotaxic apparatus. The upper incisor bar was adjusted 5.0 mm above the level of the interaural line. The electrode was placed in various parts of the brain using the coordinates of the atlas of de Groot (1959). For AH-POA stimulation the electrode was placed 0.5 mm lateral to the midline, 7.5 mm anterior and 3.5 mm

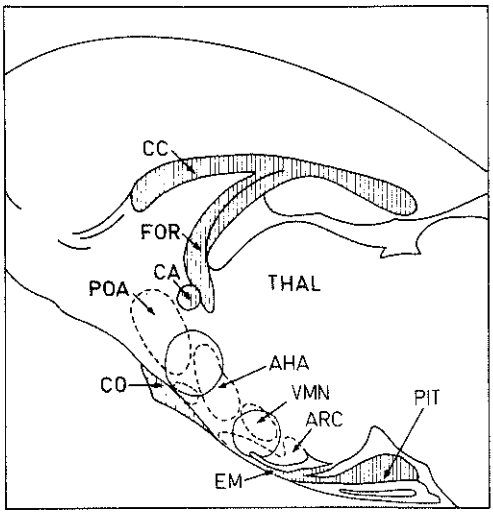


Fig. 6-1 Sagittal section of the rat brain, 0.2 mm lateral of the midline, indicating the areas of stimulation. Abbreviations used in the figure: CC = corpus callosum, FOR = fornix, CA = commissura anterior, THAL = thalamus, CO = chiasma opticum, POA = preoptic area, AHA = anterior hypothalamic area, VMN = ventromedial nucleus, ARC = arcuate nucleus, EM = eminentia mediana, PIT = pituitary. Redrawn from de Groot's atlas.

superior to the interaural line. For ARC-VMN stimulation the coordinates were: 0.25 mm lateral to the midline, 6.5 mm anterior to the interaural line and 0.5 mm above the base of the skull (Fig. 6-1). The electrode was a unipolar stainless steel rod with a non-insulated tip of approximately 0.2 mm in diameter and 0.25 to 0.3 mm long. The ECS was performed unilaterally by passing a DC current in periods of 25 sec on and 25 sec off. Stimulus intensity and duration are given in Table 6-3.

The animals were killed the following morning and ova in the fallopian tubes were counted.

The statistical analyses of results were performed using the χ^2-test or the Fischer test.

6-3 Experiments and results.

6-3-1 Induction of ovulation by HCG or LH-RH.

Since the effectiveness of ECS of the hypothalamic areas is judged by the ovulatory response of the animals two conditions have to be fulfilled:

1. The ovarian sensitivity to an ovulatory stimulus has to be comparable in both types of rats.
2. The pituitaries of the two groups have to be equally responsive to LH-RH.

Both ovarian and pituitary sensitivity are of importance since in the previous chapter it was demonstrated that at least at the age of 30 days the ovaries of the 1250 µg TP-treated rats are less responsive to the combined action of PMSG and HCG, whereas the pituitaries of 1250 µg TP-treated rats contained less LH so that these could be less responsive to LH-RH.

To test the ovaries and the pituitaries varying doses of HCG and of LH-RH were injected intravenously in high and low dose TP-treated rats. The doses of LH-RH were calculated on the base of 100 g body weight.

The results show no significant differences between the two treatment groups in the percentages of ovulating rats after treatment with either HCG (Table 6-1) or LH-RH (Table 6-2)

Table 6-1 Induction of ovulation by HCG in rats made persistently oestrous by neonatal administration of 5 or 1250 µg testosterone propionate.

dose of HCG (IU)	5 µg TP on day 5			1250 µg TP on day 5			
	rats that ovulated number	%	ova per ovulating rat (mean)	rats that ovulated number	%	ova per ovulating rat (mean)	significance* Fisher-Test
1	0/4	0	-	0/4	0	-	n.s.
2	2/8	40	5.5	0/8	0	-	n.s.
4	8/8	100	9.3	8/10	80	9.6	n.s.
8	4/4	100	8.5	4/4	100	8.5	n.s.

