EFFECTS OF SEX STEROIDS ON THE CHOLINE ACETYLASE ACTIVITY IN THE HYPOTHALAMUS OF FEMALE RATS*

TAKASHI KOBAYASHI, TAKURO KOBAYASHI, JUNZO KATO AND HIROSHI MINAGUCHI

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo

The central nervous system, especially the hypothalamus, exerts an influence on the gonadotropin secretion from the anterior pituitary, resulting in the stimulation of gonads. The sex steroids, in turn, react back on the central nervous system in order to adjust the anterior pituitary function for reproductive cycles (Harris, 1955a).

As reported in the previous paper (Kobayashi et al., 1963), the characteristic fluctuations of the choline acetylase activity in the hypothalamus of rats were found along with the estrous cycle and castration, the fact indicating a cholinergic mechanism of the hypothalamus intimately correlated with the gonadotropin secretion.

The effects of sex steroids on the content of acetylcholine (Gitsch and Reitinger, 1953; Kuwajima, 1957) and the activity of cholinesterase (Nagano, 1956) in the rat hypothalamus were demonstrated. However, the activity of choline acetylase, which may also represent the level of cholinergic function in tissues (Feldberg and Vogt, 1948; Hebb, 1957; Ebashi, 1958), has not yet been studied in relation to the reproductive function. Therefore, the effects of sex steroids such as estradiol, progesterone and testosterone on the activity of choline acetylase in the hypothalaminrs of rats were investigated to elucidate the details of the feedback mechanism of those gonadal hormones on the central nervous system from the enzymatic point of view.

MATERIALS AND METHODS

Adult female albino rats of the Wistar strain weighing 160±20 g were ovariectomized and used for investigation. Estradiol benzoate, progesterone and testosterone propionate were each dissolved in 0.2 ml of sesame oil and injected subcutaneously once daily; control animals were treated with the same dose of sesame oil. The animals were divided into 4 groups; the 1st group was to be autopsied on the 10th day and the 2nd, the 3rd and the 4th on the 15th, 20th and 100th day respectively after castration. In the 1st group, the steroids were administered for 10 days, beginning on the day of castration, and in the 2nd and the 3rd group, 5 successive days prior to the sacrifice of the animal. In the last group 1 μg estradiol benzoate was given for 3 successive days prior to the sacrifice. The animals were killed by decapitation 24 hrs. after

Received for publication December 25, 1963.

* This work was supported in part by Grant in Aid for Scientific Research from the Ministry of Education and from the Population Council, N.Y., (Grant No. M 63120).
the last injection. The details of the methods for dissecting the brain tissue and preparing the enzyme were described in the previous paper (Kobayashi et al., 1963). The choline acetylase activity was measured by the method of Ebashi (1957) with modification (Kato, 1959 and 1960). The unit of the enzyme is expressed in μg of acetylcholine formed per g of lyophilized powder of the brain tissue after 60 mins. incubation at 38°C. The pituitaries pooled from 5 rats were subjected to the assay, because of the low activity.

RESULTS

Effect of estradiol on the choline acetylase activity in the hypothalamus and the hypophysis of the rats on the 10th day after ovariectomy

Ten μg estradiol benz. per day was administered to the castrated rats. Vaginal smear was fully cornified on the day of sacrifice. As indicated in Table 1, the rats treated with estradiol showed a lowered enzyme activity in the hypothalamus, compared with that of the untreated castrated animals (P < 0.01) and of the castrated animals treated with sesame oil (P < 0.05), while any demonstrable difference in the enzyme activity in the hypophysis was not observed among these groups.

Table 1. Effects of gonadal hormones on the choline acetylase activity in the hypothalamus and hypophysis of the spayed rats†

<table>
<thead>
<tr>
<th>Control</th>
<th>With gonadal hormones administered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castrated</td>
<td>Castrated with sesame oil††</td>
</tr>
<tr>
<td>No. of animals</td>
<td>14</td>
</tr>
<tr>
<td>Enzyme activity in hypothalamus (mean ± stand. deviat.)</td>
<td>1086±216</td>
</tr>
<tr>
<td>Enzyme activity in hypophysis (mean ± stand. deviat.)</td>
<td>153</td>
</tr>
</tbody>
</table>

† The rat on the 10th day of castration
†† Sesame oil in 0.2 ml, ††† doses of 10 μg of estradiol benzoate, †††† 1 ml of progesterone and ††††† 10 μg of testosterone propionate in 0.2 ml sesame oil administered subcutaneously 10 days, beginning on day of ovariectomy, respectively
* P < 0.01, compared with the value for castrated group and P < 0.05, compared with the value for castrated rats administered with sesame oil

