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Corpus callosum: ovarian hormones and feminization

Roslyn H. Fitch, Patricia E. Cowell, Lisa M. Schrott and Victor H. Denenberg

Biobehavioral Sciences Graduate Degree Program, U-154, University of Connecticut, Storrs, CT 06269 (U.S.A.)

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The rat's corpus callosum is sexually dimorphic with the male's being larger. This difference appears to depend in part on the neonatal presence of testosterone in the male and ovarian hormones in the female. To further investigate the possibility that ovarian hormones participate in the differentiation of the rat's callosum, females received one of the following treatments on postnatal day 8, 12 or 16: (1) ovariectomy (Ovx); (2) 1 mg of testosterone propionate (TP); or (3) sham surgery. All animals were handled daily from birth until weaning. They were sacrificed at 110 days and a mid-sagittal section of the callosum was obtained. From this section measures of callosal area, perimeter, length, and 99 widths were derived. Widths were averaged into 7 factors as defined by prior factor analysis. Ovariectomy, whether on day 8, 12 or 16, enlarged callosal area and 3 of the callosal width factors. TP had no effect on any callosal variable when administered on day 8, 12 or 16. A comparison of control males and females replicated our prior findings of sexual dimorphism. We conclude that ovarian hormones act to feminize the female callosum, and that their removal results in defeminization. Furthermore, the fact that ovariectomy was effective as late as day 16, while TP treatment on day 8 or later had no effect, suggests that masculinization and feminization of this structure constitute separate processes with distinct sensitive periods.

INTRODUCTION

Recently, Berrebi et al.² reported that the mid-sagittal area of the adult male rat's corpus callosum is significantly larger than the female's, with the largest differences being found in the anterior and posterior callosal regions. In addition, rats handled in infancy showed greater male–female differences than non-handled animals. In a later report, Fitch et al.¹¹ found that 1 mg testosterone propionate (TP) administered to handled female rats on day 4 increased the size of their callosa in adulthood to that of males, although TP-treated females did not differ from control females in brain weight. The anterior and posterior regions of the callosum were particularly sensitive to the TP treatment. These data were interpreted as evidence of callosal masculinization.

Fitch et al. also reported that giving 10 μ g of the estrogen-blocker tamoxifen to female rats on day 4 increased the size of their callosa when measured in adulthood, especially in the anterior and posterior callosal regions. These findings suggested that estrogen may contribute to an active process of feminization during the development of the female rat brain, and that defeminization occurs when estrogen is removed. Tamoxifen, an estrogen receptor blocker, has previously been shown to exert defeminizing effects on the sexually dimorphic

nucleus of the preoptic area of the female rat (SDN-POA)^{9,10}, and postpubertal treatment with estrogen and progesterone has been shown to feminize several dimorphic hypothalamic nuclei in gonadectomized male rats³. Further, a number of studies indicate that neonatal ovariectomy of the rat defeminizes sexual receptivity¹³, open-field activity¹⁷, and overall cortical thickness^{7,15,16} in the adult. Neonatal ovariectomy has also been found to alter adult cortical thickness pattern⁷, although others have failed to replicate these findings¹⁸.

If the tamoxifen effects observed by Fitch et al. were mediated by the removal of estrogen, then neonatal ovariectomy should result in similar callosal enlargement. The major purpose of this paper was to test this hypothesis. A second objective was to evaluate the sensitive period for the potential effects of ovariectomy, as well as the sensitive period for the effects of TP treatment.

MATERIALS AND METHODS

Purdue–Wistar rats from our closed colony were mated and placed into maternity cages. The experimental design assigned treatments to subjects within litters. Since there were 11 treatments and 8 pups per litter, two combinations of treatments were created, and litters were randomly assigned to an A or B condition. On day 1 of life (birth occurred on day 0) 6 litters were culled to 7 females

Correspondence: V.H. Denenberg, Biobehavioral Sciences Graduate Degree Program, U-154, University of Connecticut, Storrs, CT 06269-4154, U.S.A.

and one male (condition A), and 7 litters were culled to 6 females and two males (condition B). The treatments for the females in these two conditions are shown in Table I.