* significance between percentage of ovulations in the two groups of rat (n.s.: not significant $P>0.05$)

Table 6-2 Induction of ovulation by synthetic LH-RH in rats made persistently oestrous by neonatal administration of 5 or 1250 µg testosterone propionate.

dose of LH-RH (ng/100 g b.w.)	5 µg TP on day 5			1250 µg TP on day 5			significance* Fisher-Test
	rats that ovulated number	%	ova per ovulating rat (mean)	rats that ovulated number	%	ova per ovulating rat (mean)	
20	0/4	0	-	0/4	0	-	n.s.
50	0/4	0	-	0/5	0	-	n.s.
100	1/6	17	4	1/6	17	3	n.s.
200	3/6	50	12	4/7	57	8	n.s.
500	5/5	100	8	6/6	100	6.5	n.s.

* significance between percentages of ovulations in the two groups of rats (n.s.: not significant $P>0.05$)

It is concluded that the ovaries and pituitaries of 5 and 1250 µg TP-treated rats were equally responsive to the ovulation inducing influence of HCG and of LH-RH.

6-3-2 Electrochemical stimulation of the AH-POA.

ECS of the AH-POA was performed in high and low dose TP-treated rats. The results are given in Table 6-3.

Table 6-3 Effect of neonatal androgen-treatment on the induction of ovulation by electrochemical stimulation of the anterior hypothalamic-proptic area (AH-POA) and arcuate-ventromedial nulear complex (ARC-VMN).

site of stimulation	total charge sec x µamp. (millicoulomb)	5 µg TP on day 5			1250 µg TP on day 5			significance* χ^2-Test
		rats that ovulated number	%	ova per ovulating rat (mean)	rats that ovulated number	%	ova per ovulating rat (mean)	
AH-POA	75 x 30 (2.2)	11/19	58	9.6	1/8	12.5	12	s
	75 x 60 (4.5)	19/22	86	10.3	6/12	50	13.3	s
	75 x 120 (9)	8/9	89	12.5	11/14	78	11.8	n.s.
	150 x 120 (18)	8/8	100	12.5	9/9	100	9.8	n.s.
ARC-VMN	75 x 80 (6)	2/12	17	14.5	4/17	24	12	n.s.
	75 x 160 (12)	8/10	80	9.4	8/8	100	10.4	n.s.

* significance between percentages of ovulations in the two groups of rats (s: significant, $P\leq 0.05$)

A total charge of 2.2 milicoulomb (mc) induced ovulation in 12.5% of the 1250 µg TP-treated rats and in a significantly higher percentage (58%) of the 5 µg TP-treated group. The percentage of ovulating rats increased in both treatment groups after doubling the total charge. A significant difference in the percentage of ovulating rats between the 1250 µg TP-treated group (50%) and the 5 µg TP-treated group (86%) persisted. After a further increase of the total charge no difference was observed between the treatment groups. Finally after a stimulus of 18 mc a 100% response was obtained. The mean number of tubal ova per ovulating rat varied between 9.6 and 13.3 and showed no difference between the two treatment groups. These numbers of ova are similar to those found at oestrous in normal rats of this strain.

6-3-3 Electrochemical stimulation of the ARC-VMN.

ECS of the ARC-VMN was performed in high and low dose TP-treated rats. Table 6-3 shows that a total charge of 6 mc induced ovulation in about 20% of the animals both in the 5 µg TP group (2 out of 12) and in the 1250 µg TP group (4 out of 17). Doubling of the total charge increased the response in both treatment groups. However, also after this charge there was no significant difference between the responses in the two treatment groups. The number of ova shed was similar in both groups and similar to that found after stimulation in the AH-POA.

6-4 Discussion.

The present experiments demonstrate that ECS of the AH-POA is less effective in inducing ovulation in 1250 µg TP-treated rats than in 5 µg TP-treated rats. Barraclough and Gorski (1961) and Gorski and Barraclough (1963) studying the effect of ES of the AH-POA in 1250 and 10 µg TP-treated rats, observed that both treatment groups appeared refractory to ES. After an injection of progesterone prior to ES, a modest