Inhibitory effect of estradiol at various doses

The minimal effective dose of estradiol to prevent an increase in the choline acetylase activity in the hypothalamus following gonadectomy was studied. The enzyme activity was decreased significantly in the rat treated with 10 μg (P < 0.05), 1.0 μg or 0.1 μg (P < 0.01) of estradiol, compared with that of untreated castrated rats (Table 2). Among the groups with estradiol a statistically significant difference in the activity was not revealed.
Table 2. Inhibitory effect of estradiol benzoate at various doses on the choline acetylase activity in the hypothalamus of the rat 10 days after ovariectomy

<table>
<thead>
<tr>
<th>Dose of Estradiol Benzoate (µg)</th>
<th>No. of Animals</th>
<th>Enzyme Activity (mean ± stand. deviat.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 µg</td>
<td>5</td>
<td>1021 ± 88</td>
</tr>
<tr>
<td>1 µg</td>
<td>5</td>
<td>811 ± 99*</td>
</tr>
<tr>
<td>10 µg</td>
<td>11</td>
<td>789 ± 54*</td>
</tr>
</tbody>
</table>

† 0.2 ml of sesame oil administered subcutaneously
†† Doses of estradiol benzoate administered subcutaneously 10 days, in 0.2 ml of sesame oil, beginning on the day of ovariectomy
* P<0.01, compared with the castrated control
** P<0.05, compared with the castrated control

Effect of estradiol on the choline acetylase activity in the anterior and posterior hypothalamus

In order to analyse the actual site of hypothalamus in which the choline acetylase activity is influenced by estrogen injected, the enzyme activities in the anterior and posterior hypothalamus were measured in the rats treated with estradiol.

In the anterior hypothalamus, no statistically significant difference was observed between the castrated controls and the castrated animals with estradiol, whereas in the posterior hypothalamus the difference was noticeable from the statistical point of view, P<0.01 (Table 3). It was found that the posterior hypothalamus was mainly responsible for the inhibitory effect of estradiol on the choline acetylase activity in the hypothalamus.

Table 3. Inhibitory effect of estradiol benzoate on the choline acetylase activity in the posterior hypothalamus of the spayed rat†

<table>
<thead>
<tr>
<th></th>
<th>Uncastrated controls††</th>
<th>Castrated controls</th>
<th>Castrated with estradiol benz,††</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity in anterior hypothalamus</td>
<td>776 ± 143</td>
<td>751 ± 154</td>
<td>756 ± 134</td>
</tr>
<tr>
<td>Enzyme activity in posterior hypothalamus</td>
<td>930 ± 218</td>
<td>1203 ± 275</td>
<td>1001 ± 137*</td>
</tr>
</tbody>
</table>

† The rat on the 10th day of castration
†† Doses of 10 µg of estradiol benzoate in 0.2 ml sesame oil administered subcutaneously 10 days, beginning on the day of ovariectomy
††† From the data in Table 3 in the previous paper (Kobayashi et al., 1963)
* P<0.01, compared with the value for castrated controls
Effect of estradiol on the choline acetylase activity in the hypothalamus of the rats on the 15th day after castration

Doses of 10 µg or 50 µg of estradiol benz. were administered to the rats. Vaginal smear was fully cornified on the day of sacrifice. Mean value of the enzyme activity in the rat treated with 10 µg of estradiol was lower than that of the control treated with the vehicle, but the difference between them was not statistically significant. It is to be noted that 10 µg of estradiol caused a statistically significant change in the variance of mean value, indicating some effect of estradiol in that dose on the hypothalamic enzyme activity. The clear inhibitory effect of estradiol was observed by the administration of 50 µg of the steroid, compared with the controls with the vehicle (P < 0.01). (Table 4)

Table 4. Effects of gonadal hormones on the choline acetylase activity in the hypothalamus of the rat on the 15th day of castration

<table>
<thead>
<tr>
<th>Dosage /day</th>
<th>Control</th>
<th>Estradiol benz.†††</th>
<th>Progesterone†††</th>
<th>Testosterone prop.†††</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castrated</td>
<td>Castrated with sesame oil†††</td>
<td>10 µg†</td>
<td>50 µg†</td>
<td>1 mg†</td>
</tr>
<tr>
<td>No. of animals</td>
<td>14</td>
<td>11</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td>963±210</td>
<td>946±77</td>
<td>837±184</td>
<td>768±103*</td>
</tr>
</tbody>
</table>