In A litters, 3 randomly selected females received ovariectomy (Ovx) surgery on day 4, 8 or 12, while two females received sham surgery on day 8 or 12. The remaining two females received an injection of 1 mg TP in 0.05 ml sesame oil on day 8 or 12. The male served as a control. In B litters, two randomly selected females received Ovx surgery on day 8 or 16, while two females received sham surgery on day 8 or 16. The remaining two females received an injection of 1 mg TP on day 8 or 16. The two males served as controls.

Surgery on days 4 and 8 was done under cryogenic anesthesia, while surgery on days 12 and 16 was done under ether. For Ovx surgery, a single dorsal skin incision was made, and two smaller lateral incisions were made in the underlying muscle. These incisions were positioned slightly below the kidneys. Each ovary was externalized with microforceps, and removed along with the tip of the uterine horn. Both the skin and muscle were sutured. Sham surgery consisted of a skin incision only.

All animals were handled daily, since this procedure maximizes the male-female callosal difference². Handling consisted of removing the litter of pups from the nest cage, leaving the mother in the cage, and placing each pup separately into a 1-gallon tin can containing shavings. The pups remained in the cans for 3 min and were then returned to their home cage⁴. This procedure continued through day 20 with weaning on day 21, when animals were housed in same sex and treatment pairs.

At 110 days of age animals were deeply anesthetized, and were perfused with a mixed-aldehyde fixative through the ascending aorta. Brains were removed and immersed in sucrose-formalin for cryoprotection. The olfactory bulbs were removed and a transverse cut was made just caudal to the hemispheres, leaving the forebrain and midbrain. These brains were then weighed and frozen-sectioned in the sagittal plane at a thickness of 45 μ m. The 12 sections adjacent to the midline in each hemisphere were mounted onto glass slides from a gelatin-alcohol medium, stained with Cresyl violet, and cover-slipped.

Using a projection microscope set at a magnification of 23 \times , the closest intact section to midline in the right hemisphere was chosen for each subject. A magnified drawing of the callosum was made from this section. The drawing was then traced onto a digitizing tablet connected to a Macintosh Plus computer, and the software package Stereology was used to obtain measures of callosal parameters⁵.

RESULTS

During daily handling we observed that the pups ovariectomized on day 4 were markedly delayed in development and were approximately half the size of their littermates at weaning. Therefore, these subjects were not included in subsequent data analyses.

The Stereology software package yielded measures of callosal area, perimeter, length, and 99 widths (taken at equally spaced perpendiculars to the longitudinal axis) for each drawing. The width measures were grouped into the 7 region-specific factors as defined by prior factor analyses⁵. These regional width factors, starting from the genu (anterior) to the splenium (posterior), are: width 1-5 (W1-5), W6-17, W24-36, W46-57, W62-72, W79-95 and W96-99. The final measures for the experimental groups consisted of brain weight (forebrain and midbrain less olfactory bulbs), callosal area, length, perimeter, and the 7 regional widths.

TABLE I

Treatment schedules for A and B female littermates

Age in days	Ovx	Sham	TP
4	A*		
8	A,B	A,B	A,B
12	A	A	A
16	B	B	B

* Due to developmental delays, this group was dropped from subsequent analyses.

The 3 objectives of this study were to evaluate effects of age at which TP was injected or ovariectomy performed; to determine the effects of TP and ovariectomy upon callosal parameters; and to compare male and female controls for sex differences. Since different groups of animals were involved in these comparisons, separate analyses of variance (ANOVAs) were carried out, as discussed below. It should be noted that the standard errors reported in the tables underestimate significance of the within-litter treatments because they do not take into account the littermate correlations.

Age at treatment

Age at testosterone administration. The effects of TP treatment to females on day 8, 12 or 16 were not found to differ significantly for any callosal parameter. Thus TP values were pooled across ages at treatment within litters.