ovulatory response was found only in the 10 µg TP-treated rats. The basic finding of Barraclough and Gorski, i.e., that ES of the AH-POA is less effective in 1250 µg TP-treated rats than in 10 µg TP-treated rats, is in agreement with our results after ECS and indicates that after attainment of the anovulatory state low dose TP-treated rats are still different from high dose TP-treated rats. However, the difference between high and low dose TP-treated rats is larger after ES than after ECS. The larger ovulatory response in our experiments than in those of Barraclough and Gorski, is in line with the findings of Everett and Radford (1961) and Terasawa and Sawyer (1969) that ECS is much more effective in inducing ovulation than ES. Terasawa et al (1969) comparing both ES and ECS of the AH-POA in normal and 50 µg TP-treated rats observed in androgenized rats a lower response to ES but no difference in the response to ECS. Their findings in TP-treated rats differ from our results in that a much lower total charge was effective in all animals. Everett et al (1970) observed a POA threshold in rats treated with 1250 µg TP which was close to that of cyclic rats. In another strain of rats (the C.D. strain) a larger difference between normal and TP-treated rats was found. Since TP-treated rats of this strain secreted large amounts of prolactin, it was suggested that the observed elevation of the POA threshold was due to an action of that hormone on the brain (Everett et al, 1970).

A difference in the response to stimulation of the ARC-VMN between both types of rats as reported by Barraclough and Gorski after ES was not observed in the present experiments after ECS.

From the present data and those of Barraclough (1966[a]) it cannot be concluded that the absence of an ovulatory LH surge in TP-treated rats is the result of a decreased excitability of neurous in the AH-POA, since no comparison has been made between cyclic and anovulatory animals. However, such a comparison would be difficult. In the first place because of the inequality of the hormonal evironment. It has been

demonstrated that ovarian steroids can influence the effectiveness of ECS (McDonald and Gilmore, 1971). Furthermore, the number of ovulating animals (Holsinger and Everett, 1970) and the amount of LH released (Kalra and McCann, 1973) following ECS of the preoptic area varies during the oestrous cycle.

General discussion

Summary of findings.

Before discussing the results described in the chapters 3 to 6, they will be summarized briefly.

Chapter 3. Female rats treated with 1250 µg TP on day 5 failed to ovulate from the time of vaginal opening. In contrast, animals treated with 5 µg TP on day 5 often showed ovarian cyclicity for more than three months. The ovaries of anovulatory animals contained large follicles but no corpora lutea and vaginal smears were permanently cornified.

Chapter 4. The influence of neonatal TP on various parameters, related to the onset of reproductive function, was studied during the prepuberal period. An immediate effect of the TP injected was a decrease in FSH concentration in the blood. In animals treated with 1250 µg TP this effect lasted 10 to 15 days, resulting in the absence of the high serum FSH concentrations around day 15. Compared to control rats, the 1250 µg TP-treated rats showed less ovarian weight gain till day 30 and smaller numbers of small antral follicles on days 20 and 30. It is possible that the retarded follicular growth was due to the absence of a FSH peak around day 15. On day 35 the numbers of follicles in the largest volume class were higher in the 1250 µg TP-treated rats than in 5 µg TP- or oil-treated rats. Also, uterine weights were higher and vaginal opening, although of an abnormal character, occurred at an earlier age. These findings suggest that follicular growth was accelerated at that age in rats treated neonatally with a high dose of TP.

In rats treated with 5 µg TP only a small decrease in serum FSH concentration was noticed. The treatment had no significant effect on ovarian or uterine weights, follicular development or the time of vaginal opening. In all these animals

regular 5-day cycles were found after vaginal opening and the number of ova shed at oestrus was not different from that of control rats.

Chapter 5. In this chapter the ovulatory response to an injection of PMSG in prepuberal TP-treated animals was studied. After an injection of 30 IU PMSG on day 30 all oil-treated animals showed an increase in serum LH on the afternoon of day 32 and fresh tubal ova on day 33. No such increase in serum LH concentrations was found on day 32 in 5 and 1250 µg TP-treated rats. This probably explains that no ovulation occurred in the 1250 µg TP-treated rats. However, a number of 5 µg TP-treated rats ovulated. The absence of increased serum LH concentrations in these rats might be explained by the insensitivity of the LH assay system. Pituitary LH content was reduced in the high dose TP-treated rats. It was found that in the 5 µg TP-treated rats an injection of progesterone 52 hrs after PMSG administration increased the ovulatory response. With this combined PMSG and progesterone treatment a dose- and an age-response curve was constructed for the three treatment groups. At every age and dose tested 1250 µg TP-treated rats either did not ovulate or responded with only a very small number of ova. The response of the 5 µg TP-treated rats was intermediate between control and high dose TP-treated rats, but the pattern of the dose- and age-response curves resembled that of control rats. A clearly decreased sensitivity of the ovaries was found only for the high dose TP-treated animals.

