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# INFLUENCE OF INTRAHYPOTHALAMIC IMPLANTS OF ANTIOESTROGEN OR AROMATASE INHIBITOR ON DEVELOPMENT OF STERILITY FOLLOWING NEONATAL ANDROGENIZATION IN FEMALE RATS

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## SUMMARY

The validity of the hypothesis that aromatization of androgens in the hypothalamus is a prerequisite for induction of sterilization in female rats androgenized neonatally was tested by implanting paraffin micropellets containing 16$\beta$-ethylestradiol-17$\beta$ (EED) or 1,4,6-androstatriene-3,17-dione (ATD), directly into the hypothalamus of neonatal female rats 6 h prior to a single subcutaneous injection of testosterone propionate (TP). The antagonists did not impair the sterilizing action of TP but enhanced the induction of sterility. By contrast, intrahypothalamic implantation of paraffin micropellets containing 1% TP together with 50% MER-25, an antioestrogen, brought about a significant suppression of induction of sterility. The reasons why intrahypothalamic implants of antioestrogen or aromatase inhibitor failed to suppress the sterilizing effect of TP subcutaneously injected were discussed.

## INTRODUCTION

As early as 1936, Pfeiffer[1] reported that the acyclic secretion of pituitary gonadotropins in male rats is determined by the secretion of testicular hormone during the neonatal period. It is not the purpose of the present paper to review a large number of investigations carried out to try to find the mechanisms involved in the sexual differentiation of the pituitary gonadotropin secretion. However, a few pertinent experiments should be mentioned here. Confirming the Pfeiffer's report, Takasugi[2] and Barraclough and Gorski[3, 4] induced the sterility syndrome characterized by anovulation and persistent oestrus in female rats by neonatal injections of sex steroids such as testosterone or oestrone. Wagner *et al.*[5] obtained similar results by implanting small amounts of testosterone crystals into the hypothalamus of neonatal female rats. If implanted subcutaneously, the same amount of testosterone was without effect in inducing the syndrome. With an improved technique of intracranial implantation of micropellets of testosterone propionate (TP)-paraffin mixture, Nadler[6, 7] suggested that the site of action of TP in the hypothalamus of neonatal rats was located in the middle hypothalamus including the arcuate and ventromedial hypothalamic nuclei but not in the anterior hypothalamus including the preoptic and anterior hypothalamic nuclei. However, Hayashi and Gorski[8] induced sterility by a timed implantation of TP into the anterior as well as the middle hypothalamus. Döcke and Dörner[9] who implanted oestradiol micropellets in the hypothalamus of neonatal female rats failed to find any regional difference in effects, while Hayashi[10] reported that implantation of oestradiol in an adequate amount into the anterior hypothalamus evoked anovulatory sterility but placement of the same amount of the steroid in the middle hypothalamus was ineffective. Thus, the site of sterilizing action of sex steroids in the hypothalamus of neonatal female rats has not yet been definitely determined.

On the other hand, McDonald and Doughty[11] reported that neonatal administration of 5$\alpha$-dihydrotestosterone (DHT), an active, non-aromatizable androgen, could not provoke the sterility syndrome. They also blocked the sterilizing effects of neonatal androgen administration by simultaneous injections of anti-oestrogen, ethamoxytriphetol (MER-25) [12, 13]. Naftolin *et al.*[14, 15] who demonstrated the aromatase activity in the hypothalamus, postulated that the conversion of testosterone to oestrogen by brain aromatase is a prerequisite for androgen-sterilization. The presence of the aromatase activity in the hypothalamus of neonatal rats lent support to this hypothesis [16]. Recently, McEwen *et al.*[17] and Vreeburg *et al.*[18] showed that the sterilizing effects of neonatal androgen injections could be abolished by administration of an aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD). However, it should be added that a few workers are still hesitant in agreeing to the aromatization concept [19–21] (*vide infra*). In all the experiments yielding results supportive of this hypothesis, antioestrogens or aromatase inhibitors were given subcutaneously. In the experiments reported here, anti-substances were directly placed in the hypothalamus of newborn rats.

## MATERIALS AND METHODS

Female rats of the Sprague–Dawley strain were used. In experiments I and II, 5-day-old female rats

(day of birth = day 1) received bilateral implants of an antioestrogenic steroid, 16β-ethyloestradiol-17β (EED) or an aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD, ICN K & K Lab. Inc. U.S.A.), followed by a single subcutaneous injection of 50 μg testosterone propionate (TP) dissolved in 0.02 ml sesame oil 6 h later. In experiment III, a mixture of TP and the antioestrogen, ethamoxytriphetol (MER-25), was implanted to 2-day-old rats. Procedures of intrahypothalamic implantation were the same as those described previously [19]. Rats were given implants under cold anesthesia. The head was stabilized in a head holder made of dental plastics and attached to a stereotaxic apparatus. The head holder was adequately soft to keep pup's head tight enough during implantation. It was so adjusted that the interaural line of the pup was aligned with the ear bars of the apparatus. Appropriate adjustment of the tooth bar made the basal surface of the hypothalamus horizontal.