† Dosage /day
††† 0.2 ml of sesame oil administered subcutaneously
††††Doses of estradiol benzoate, progesterone, and testosterone propionate in 0.2 ml sesame oil administered subcutaneously 5 days, beginning on the 10th day postoperatively
* P < 0.01, compared with the value for the castrated with sesame oil group

Effect of estradiol on the choline acetylase activity in the hypothalamus of the rats on the 20th day after castration

The experiment of this group is aimed at the investigation of whether estrogen exerts an influence upon the choline acetylase activity in the hypothalamus of the rat on the 20th day after the castration, which has been shown to have returned to the initial value of the uncastrated controls (Kobayashi et al., 1963). Ten µg of estradiol benz. were injected to the rats. Vaginal smear was fully cornified on the day of sacrifice.

As indicated in Table 5, the test showed no significant difference in the mean value of the choline acetylase activity among the groups of spayed control with or without sesame oil and spayed animals with estradiol. Greater variation in standard deviation was observed in rats with estradiol than that in the controls, indicating some effect of estradiol on the enzyme activity in the hypothalamus of the rats on the 20th day after castration. It was found that in the rats 20 days after castration the activity of choline acetylase in the hypothalamus was not affected by the administration of estradiol.
Table 5. Effects of gonadal hormones on the choline acetylase activity in the hypothalamus of the rat on the 20th day after castration (mean ± stand. deviat.)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Castrated with sesame oil</th>
<th>With gonadal hormones</th>
<th>Castrated with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Castrated</td>
<td>0.2 ml of sesame oil administered subcutaneously</td>
<td>Estradiol††</td>
<td>Progesterone†††</td>
</tr>
<tr>
<td>No. of animals</td>
<td>12</td>
<td>11</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td>765±116</td>
<td>722±62</td>
<td>809±235</td>
<td>766±88</td>
</tr>
</tbody>
</table>

† 0.2 ml of sesame oil administered subcutaneously
†† Doses of 10 µg of estradiol benzoate, ††† of 1 mg of progesterone and †††† of testosterone propionate in 0.2 ml of sesame oil administered subcutaneously 5 days, beginning on the 15th day after castration
* P<0.05, compared with the value for the control group

**Effect of estradiol on the choline acetylase activity in the hypothalamus of the rats on the 100th day after castration**

No significant difference was revealed between the controls and the animals with estradiol (Table 6). It was found that in the rats which had long been castrated the activity of choline acetylase in the hypothalamus was not affected by the administration of estradiol.

Table 6. Effect of estradiol benzoate on the choline acetylase activity in the hypothalamus of the rat on the 100th day after castration (mean ± stand. deviat.)

<table>
<thead>
<tr>
<th></th>
<th>Castrated control</th>
<th>Castrated with estradiol benz.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td>760±108</td>
<td>702±75</td>
</tr>
</tbody>
</table>

† Doses of 1 µg of estradiol benzoate administered subcutaneously 3 successive days prior to the sacrifice

**Effect of progesterone on the choline acetylase activity in the hypothalamus of the spayed rats**

The effect of progesterone on the choline acetylase activity in the hypothalamus of rats was studied simultaneously to disclose the possible mechanism of progesterone effect on the central nervous system.

One mg of progesterone did not evoke significant variations in the enzyme activity in the hypothalamus nor in the hypophysis of the rats on the 10th day after the castration (Table 1). Progesterone had no marked effect on the choline
acetylase activity in the hypothalamus and the hypophysis 10 days after the castration. The effects of 1 mg, 5 mg and 10 mg of progesterone on the enzyme activity in the hypothalamus of the rats on the 15th day after the castration were shown in Table 4. Any significant difference in mean value was not observed among these groups and the control, but in the animals with 1 mg of progesterone the standard deviation of the value was significantly greater than that of the controls with sesame oil (P < 0.01). Therefore, progesterone had no definite effect on the choline acetylase activity in the hypothalamus on the 15th day after the castration, except for the presence of considerable variations in the activity in the animals treated with doses of 1 mg.

Among the controls with or without sesame oil and the rats of 1 mg of progesterone on the 20th day after the castration, any significant difference in the activity of choline acetylase in the hypothalamus was not observed (Table 5). It seems unlikely that progesterone has some effect on the enzyme activity in the hypothalamus of the rat 20 days after the castration.

Effect of testosterone on the activity of choline acetylase in the hypothalamus of the spayed rat

It has been reported that androgen may act on the level of the hypothalamus or the hypophysis in female rats (Nelson and Merckel, 1937; Wolfe and Hamilton, 1937; Miyamoto, 1959).