Chapter 6. After attainment of the anovulatory state in adulthood, the ovulatory response to ECS of the brain was studied. A preliminary experiment showed that the pituitaries and the ovaries of high and low dose TP-treated rats were equally responsive to the ovulation inducing influence of LH-RH and HCG respectively. Animals made anovulatory with 1250 µg TP appeared less responsive to ECS of the AH-POA than animals made anovulatory with 5 µg TP. However, ECS of the ARC-VMN was equally effective in 5 and 1250 µg TP-treated rats.

Conclusions and a concept of the change with age of the "potential" of the brain to induce an LH surge in normal and androgenized rats.

The observation of the delayed onset of the anovulatory state after a low dose of TP during the "critical period" confirms earlier findings (van der Werff ten Bosch, 1964[a,b]; Gorski, 1968). From the data of these authors it was concluded that the degree of alteration of the cyclic LH release mechanism depended on the dosage of androgen administered. However, differences in ovarian cyclicity between 5 µg TP-treated rats and normal rats before these former animals have become anovulatory have not been described, and the observed difference with 1250 µg TP-treated rats consisted mainly in the time of the onset of the anovulatory state. The results of the present studies show that

1. Even before puberty the mechanism which induces an ovulatory surge of LH is deficient in 5 µg TP-treated rats and that such a mechanism is never operative at all in 1250 µg TP-treated rats.
2. After attainment of the anovulatory state, 5 µg TP-treated rats are still different from 1250 µg TP-treated rats in that the former group is more responsive to ECS of the AH-POA. This last finding is in agreement with that obtained by Barraclough and Gorski with ES (Barraclough, 1966[a]).

The diagram in Fig. 7-1 depicts the development of the "potential" of the brain to induce an LH surge and the effect of neonatal TP on this "potential". This overview is based on the following considerations.

(1) Normal female rats, which have passed the critical period unaffected by androgenic hormones, show an increase of this "potential" before puberty. Arguments, which support this view are the following.

From day 20 onwards the brain is able to induce an ovulatory LH surge after appropriate hormonal stimulation, i.e., after PMSG (Zarrow and Quinn, 1963; McCormack and Meyer, 1964), after OB (Ying and Greep, 1971[b]) and after progesterone in

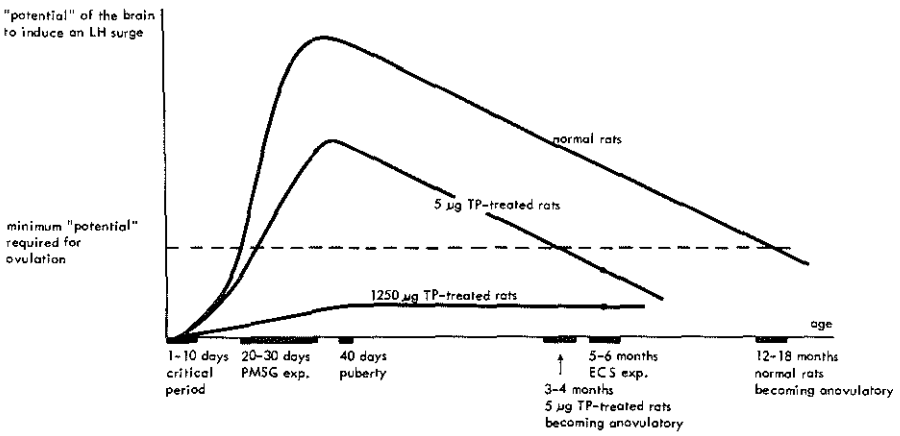


Fig. 7-1 Diagram illustrating the change with age of the "potential" of the brain to induce an LH surge and the effect of testosterone propionate administered neonatally of this "potential". In the area below the dotted line the "potential" of the brain is too low to induce an LH surge. In this area ovulation can only be induced by electrical or electrochemical stimulation of the brain.

ovariectomized OB-primed rats (Caligaris et al, 1972). These authors all showed that the response to the various stimulating agents increases with age and reaches a maximum at around day 28. Although the increase with age of the ovulatory response to PMSG and OB may be due to an increased follicular growth, the gradually increasing LH surges evoked by progesterone in the ovariectomized OB-primed rats (Caligaris et al, 1972) suggest an increase with age of the "potential" of the brain to induce an LH surge.