In experiments I and II, a mixture of EED or ATD, carbon marker and paraffin (50:5:45, by wt.) was warmed on a glass slide placed on a heater. Two L's of stainless steel wire, 0.5 mm in diameter, were placed on the glass to get a 0.5-mm thick sheet of the mixture. Another glass slide was then put on the melted mixture and cooled. The mixture was tamped into a 22 gauge tubing (0.4 mm i.d.) by pressing the tip of the tubing into the sheet. The tubing was inserted into the brain toward the desired site and the pellet at the tip was extruded by means of a stylet. In experiment III, paraffin mixture containing 1% TP and 5% carbon marker, and another one containing 1% TP, 5% carbon marker and 50% MER-25 were prepared for implantation. Sham-operated rats received micropellets of paraffin-5% carbon marker mixture. Some rats were implanted subcutaneously with micropellets.

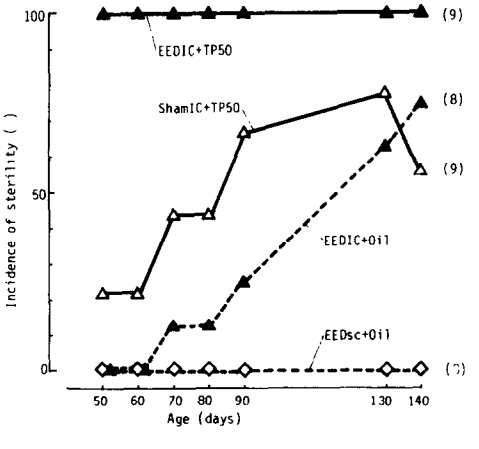


Fig. 1. Development of sterility after implantation of micropellets of EED-paraffin mixture followed by a subcutaneous injection of 50 μg TP dissolved in 0.02 ml oil (TP50) or oil vehicle (Oil) in newborn female rats. IC, sc and Sham designate intrahypothalamic, subcutaneous and sham-implantation, respectively. Numbers of rats are given in parentheses.

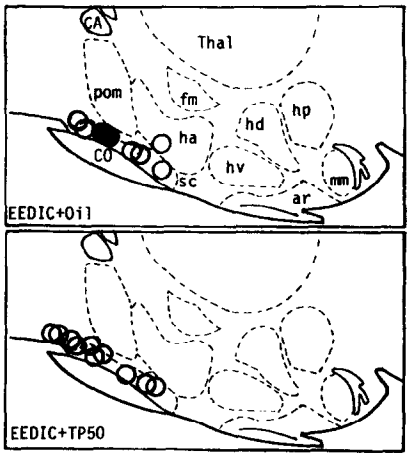


Fig. 2. Diagrams showing loci of intrahypothalamic micropellets of EED-paraffin mixture in rats given a subcutaneous injection of oil (Oil) or 50 μg TP (TP50). Cross-hatched and open circles indicate loci of micropellets in rats with (CL+) and without corpora lutea (CL−) in their ovaries, respectively. Abbreviations: ar, arcuate nucleus; CA, anterior commissure; CO, optic chiasma; fm, paraventricular nucleus; ha, anterior hypothalamic nucleus; hd, dorsal hypothalamic nucleus; hp, posterior hypothalamic nucleus; hv, ventromedial hypothalamic nucleus; mm, medial mammillary nucleus; pom, medial preoptic nucleus; sc, suprachiasmatic nucleus; Thal, thalamus.

The operated pups were returned to their nursing mothers after regaining conciousness, and weaned at 22 days of age. Vaginal smears were examined daily from the day of vaginal opening onward until 140 days of age in experiments I and II and until 60 days in experiment III. Sacrifices were performed at 140 days of age in experiments I and II and at 100 days in experiment III. Ovaries were weighed and studied histologically. Animals which showed persistent vaginal cornification for more than 10 consecutive days and/or had no corpora lutea in their ovaries were considered as sterilized. Exact loci of implanted micropellets were checked histologically.

Six to 12 control micropellets were weighed by an ultramicrobalance (Mettler ME-22) to the nearest 0.1 μg. Calculated amounts of EED, ATD, TP and MER-25 per rat averaged 50, 87, 1.5 and 82 μg, respectively.