In the rat 10 days after ovariectomy, 10 μg of testosterone propionate caused any significant changes in the activity of choline acetylase neither in the hypothalamus nor in the hypophysis (Table 1), and also dosage of 1 mg of testosterone did not exert a clear effect on the activity in the hypothalamus of the rat on the 15th day after the castration (Table 4). On the other hand, a significant increase in the enzyme activity in the hypothalamus was obtained by the administration of 10 μg of the steroid to the rats 20 days after the castration (Table 5).

DISCUSSION

Elevation of the activity of choline acetylase in rat hypothalamus following ovariectomy was reduced by the administration of estradiol to the level of the normal controls (Table 1). This is consistent with the well established fact (Nelson, 1933; Schoeller et al., 1936) that the development of castration cells in the hypophysis of rats that accompanies an increased gonadotropic potency is prevented by the administration of estrogen. Nagano (1956) reported that the cholinesterase activity in spayed rat hypothalamus was depressed with a large amount of follicular hormone. Furthermore, definite effects of estrogen on the content of acetylcholine in rat hypothalamus were recognized by other workers (Gitsch and Reitinger, 1953; Kuwajima, 1957). Along with those results concerning cholinesterase and acetylcholine in this laboratory (Nagano 1956; Kuwajima 1957), our data indicate that a cholinergic mechanism in spayed rats may be sensitive to estradiol. Hohlweg and Junkmann (1932) and Hohlweg (1935) postulated the existence of the "Sexualzentrum" in the hypothalamus, a center which is affected by a blood level of estrogen. It is possible that estrogens exert some direct action on the adenohypophysis, but there can be little doubt that the main action of
estrogens in the regulation of estrous cycle is exerted on the central nervous system (Harris, 1955b). Furthermore, Flerkó and Szentágothai (1957) and Lisk (1960) has insisted that the estrogen sensitive structures appeared to lie in the hypothalamus. In consideration of these arguments it is reasonable to presume that the estrogen sensitive structure of cholinergic nature in the hypothalamus is closely associated with the feedback mechanism of estrogen on the sex centre.

As indicated in Table 2, the minimal effective dose per day of estradiol benz. to prevent an increased gonadotropic potencies in the hypophysis was less than 0.1 μg. This fact suggests that the enzyme activity in the hypothalamus may be so highly sensitive to such a small amount of estrogen. Spayed rats did not develop castration cells in the hypophysis by daily injections of 0.4 μg of estradiol benz. (Schoeller et al., 1936). It is noticeable that the effective dose of estradiol to prevent an increase in the choline acetylase activity in the hypothalamus was to the same degree as to the prevention of castration cell changes in the hypophysis. This finding supports the evidence that there is a close correlation between the cholinergic mechanism in the hypothalamus and the secretion of gonadotropins from the hypophysis.

A striking increase in the activity of choline acetylase in the posterior hypothalamus following ovariectomy was prevented by the administration of estradiol (Table 3). In our previous paper (Kobayashi et al., 1963), it was demonstrated that the posterior hypothalamus might be responsible for the change in the choline acetylase activity in the hypothalamus following castration. Flerkó et al. (Flerkó, 1957; Flerkó and Illei, 1957; Flerkó and Szentágothai, 1957) proposed that in the anterior hypothalamus existed “steroid receptors”, but recently Bogdanove (1963) published that in rats with anterior hypothalamic lesions castration cell development can result from ovariectomcy and be prevented by estrogen replacement. Although there is a diversity of opinion as to the exact location of the steroid sensitive structure in the hypothalamus, our results suggest that in the posterior hypothalamus in rats may exist a estrogen sensitive structure, cholinergic in nature.

Table 4 revealed that the choline acetylase activity in the rat hypothalamus on the 15th day after castration was reduced to the level of the normal control only with as much as 50 μg of estradiol. Along with the data that estradiol prevented the elevation in the acetylcholine content in the rat hypothalamus on the 15th day after castration (Kuwajima, 1957), it is clear that estradiol has the inhibitory effect on the level of the cholinergic function in the hypothalamus of rats on the 15th day after castration. When 10 μg of estradiol was administered, however, the difference in mean value of the enzyme activity in the hypothalamus was not statistically significant, but that in variance of the value was statistically significant between the control and the animals with estradiol. Therefore, the administration of 10 μg of estradiol may also have some effect on the enzyme activity of the choline acetylase in the hypothalamus. It is found that estradiol has an inhibitory effect on the elevated activity of choline acetylase in the hypothalamus. This finding is also in good agreement with the fact (Nelson, 1933; Schoeller et al., 1936) that estrogen prevents the castration cell changes in the hypophysis. However, in the rat 20 days and 100 days after castration the acti-
vity of choline acetylase in the hypothalamus was not affected by the administration of estradiol (Tables 5 and 6). Accordingly, it seems unlikely that estradiol has some effect on the activity of hypothalamic choline acetylase in rats 20 days and 100 days after castration which is lowered to a level of the uncastrated rats.