(2) The "potential" decreases gradually till at the age of 12 to 18 months it falls below the threshold which is required to induce spontaneous ovulations. Arguments supporting this view are the observations that female rats become anovulatory at old age (Wolfe et al, 1940; Ingram, 1959; Mandl, 1961). This anovulatory state seems to be due to the failure of the brain to induce an ovulatory release of LH, since transplantation of ovaries (Ascheim, 1964/1965)

or pituitaries (Peng and Huang, 1972) from "senile" rats to cyclic females can be followed by resumption of regular oestrous cycles.

The "potential" of the brain to induce an LH surge will be proportional to the amount of gonadotrophin released when the enteroceptive and exteroceptive stimuli which evoke the LH surge do not vary. This would mean that in normal rats the magnitude of the LH peak becomes smaller as the animals grow older. Some findings favour this idea. In a study in which serum LH concentrations were measured during the day before first ovulation an LH peak was observed which seemed to be higher than that on the afternoon of prooestrus in adulthood (Meijs-Roelofs and Uilenbroek, unpublished observations). Furthermore, it has been demonstrated, with timed sodium pentobarbitone injections on the afternoon of prooestrus, that less than the normal peak level of LH successfully elicits ovulation (Greig and Weisz, 1973). A gradual decrease of the LH peak height with higher ages will therefore not immediately result in anovulation. An argument for the view that the cyclic period ends because the prooestrus LH peak fails to reach an ovulatory level can be deduced from the finding of atretic large follicles at the time of the expected onset of anovulation in ovarian grafts in neonatally castrated male rats (van der Schoot, 1973). Since it has been demonstrated that preovulatory follicles become atretic after subovulatory gonadotrophin stimulation (Vermeiden and Zeilmaker, in preparation), the presence of atretic follicles at the expected time of ovulation in neonatally castrated male rats may indicate that the peak level of LH was insufficient for induction of ovulation.

The available data support the idea that animals treated with 5 µg TP on day 5 have a lower "potential" of the brain to induce an LH surge than untreated rats at all ages (Fig. 7-1). However, their pattern is not different from that of normal rats. The lower ovulatory response after PMSG in the prepuberal period and the early onset of the anovulatory state in the postpuberal period are indications that the

brain "potential" is subnormal. The parallel between the cessation of ovulatory cycles in "senile" rats and in low dose TP-treated rats has been drawn earlier by van der Werff ten Bosch et al (1971). They speculated that one effect of neonatal TP-treatment might be an acceleration of the ageing of the central nervous system mechanism which controls the gonadotrophin release responsible for ovulation. The present data indicate that neonatal TP-treatment causes an underdevelopment of this mechanism.

In contrast to that of 5 µg TP-treated rats, the brain of 1250 µg TP-treated rats never reaches the "potential" for spontaneous induction of an ovulatory surge of LH. Also in response to different hormones, an LH release fails to occur as was demonstrated after PMSG (this thesis) and after OB (Dörner and Döcke, 1964) in prepuberal TP-treated rats, and after OB in adult ovariectomized TP-treated rats (Taleisnik et al, 1971). The difference in responsiveness between 5 and 1250 µg TP-treated rats to ECS of the AH-POA indicates that the brain of 1250 µg TP-treated rats has been more intensively affected by neonatal androgen treatment than the brain of 5 µg TP-treated rats.

Concepts to explain the sterilizing effect of early TP-treatment.

