

BRESO 51569

Ovarian estrogen acts to feminize the female rat's corpus callosum

C.M. Mack^a, R.H. Fitch^b, P.E. Cowell^a, L.M. Schrott^a and V.H. Denenberg^c

^a Biobehavioral Sciences Graduate Degree Program, University of Connecticut, Storrs, CT 06269 (USA),

^b Center for Molecular and Behavioral Neuroscience, Rutgers University, Newark, NJ 07102 (USA)

and ^c Biobehavioral Sciences Graduate Degree Program and Department of Psychology, University of Connecticut, Storrs, CT 06269 (USA)

(Accepted 1 September 1992)

Key words: Estrogen; Sensitive period; Ovariectomy; Sex difference; Cerebral cortex

The rat corpus callosum (CC) is sexually dimorphic, with the male CC being larger. Ovariectomy (Ovx) on day 12 has been shown to eliminate this sex difference, with callosal values of Ovx females approaching those of male controls. This suggested that postnatal ovarian estrogen affects the size of the female CC. In the present experiment, one group of female rats received Ovx on day 12, and a second group received Ovx followed by chronic implantation of a silastic tube containing β -estradiol on day 25. Unmanipulated males and sham females served as controls. Examination of the CC at 110 days confirmed our prior findings that males have larger callosa than females and that the Ovx group had increased CC's compared to sham controls. Our new finding was that estrogen treatment was capable of reversing the effects of Ovx. Ovx + estrogen-treated females had decreased CC size as compared to Ovx alone. Indeed, they also had smaller CC values than control females. These findings indicate that ovarian estrogen plays a role in determining CC morphology and that estrogen in the female acts to inhibit overall callosal growth as measured by changes in gross callosal size.

INTRODUCTION

We have reported sexual dimorphism of the rat corpus callosum (CC), with the adult male CC being larger than the female^{1,7}. Zimmerberg has independently confirmed our findings in both the newborn and the adult^{22,23}. More recent studies have demonstrated that exposure to gonadal steroids during prenatal through prepubertal development mediate this dimorphism. Thus, in the male, androgen antagonism via prenatal flutamide treatment and subsequent castration at birth eliminated the sex difference seen in adulthood⁹. However, castration alone on day 1 had no effect⁷. These findings suggest (1) a prenatal sensitive period for androgen action which ceases around the time of birth, (2) the postnatal testosterone surge occurring 2 h after birth contributes to adult CC organization, or (3) the two processes interact.

Androgenic effects on the CC were also found in the female. Testosterone propionate (TP) treatment on day 4, when combined with postnatal handling stimulation, enlarged the female CC in adulthood to equal

that of the male⁷. This sensitivity of the female CC to androgens ceased sometime between postnatal day 4 and day 8¹⁰.

Gonadal steroid influence on the female callosum is not restricted to androgens. There is ample evidence that ovarian hormones during postnatal development play a role in determining adult callosal morphology. Fitch et al.¹⁰ reported that ovariectomy (Ovx) on day 8, 12, or 16 enlarged the female CC, with adult callosal parameters equal to or approaching male control values. This finding has since been replicated¹⁴. These reports parallel those of others who have found neuroanatomical alterations in the rat cerebral cortex following Ovx^{5,15,17}.

This increase in callosal size following Ovx can be attributed to the absence of estrogen, progesterone, or both steroids, either via removal of direct action on steroid receptors or through secondary processes. A study by Fitch et al.⁷ provides indirect evidence for estrogenic influence since administration of the estrogen blocker tamoxifen to female pups on day 4 increased adult callosal size. The above findings, in addi-

tion to reports of estrogenic effects on a variety of cortical parameters^{12,17,18}, suggest that estrogen may play a major role in female CC development. Thus, the purpose of the current study was to evaluate the independent contribution of ovarian estrogen to the morphology of the adult female callosum.

MATERIALS AND METHODS

Purdue-Wistar rats were bred in our closed colony. On day 1 (birth occurred on day 0) litters were culled to 5 females and 3 males. Three females and 2 males from each of 13 litters were used. All animals were handled from day 1 to 21 since this procedure enhances the male-female callosal difference¹. Handling consisted of removing the pups from their home cage and placing them singly into one-gallon tin cans filled with shavings. The pups remained in the can for 3 min and were then returned to their home cage³. Following weaning on day 21, animals were housed in same sex-treatment pairs until sacrificed.

Treatment assignments were made within litters. All surgery was performed under ether anaesthesia. Since no differences in CC size had previously been found among females receiving Ovx on days 8, 12, or 16, and a low survival rate was found among day 4 Ovx¹⁰, all surgeries were performed on day 12. Two females from each litter received Ovx. Two dorsolateral incisions were made in the skin and peritoneum and the ovaries and the tips of the uterine horn were removed. A third female served as an unoperated control.