Age at ovariectomy. The day 8 and day 12 Ovx values were compared within A litters, and the day 8 and day 16 Ovx values were compared within B litters. Age at Ovx effects were not found for callosal widths, callosal area, or for brain weight. Therefore, Ovx female values were averaged across days within litters for these measures. Values from control female littermates, and male litter-

TABLE II

Mean \pm S.E.M. corpus callosum parameters (mm) and brain weights (g) for treatment groups

Variable	Sham female	Ovx female	Male
Area	3.123 \pm 0.053	3.342 \pm 0.065**	3.377 \pm 0.067***
Brain weight	1.307 \pm 0.012	1.403 \pm 0.011***	1.480 \pm 0.018***
W1-5	0.745 \pm 0.014	0.784 \pm 0.012*	0.782 \pm 0.015*
W6-17	0.733 \pm 0.011	0.780 \pm 0.014**	0.784 \pm 0.013***
W24-38	0.425 \pm 0.012	0.454 \pm 0.013*	0.441 \pm 0.007
W46-57	0.336 \pm 0.010	0.359 \pm 0.010 [†]	0.360 \pm 0.009*
W62-72	0.310 \pm 0.009	0.326 \pm 0.009	0.315 \pm 0.006
W79-95	0.507 \pm 0.009	0.520 \pm 0.012	0.520 \pm 0.011
W96-99	0.552 \pm 0.008	0.565 \pm 0.010	0.593 \pm 0.020*
n	13	13	13

All statistical comparisons noted above are against the female control group: [†] $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE III

Callosal perimeter and length (mean \pm S.E.M. in mm) by age at Ovx

Variable	Sham female	Day 8 Ovx	Day 12 Ovx	Day 16 Ovx	Control male
Perimeter	14.96 \pm 0.075	14.910 \pm 0.200	15.611 \pm 0.154**	15.172 \pm 0.052*	15.55 \pm 0.132***
Length	6.676 \pm 0.034	6.625 \pm 0.079	6.933 \pm 0.079*	6.791 \pm 0.036*	6.929 \pm 0.058***
n	13	6	5	6	13

All statistical comparisons noted above are against the female control group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

mates in B litters, were also averaged. This yielded one control female value, one Ovx value, and one male value for each parameter in each of 13 litters. Table II summarizes these data.

Age at ovariectomy effects were found for callosal perimeter and length. Therefore, these measures were analyzed separately against controls for each Ovx age. These data are summarized in Table III.

Treatment effects

Testosterone effects. When mean TP values were compared against control female values, with 12 df, no effects of treatment were found for any callosal parameter or for brain weight. It should be noted, however, that females treated with TP on day 8, but not day 12 or 16, had significantly higher body weights than control females (day 8 TP females, 263 g; control females, 250 g; $P < 0.05$). This is consistent with the findings of Barraclough¹, and affirms the potency of the TP injections.

Ovariectomy effects. The ANOVAs comparing Ovx and control female littermates had 12 df. Significant differences were found for callosal area, brain weight, W1-5, W6-17, and W24-38, with Ovx female values being larger than those of control females (Table II). A near-significant effect was also found for W46-57 ($P < 0.09$).

Since a significant age at ovariectomy effect was found for length and perimeter, these measures were analyzed separately against controls for each Ovx age (Table III).

Significant effects were found for both measures only for ages 12 and 16 days, with the Ovx female values being larger than those of controls.

Sex effects in controls. The comparison of our control males and females (Table II) served as an independent test of our prior reports of callosal sexual dimorphisms^{2,5,11}. These ANOVAs had 12 df, with one control male and female value for each of 13 litters. Significant sex effects were found for callosal area, brain weight and callosal regions W1-5, W6-17, W46-57 and W96-99, with male values being larger. Significant sex effects favoring males were also found for callosal perimeter and length (Table III). These sex effects fully replicate our earlier reports^{2,5,11}.

Correlations of brain weight and callosal width factors

To investigate the possibility that the increase in callosal size for Ovx females reflected a general increase in brain size, correlations between the 7 callosal width factors and brain weight were run within each of the treatment groups (control females, control males, and Ovx females). These are shown in Table IV. No consistent correlations were found between brain weight and the width factors within the experimental groups.

DISCUSSION

The results of this study show that: (1) ovariectomy of the female rat between postnatal days 8 and 16 leads to a significant increase in the cross-sectional size of the corpus callosum in adulthood; (2) females ovariectomized during this period have callosa approximately the size of males; and (3) treatment with 1 mg TP, which has previously been shown in two separate experiments to exert profound masculinizing effects on the adult female rat callosum when administered on postnatal day 4¹¹, had no effect on female callosal size when given on day 8, 12 or 16.