It is still unclear why the brains of androgenized rats are unable to induce cyclic release of LH. It is possible that neuronal connections indispensable for the induction of an ovulatory release of LH fail to develop. Morphological differences correlated with the neonatal presence or absence of androgens have been described by Raisman and Field (1973). They observed a lower incidence of synapses on dendritic spines from other areas than from the stria terminalis in the preoptic area of normal males and of females treated with 1250 µg TP on day 4. Conversely a high incidence of synapses on dentritic spines was observed in normal females and in males castrated within 12 hrs of birth. However, one can only speculate about the relationship between these synapses and

the machanism which induce LH release.

One of the most popular explanations of the inability of the brain to induce cyclic LH release states that the sensitivity of the brain to oestrogen is decreased (Flerkő, 1971; Gorski, 1971). It has been suggested that exposure of the developing brain to androgen alters the formation of steroid receptor mechanism necessary for the induction of ovulation (Gorski, 1971). It has been shown that the uptake of ^{3}H-oestradiol in the uterus, pituitary and the hypothalamus of androgen-sterilized rats is decreased (Flerkő et al, 1969,1971; Anderson and Greenwald, 1969; McGuire and Lisk, 1969; McEwen and Pfaff, 1970 and Vértes and King, 1969). In rats treated with 1000 µg TP on day 2 Vértes and King (1971) observed a decreased oestrogen binding in both nuclear and cytoplasmatic fractions from the anterior hypothalamus at day 60, but only in the nuclear fraction at day 28.
A decreased sensitivity of the brain to the inhibitory feedback action of oestrogen has been reported by Petrusz and Nagy (1967) and by Barraclough and Haller (1970). Petrusz and Nagy observed that more oestrogen was required to suppress ovarian compensatory hypertrophy in androgenized rats than in normal rats. However, this experiment is not conclusive because the endpoint studied, the ovary, is dissimilar in the two groups of animals; while corpora lutea were present in control animals, they were lacking in androgenized rats. This difficulty was avoided by Barraclough and Haller (1970). They estimated the effect of oestrogen on plasma LH levels in ovariectomized rats and observed that five times more OB was needed to suppress plasma LH concentrations in TP-sterilized rats than in normal rats. However, the decrease in plasma LH concentrations of TP-treated rats after ovariectomy in these experiments is in contrast to the observations of other investigators (Schiavi, 1969; Labhsetwar, 1970; Uilenbroek, 1972).

A single injection of oestradiol benzoate on day 5 also has a sterilizing effect (Gorski, 1963) and neonatal administration of an antioestrogen (MER-25) inhibits the sterilizing

effect of TP (McDonald and Doughty, 1972[a]). In addition to the assumption that the neonatally administered testosterone acts directly on the developing brain as such, it is conceivable that the testosterone injected is converted to oestrogen and that this oestrogen then acts on the brain. It has been demonstrated that aromatization of androstenedione can occur in the foetal hypothalamus (Naftolin et al, 1971). Furthermore, dihydrotestosterone which cannot be converted to oestrogen has no sterilizing effect when administered neonatally to female rats (Luttge and Whalen, 1970; Arai, 1972; McDonald and Doughty, 1972[b]).

An alternative hypothesis, which explains the sterilizing action of both testosterone and oestrogen, has been proposed by Sheridan et al (1973[b]). These investigators suggested that not the presence of high amounts of steroids in itself but that the absence of high concentrations of serum gonadotrophins during the neonatal period might induce sterilization. Indeed, the correlation between serum levels of FSH during the neonatal period and the type of gonadotrophin regulation in adulthood is striking. High concentrations of serum FSH neonatally - present in normal females (Weisz and Ferin, 1970; Meijs-Roelofs et al, 1973[b]) and in neonatally castrated males (Goldman et al, 1971) - correlate with cyclic release of gonadotrophins in adulthood. Low concentrations of serum FSH neonatally - present in normal males (Weisz and Ferin, 1970) and in TP-treated females (Weisz and Ferin, 1970; Goldman and Gorski, 1971 and this thesis) - correlate with the absence of cyclic variations in gonadotrophin secretion in adulthood. However, dihydrotestosterone, which has a strong antigonadotrophic action both in adult rats (Beyer et al, 1972; Swerdloff et al, 1972; Verjans et al, in preparation) and in prepuberal rats (Feder, 1971; Rosekrans and Aafjes, unpublished results) is unable to induce sterilization when given neonatally. If the hypothesis of Sheridan et al is correct any agent which suppresses gonadotrophin secretion would eliminate the capacity of ovarian cyclicity. It therefore seems likely that the

observed temporary decrease in serum FSH concentrations which follows neonatal TP-treatment is of no decisive importance for the development of the early-androgen syndrome.