Tests for purity of EED and ATD before use by means of melting point, I.R. spectrum, mass spectrometry, $^{12}$C-nuclear magnetic resonance and high pressure liquid chromatography revealed no significant amounts of contaminants. Up to 85–88% of aromatase activity in immature rat ovaries which had been stimulated by pregnant mare's serum gonadotropin was inhibited *in vitro* by ATD [22].

## RESULTS

### I. *Effect of EED on androgen sterilization*

Of 8 rats given intrahypothalamic implants of EED followed by a subcutaneous injection of seasame oil

6 h later, 1 rat became sterilized by 70 days of age, 2 by 90 days, 5 and 6 by 130 and 140 days, respectively. Thus, EED-paraffin mixture implanted in the hypothalamus induced sterility in the recipients. The sterilizing potency of intrahypothalamic micropellets of EED-paraffin mixture was still more evident when the implantation was followed by subcutaneous injection of 50 μg TP. Nine rats so treated became persistent-oestrous from the day of vaginal opening onwards, while only 2 of the 9 rats receiving sham-operation or TP injection neonatally were sterile at 50 days of age (Fig. 1). The micropellets were located in the anterior hypothalamus (Fig. 2).

II. *Effect of ATD on androgen-sterilization*

Subcutaneous injection of 1 mg ATD dissolved in 0.02 ml oil neither exerted any sterilizing effect nor inhibited the sterilizing effect of 10 or 50 μg TP (data not shown).

By contrast, intrahypothalamic implants of micropellets of ATD-paraffin mixture induced sterility in 4 out of 10 rats by 90 days of age. The sterilizing effects of micropellets of ATD-paraffin mixture placed in the hypothalamus was more evident when the implantation was followed by subcutaneous injection of 50 μg TP 6 h later. Two of the 9 rats receiving sham-implantation and 50 μg TP were sterilized at 50–60 days of age, while 7–8 of the 10 rats, given intrahypothalamic ATD pellets and injection of 50 μg TP, developed sterility at 50 and 90 days of age, respectively (Fig. 3). The micropellets of ATD-paraffin mixture were located in the anterior hypothalamus (Fig. 4).

III. *Effects of intrahypothalamic implantation of TP-paraffin and TP-MER-25-paraffin micropellets*

At 60 days of age, intrahypothalamic paraffin micropellets containing 1% TP induced sterility in 11 out of 16 rats (69%), while the same amount of TP developed sterility in only 1 out of 16 rats (6%) if the intrahypothalamic micropellets contained MER-25

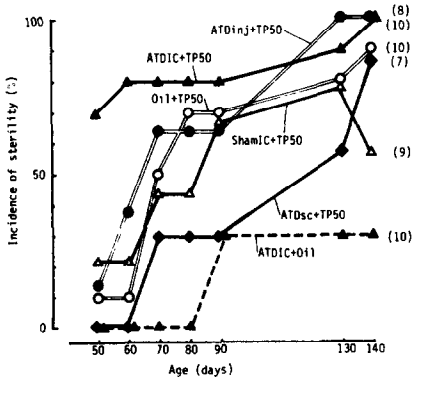


Fig. 3. Development of sterility after administration of ATD-paraffin mixture (ATDIC) or 1 mg ATD dissolved in 0.02 ml oil (ATDinj) in combination with 50 μg TP in 0.02 ml oil (TP50), or oil vehicle only (Oil) in newborn female rats. See Fig. 1 for other abbreviations.

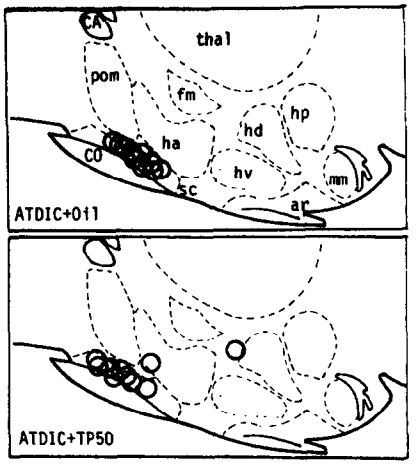


Fig. 4. Diagrams showing loci of intrahypothalamic micropellets of ATD-paraffin mixture in rats given a subcutaneous injection of 0.02 ml oil (Oil, upper) or 50 μg TP (TP50, lower) 6 h later, respectively. For abbreviations see Fig. 2.

together with TP. The difference was significant ($\chi^2$-test, $df = 2$, $\chi^2 = 13.3$, $P < 0.01$). Amount of MER-25 in the pellets was about 55 times that of TP by weight. Intrahypothalamic implantation of micropellets of MER-25-paraffin mixture or subcutaneous implantation of either one of those two types of micropellets did not induce sterility (Table 1). The micropellets were located between the anterior and posterior hypothalamus including suprachiasmatic nucleus, arcuate nucleus and medial mammillary nucleus (Fig. 5).