Progesterone failed to cause a definite change in the activity of choline acetylase in the hypothalamus of spayed rats in clear contrast to the inhibitory effect of estradiol (Tables 1, 4 and 5). This is compatible with the generally accepted fact that progesterone can not prevent the appearance of castration cells in the hypophysis of rats (Greep and Jones, 1950; Iwasaki, 1957). Kent and Liberman psychic estrus in the hamster by the administration (1949) induced of progesterone into the brain ventricle. Kobayashi (1956) confirmed that copperinduced ovulation in rabbits can be inhibited by the intravenous injection of progesterone prior to copper salt injection. Kawakami and Sawyer (1959) reported the characteristic changes in the electroencephalographic pattern following progesterone treatment in ovariectomized rabbits. Furthermore, recently, it was found by an electroencephalographic analysis that progesterone modified the activity level of the posterior hypothalamus (Kobayashi et al., 1962). It is revealed from the above data that progesterone may act on the central nervous system, especially on the hypothalamus in regard to regulation of reproductive functions. According to the result obtained by the use of progesterone-4-C^14 it seems unlikely that the hypothalamus of female rats has an ability of preferential incorporation of progesterone (Kato, 1963). Although much remains to be investigated to confirm whether or not progesterone can accumulate in the hypothalamus, there is a possibility that some progesterone sensitive mechanism of non-cholinergic nature is located in the hypothalamus.

It has been reported that the androgenic substances altered changes in the hypophysis (Nelson and Merckel, 1937; Wolfe and Hamilton, 1937). However, as can be seen in Tables 1 and 4, testosterone in a small dose did not evoke clear-cut alterations in the activity of choline acetylase in the hypothalamus of spayed rats. The clear steroid-choline acetylase activity relationship was not observed in the case of androgen. It is possible that the inhibitory effect of androgen on the development of castration cells is mediated through a way other than the cholinergic mechanism. It is of interest that 10 μg testosterone propionate increased the choline acetylase activity in the hypothalamus of rats on the 20th day after castration (Table 5). This fact is in favor of observations of Hoogstra and Paesi (1957) that the increase of pituitary FSH occurred in gonadectomized males and females with rather small dose of androgen.

**SUMMARY**

The effects of gonadal hormones—estradiol, progesterone and testosterone on the activity of choline acetylase in relation to the synthesis of acetylcholine in the hypothalamus of the spayed rats were studied to clarify the feedback mechanism of those gonadal steroids on the central nervous system. The following results were obtained: The elevation in the choline acetylase activity in the hypothalamus following ovariectomy was reduced to the level of the normal control by
daily injection of estradiol benzoate in a range of doses of 0.1 µg to 10 µg. Attention should be given to the finding that the posterior hypothalamus is mainly responsible for the inhibitory effect of estradiol. However, no definite effect of estradiol was observed in the rats on the 20th day and the 100th day after castration which showed the lowered enzyme activity in the hypothalamus. Progesterone or testosterone had no demonstrable effect on the choline acetylase activity in the hypothalamus except that as small as 10 µg of testosterone caused an increase in the activity of the rat on the 20th day after castration. It seems reasonable to conclude from the results mentioned above and those from the studies on cholinesterase and acetylcholine in this laboratory that there is the estrogen sensitive structure of cholinergic nature in the hypothalamus, especially in its posterior part, and the structure is closely involved in the mechanism of the secretion of gonadotropins as well as in the feedback mechanism of the steroid.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Hiroshi Kumagai, M. D. and Setsuro Ebashi, M. D., Professors of Department of Pharmacology, Faculty of Medicine, University of Tokyo, for unlimited assistance and valuable advice, and to the staffs of the endocrinological laboratory in our department for their sustained assistance during the course of this investigation. The invaluable help of Dr. Fumiko Ebashi, Department of Pharmacology, University of Tokyo, was greatly appreciated.

REFERENCES

Harris, G.W. Ibid. p. 91, (1955b).