Since Ovx females had significantly greater brain weights than controls (see Table II), we considered the possibility that the callosal enlargement observed in Ovx females resulted from a general increase in brain size. Several findings argue against this interpretation. First, the difference in brain weight for Ovx females and

TABLE IV

Correlation of width factors against brain weight

Factor	Control males	Control females	Ovx females
W1-5	0.464*	0.430*	0.259
W6-17	0.572**	0.363	0.157
W24-38	0.026	0.218	0.457*
W46-57	0.245	0.451*	0.277
W62-72	0.168	0.224	0.074
W79-95	0.239	0.352	-0.015
W96-99	0.174	0.500*	0.426
n	19	20	17

* $P < 0.05$; ** $P < 0.01$.

controls was only about half of the male–female difference. In fact, brain weights for Ovx females were still significantly less than those of males. In contrast, callosal widths for Ovx females were equal to or greater than callosal widths of males for all but the posterior-most factor (W96–99). Second, brain weight and callosal widths correlated only sporadically within the experimental groups, and the callosal region which showed the most profound Ovx effect, W6–17, did not show even a marginal correlation to brain weight in the Ovx group (Table IV). Also, the second-highest correlation in the Ovx group (0.426), was obtained in a region in which Ovx and control females did *not* differ (W96–99). These findings are in accord with prior factor analysis findings from a large sample of rat callosal data showing that brain weight and callosal widths did not load on a common factor⁵, as well as the report that callosal size can be significantly increased in day 4 TP-treated females with no increase in brain weight¹¹. The current data, in combination with our prior findings, support the conclusion that the increase in callosal size for ovariectomized females cannot be attributed to a general increase in brain size.

The findings of the current study support the hypothesis that removal of ovarian hormones resulted in callosal enlargement, an effect we have interpreted as callosal defeminization¹¹. This defeminization effect is distinct from our prior finding that TP on day 4 masculinized females' callosa, even though the endpoint of measurement (increased callosal size) is the same. There are two sets of findings supporting this interpretation. First, ovariectomy does not bring about an increase in plasma androgen in female rats, so one cannot argue that the increase in callosal size in Ovx females is androgen-mediated¹². Second, there are distinctly different sensitive periods for the effects of TP treatment and ovariectomy: while callosal sensitivity to TP in the female rat appears to end between days 4 and 7, the sensitive period for ovariectomy lasts at least until day 16. Ultimately, it will be of interest to assess the relative effects of TP treatment and ovariectomy on the callosum at the cellular level, where differences not detectable at the global level may be observed, particularly since sex differences at the EM level have been reported for the rat callosum under

certain environmental conditions¹⁴.

Our ovariectomy findings are consistent with other studies examining the effects of gonadectomy on sexual differentiation of the female rat. These have shown that ovariectomy, or low-dose estrogen replacement following ovariectomy, is effective in altering sexually dimorphic parameters until near-puberty^{13,17}. Indeed, Bloch and Gorski³ have even reported feminizing effects of estrogen and progesterone on the hypothalamic nuclei of post-pubertal gonadectomized male rats. The time-frame for the onset of this sensitive period, however, is less clear. Indeed, the ability to obtain a defeminizing effect on the callosum with a one-time dose of tamoxifen on day 4¹¹ seems inconsistent with this extended sensitive period. It is possible that tamoxifen administered on day 4 permanently altered ovarian secretion. In support of this hypothesis, daily postnatal treatment with as little as 4 μg of tamoxifen on days 1–5 is sufficient to cause permanent anovulatory sterility (PAS) in female rats⁹.

Given this latter interpretation of our tamoxifen effect, it will be necessary to determine whether our ovariectomy effect is mediated by the removal of estrogen, progesterone or both. Pappas et al.¹⁵ found a significant increase in cortical thickness in the rat following neonatal ovariectomy, which could be countered with ethinyl-estradiol replacement but not progesterone. Thus we might hypothesize that the callosal effect is estrogen-dependent as well. Furthermore, the report by Pappas et al. included the finding that Krieg's cortical area 4 was particularly sensitive to ovarian hormones, and this may relate to our finding of increased thickness in the anterior body of the rat callosum.

Finally, the findings discussed in this paper derive from animals that were handled in infancy. We have recently found that handling is necessary for TP to masculinize the female's callosum, since non-handled controls given an injection of 1 mg TP on day 4 did not have an enlarged callosum, while handled females did⁶. We do not know whether handling is necessary for the ovariectomy effect to express itself. However, we can conclude that the administration of testosterone and the removal of ovarian hormones exert distinct effects on the callosum, with different sensitive periods.