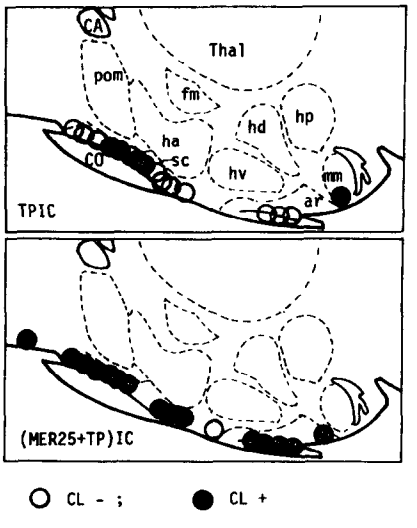


Fig. 5. Diagrams showing loci of intrahypothalamic micropellets of TP-paraffin mixture (upper) and MER-25-TP-paraffin mixture (lower). Cross-hatched and open circles indicate loci of micropellets in rats with (CL+) and without CL (CL−) in their ovaries, respectively. For abbreviations see Fig. 2.

Table 1. Sterilization at 60 days of age after implantation of micropellets TP-paraffin, TP-MER-25-paraffin or MER-25-paraffin mixture neonatally.

| Group | Number of rats CL− | CL+ | Total | Sterilization (%) |
|---|---|---|---|---|
| 1%TP, intrahypothalamic | 11 | 5 | 16 | 69* |
| 1%TP, subcutaneous | 0 | 7 | 7 | 0 |
| 1%TP + 50%MER-25, intrahypothalamic | 1 | 15 | 16 | 6* |
| 1%TP + 50%MER-25, subcutaneous | 0 | 6 | 6 | 0 |
| 50%MER-25, intrahypothalamic | 0 | 18 | 18 | 0 |

* Significantly different ($P < 0.01$).
Rats bearing no CL (CL−) in their ovaries were considered as sterilized.

## DISCUSSION

The results of experiment III supported the hypothesis that aromatization of testosterone into oestradiol is a prerequisite for sterilization of female rats given neonatal administration of the androgen. Since bilateral intrahypothalamic implantation of micropellets of MER-25-paraffin mixture containing 42 μg of the antioestrogen per pellet in 4-day-old female rats failed to inhibit the sterilizing effect of a subcutaneous injection of 30 μg TP 24 h later, the present author suggested the possibility that unconverted testosterone might have a sterilizing action on the neonatal brain [20]. In later experiments, however, the author observed that intrahypothalamic implants of 5α-dihydrotestosterone (DHT)-paraffin mixture was ineffective in inducing sterilization [23], confirming the report of McDonald and Doughty[11]. Failure of neonatal intrahypothalamic implants of MER-25 to block sterilization by TP in the author's previous experiments might be attributed to the fact that the amount of the antioestrogen was too small to block the action of the oestrogen converted from testosterone injected subcutaneously or that the effect of the antioestrogen pellets was restricted to the close proximity to the pellets so that oestrogen converted from androgen in the other parts of the hypothalamus was still capable of inducing sterilization, since the sites of oestrogen action involved in the androgen-sterilization may be distributed in a wide area of the brain.

In the experiments I and II, EED and ATD did not inhibit but even stimulated the sterilizing effect of TP. According to Takikawa[24], the uterotropic potency of EED was only 0.01% of that of oestradiol. The present author reported previously that paraffin micropellets containing 0.4 μg oestradiol implanted into the anterior hypothalamus of a newborn female rat induced sterility, while similar micropellets placed in the other parts of the hypothalamus were without effect in this respect [10]. Accordingly, when applied to the hypothalamus, 0.4 μg oestradiol/rat appears to be the threshold amount for inducing sterilization. Since the amount of EED given to each rat was 50 μg, this may be as active as 0.005 μg oestradiol in uterotropic function, or 1/80 of the threshold amount of oestradiol for inducing sterilization when placed directly in the hypothalamus of neonatal rats. It is not known whether EED has an intrinsic differentiative effect on the neonatal rat brain.

Induction of sterility by intrahypothalamic implants of ATD does not seem to be due to any impurity, since $^{12}$C-NMR analysis and high pressure liquid chromatography demonstrated no significant contaminants. The present result does not agree with the report of McEwen *et al.*[17] that the neonatal rat brain was protected from the effects of exogenous or endogenous testosterone by ATD given in Silastic capsules. This discrepancy may be due to the difference in method of application of ATD or to the strain difference.

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