Summary

Female rats, treated with androgens during the early post-natal period, may become anovulatory in adulthood. In this thesis the development of this so called "early-androgen syndrome" has been described for rats treated on day 5 with a high (1250 µg) or with a low (5 µg) dose of testosterone propionate (TP). Various indices for hypothalamo-hypophysial-ovarian activity were monitored: (1) during the prepuberal period, (2) at the time at which puberty occurs in normal rats and (3) in adulthood.

Following an injection of 1250 µg TP on day 5 serum FSH concentrations did not reach the high levels normally seen in untreated rats between days 10-15 (Chapter 4). From day 20 onwards the FSH pattern was not different from that of normal rats. The number of small antral follicles was lower compared to normal rats on days 20 and 30. This may have been due to the subnormal FSH levels before day 20. The development of the mechanism to induce an ovulatory surge of LH was tested by attempting to induce ovulation with PMSG (Chapter 5). In 1250 µg TP-treated rats an injection of PMSG was not followed by a surge of LH, and ovulation did not occur. It is concluded that the mechanism to induce an ovulatory surge of LH does not develop in rats with a high dose of TP. At the end of the prepuberal period the following abnormalities could be demonstrated: abnormal vaginal development, earlier presence of large follicles and a higher uterine weight (Chapter 4). The absence of spontaneous ovulations in adulthood described in Chapter 3 is in agreement with the above reached conclusion that the mechanism to induce LH surges does not develop in rats treated with a high dose of TP.

Administration of the low dose of 5 µg TP has an effect

on ovarian cyclicity that differs from the effect obtained with 1250 µg TP. Animals treated with 5 µg TP show ovarian cyclicity for some time after puberty before they become anovulatory (Chapter 3). Such a treatment on day 5 had no marked effect on serum FSH concentration and follicular development in the prepuberal period: the serum FSH concentrations were lower than in normal rats for only three days and the composition of the follicular population was not different from that of normal rats (Chapter 4). The ovulatory response to PMSG administration, however, was subnormal and a pre-ovulatory LH surge could not be demonstrated (Chapter 5). In contrast to the effect in 1250 µg TP-treated rats, the facilitating action of progesterone on the ovulatory response to PMSG was present in the 5 µg TP-treated rats. It is concluded that neonatal administration of a low dose of TP partially inhibits the development of the mechanism to induce an ovulatory surge of LH. However, the diminished "potential" of the brain to induce an LH surge in the prepuberal period is of no consequence in the early period after puberty. The animals started cycling at the same age as normal animals and continued to cycle for some time. Thereafter cyclicity stopped presumably for lack of an LH stimulus (Chapter 3).

Although animals treated neonatally with either a low or a high dose of TP eventually became anovulatory, a difference in the "potential" of the brain to induce an ovulatory surge of LH persisted since 5 µg TP-treated rats showed a lower threshold to electrochemical stimulation of the anterior hypothalamic-preoptic area (Chapter 6).

A concept to describe the change with age of the "potential" of the brain to induce an LH surge is discussed in Chapter 7.

Samenvatting

Vrouwelijke ratten, die kort na de geboorte behandeld zijn met androgenen, kunnen anovulatoir worden op volwassen leeftijd. In dit proefschrift wordt de ontwikkeling beschreven van dit zogenaamde " vroege androgen-syndroom" en wel voor ratten die op de vijfde dag behandeld zijn met een hoge (1250 µg) of met een lage (5 µg) dosis testosteron propionaat (TP). Verschillende parameters voor hypothalamus-hypophyse-ovarium activiteit zijn bestudeerd: (1) in de prepuberale periode, (2) in de tijd dat puberteit optreedt bij normale ratten en (3) bij ratten op volwassen leeftijd.