REFERENCES

- 1 Barraclough, C.A., Production of anovulatory, sterile rats by single injections of testosterone propionate, *Endocrinology*, 68 (1961) 62–67.
- 2 Berrebi, A.S., Fitch, R.H., Ralphe, D.L., Denenberg, J.O., Freidrich, V.L., Jr. and Denenberg, V.H., Corpus callosum: region-specific effects of sex, early experience, and age, *Brain Research*, 438 (1988) 216–224.
- 3 Bloch, G.J. and Gorski, R.A., Estrogen/progesterone treatment in adulthood affects the size of several components of the medial preoptic area in the male rat, *J. Comp. Neurol.*, 275 (1988) 613–622.
- 4 Denenberg, V.H., Assessing the effects of early experience. In R.D. Myers (Ed.), *Methods in Psychobiology*, Academic, New York, 1977, pp. 127–147.
- 5 Denenberg, V.H., Berrebi, A.S. and Fitch, R.H., A factor analysis of the rat's corpus callosum, *Brain Research*, 497 (1989) 271–279.
- 6 Denenberg, V.H., Fitch, R.H., Schrott, L.M., Cowell, P.E. and Waters, N.S., Corpus callosum: interactive effects of infantile handling and testosterone in the rat, submitted.

- 7 Diamond, M.C., New data supporting cortical asymmetry differences in males and females, *Behav. Brain Sci.*, 3 (1980) 233–234.
- 8 Diamond, M.C., Johnson, R.E. and Ehlert, J., A comparison of cortical thickness in male and female rats — normal and gonadectomized, young and adult, *Behav. Neural Biol.*, 26 (1979) 485–491.
- 9 Dohler, K.D., Hancke, J.L., Srivastava, S.S., Hofmann, C., Shryne, J.E. and Gorski, R.A., Participation of estrogens in female sexual differentiation of the brain: neuroanatomical, neuroendocrine and behavioral evidence. In G.J. De Vries, J.P.C. De Bruin, H.B.M. Uylings and M.A. Corner (Eds.), *Progress in Brain Research*, Vol. 61, Elsevier, Amsterdam, 1984, pp. 99–117.
- 10 Dohler, K.D., Srivastava, S.S., Shryne, J.E., Jarzab, B., Sipos, A. and Gorski, R.A., Differentiation of the sexually dimorphic nucleus in the preoptic area of the rat brain is inhibited by postnatal treatment with an estrogen antagonist, *Neuroendocrinology*, 38 (1984) 297–301.
- 11 Fitch, R.H., Berrebi, A.S., Cowell, P.E., Schrott, L.M. and Denenberg, V.H., Corpus callosum: neonatal hormones and sexual dimorphism in the rat, *Brain Research*, 515 (1990) 111–116.
- 12 Fitch, R.H., McGivern, R.F., Redei, E., Schrott, L.M., Cowell, P.E. and Denenberg, V.H., Neonatal ovariectomy and androstenedione regulation in the adult rat, submitted.
- 13 Gerall, A., Dunlap, J. and Hendricks, S., Effects of ovarian secretions on female behavioral potentiality in the rat, *J. Comp. Physiol. Psychol.*, 82 (1973) 449–465.
- 14 Juraska, J.M. and Kopcik, J.R., Sex and environmental influences on the size and ultrastructure of the rat corpus callosum, *Brain Research*, 450 (1988) 1–8.
- 15 Pappas, C.T.E., Diamond, M.C. and Johnson, R.E., Morphological changes in the cerebral cortex of rats with altered levels of ovarian hormones, *Behav. Neural Biol.*, 26 (1979) 298–310.
- 16 Pappas, C.T.E., Diamond, M.C. and Johnson, R.E., Effects of ovariectomy and differential experience in the rat cerebral cortical morphology, *Brain Research*, 154 (1978) 53–60.
- 17 Stewart, J. and Cygan, D., Ovarian hormones act early in development to feminize open field behavior in the rat, *Horm. Behav.*, 14 (1980) 20–32.
- 18 Stewart, J. and Kolb, B., The effects of neonatal gonadectomy and prenatal stress on cortical thickness and asymmetry in rats, *Behav. Neural Biol.*, 49 (1988) 344–360.