Na een injectie met 1250 µg TP op dag 5 bereikten de serum FSH concentraties niet die hoge waarden die normaal in onbehandelde ratten worden waargenomen tussen dag 10 en 15 (hoofdstuk 4). Vanaf dag 20 evenwel was het FSH patroon niet verschillend van dat van normale ratten. Het aantal kleine follikels met een antrum was op dag 20 en 30 kleiner dan in normale ratten. Dit kan het gevolg zijn van de lage FSH spiegels voor dag 20. De ontwikkeling van het mechanisme om ovulatoire LH pieken op te wekken werd getest door te proberen ovulatie op te wekken met PMSG (hoofdstuk 5). In de met 1250 µg TP behandelde ratten werd een injectie met PMSG niet gevolgd door een LH piek en ovulatie vond niet plaats. Geconcludeerd wordt dat het mechanisme om ovulatoire LH pieken op te wekken zich niet ontwikkelt in ratten behandeld met een hoge dosis TP. Tegen het einde van de prepuberale periode konden de volgende abnormaliteiten worden aangetoond: een abnormale vagina ontwikkeling, eerder optreden van grote follikels en een hoger uterus gewicht (hoofdstuk 4). De afwezigheid van spontane ovulaties op volwassen leeftijd, zoals beschreven in hoofdstuk 3, komt overeen met de bovengenoemde conclusie, dat het mechanisme om LH pieken op te wekken zich

niet ontwikkelt in ratten behandeld met een hoge dosis TP.

Toediening van 5 µg TP, de lage dosis, heeft op ovarium cycliciteit een effect dat verschilt van het effect dat verkregen wordt in ratten behandeld met 1250 µg TP. De met een lage dosis TP behandelde dieren vertonen na de puberteit gedurende enige tijd ovariële cycli voordat ze anovulatoir worden (hoofdstuk 3). Een dergelijke behandeling had geen uitgesproken invloed op de serum FSH concentraties en op de follikel ontwikkeling in de prepuberale periode: de serum FSH concentraties waren slechts gedurende 3 dagen lager dan in normale ratten en de samenstelling van de follikel populatie was niet verschillend van die van normale ratten (hoofdstuk 4). De ovulatie respons op PMSG toediening evenwel was lager dan in onbehandelde ratten en een preovulatoire LH piek kon niet worden aangetoond (hoofdstuk 5). Progesteron had een faciliterende werking op de ovulatie respons in 5 µg TP behandelde ratten na toediening van PMSG. Dat was niet het geval in 1250 µg TP behandelde ratten. Geconcludeerd wordt dat neonatale toediening van een lage dosis TP de ontwikkeling van het mechanisme om ovulatoire LH pieken te induceren gedeeltelijk remt. De verminderde "potentie" van de hersenen om LH pieken op te wekken in prepuberale ratten heeft geen invloed op de eerste periode na de puberteit. De dieren vertoonden ovariële cycli op dezelfde leeftijd als normale ratten. Na enige tijd hield de cycliciteit op, vermoedelijk door het ontbreken van een LH stimulus (hoofdstuk 3).

Ofschoon zowel de met hoge als de met lage dosis TP behandelde dieren tenslotte anovulatoir werden bleef er een verschil bestaan in de "potentie" van de hersenen om een ovulatoire LH piek op te wekken: 5 µg TP behandelde ratten vertoonden een lagere drempelwaarde voor electrochemische stimulatie van het anterior hypothalamisch-preoptische gebied (hoofdstuk 6).

In hoofdstuk 7 wordt een concept besproken dat weergeeft het verloop met de leeftijd van de "potentie" van de hersenen om ovulatoire LH pieken op te wekken.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren in 1938 te Rotterdam. Na het behalen van het diploma HBS-B aan het St. Franciscuscollege te Rotterdam begon hij in 1960 de studie biologie aan de Rijksuniversiteit te Utrecht, alwaar het kandidaatsexamen in 1964 en het doctoraalexamen met hoofdvak algemene zoölogie en bijvakken biochemie en microbiologie in 1967 werden behaald. Van september 1963 tot september 1966 was hij als assistent verbonden aan het Zoölogisch Laboratorium te Utrecht. Sinds september 1967 is hij als wetenschappelijk medewerker werkzaam aan de afdeling Endocrinologie, Groei en Voortplanting van de Faculteit der Geneeskunde, Erasmus Universiteit Rotterdam.