Silastic implants were inserted on day 25. They were constructed from Silastic tubing (Dow Corning) of 0.625 mm i.d. and 1.2 mm o.d. and were designed to approximate circulating estrogen levels in the female rat pup^{6,13}. The steroid was packed into 10 mm of tubing and sealed with silastic adhesive. After soaking in saline for 24 h, the tubing was inserted under the skin on the dorsal midline just below the neck of one of the Ovx females. The second Ovx female and the control female in each litter received a blank implant. One male served as an unoperated control and a second male received a blank implant.

At 110 days, animals were overdosed with sodium pentobarbital and perfused through the ascending aorta with a mixed-aldehyde fixative. The brains were then removed and stored in sucrose-formalin for cryoprotection. The olfactory bulbs and hindbrain were removed, leaving the forebrain and midbrain. The brain was weighed and sagittally sectioned at a thickness of 45 μ m. The 12 sections closest to midline in each hemisphere were mounted, stained with Cresyl violet, and coverslipped. Using a projection microscope, the closest intact callosum to midline from the right hemisphere was

traced at a magnification of 23 \times . Each drawing was then traced 5 times onto a digitizing tablet connected to a Macintosh Plus computer and the average taken to yield one callosal tracing for each subject. Callosal parameters were obtained using the software package Stereology⁴.

RESULTS

The computer program yielded callosal parameters of area, perimeter, length, and 99 equidistant widths perpendicular to the longitudinal axis. Callosal widths were divided into 7 region-specific factors based on previous factor analysis of the rat corpus callosum⁴. Beginning anteriorly, the 7 callosal widths factors were: widths 1-5 (W1-5), W6-17, W24-38, W46-57, W62-72, W79-95 and W96-99. The final measures were callosal area, perimeter, length, and the seven width factors; and brain weight. No differences were found between sham and control males and their data were pooled. Table I presents the means and standard errors for callosal parameters and brain weight for all subjects. Since the within-litter experimental design yielded one subject per litter per treatment, one missing value eliminated that pair from analyses. In addition, the standard errors in Table I underestimate significance since they do not take into account litter-mate correlations. Analyses of variance were performed to test for group differences.

The main objective of this study was to determine the contribution of ovarian estrogen to adult callosal size. However, before addressing this question, it was first necessary to replicate the sexual dimorphism and the Ovx effects previously reported in our laboratory.

Sex effects

Male and female callosal parameters were compared. One-tailed tests were used since the direction of

TABLE I

Mean \pm S.E.M. for callosal parameters (mm) and brain weight (g) for sham females (Sham F), sham males (Sham M), ovariectomized females (Ovx F) and ovariectomized + estrogen-treated females (Ovx + E F)

Parameter	Sham F	Sham M	Ovx F	Ovx + E F
Area	2.934 \pm 0.078	3.274 \pm 0.062 ****	3.190 \pm 0.074 **	2.733 \pm 0.072 ****
Perimeter	15.113 \pm 0.145	15.696 \pm 0.130 ***	15.298 \pm 0.101	14.753 \pm 0.119 ***
Length	6.781 \pm 0.066	7.011 \pm 0.058 ***	6.818 \pm 0.034	6.619 \pm 0.055 **
W1-5	0.743 \pm 0.012	0.806 \pm 0.018 **	0.754 \pm 0.017	0.722 \pm 0.019
W6-17	0.710 \pm 0.013	0.760 \pm 0.013 **	0.740 \pm 0.017	0.681 \pm 0.022 ***
W24-38	0.397 \pm 0.017	0.407 \pm 0.009	0.428 \pm 0.013	0.361 \pm 0.011 ****
W46-57	0.306 \pm 0.014	0.336 \pm 0.008 *	0.339 \pm 0.010 **	0.286 \pm 0.008 ***
W62-72	0.262 \pm 0.013	0.297 \pm 0.008 **	0.297 \pm 0.011 *	0.251 \pm 0.011 **
W79-95	0.465 \pm 0.016	0.511 \pm 0.010 **	0.510 \pm 0.016 **	0.451 \pm 0.013 **
W96-99	0.517 \pm 0.012	0.554 \pm 0.015 *	0.551 \pm 0.014 *	0.516 \pm 0.013 #
Brain weight	1.330 \pm 0.041	1.487 \pm 0.015 ****	1.394 \pm 0.029 **	1.240 \pm 0.029 ****
n	12	12	13	11

* $P < 0.10$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$; comparisons are against Sham F group (1-tailed tests).

$P < 0.10$; ** $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$; comparisons are against Ovx F group (2-tailed tests).

the difference was known. Males had larger CC's than females, with the genu and splenium having the largest difference (Fig. 1). Significance was obtained for CC area, perimeter, length, and for brain weight (F 's_{1,10} = 21.74, 12.84, 11.36, 15.48; all P 's < 0.01). A 7×2 repeated ANOVA of callosal region \times sex found significant effects for sex and width (P 's < 0.01). Because of our interest in regional specificity, separate analyses were run for each region. Significance was obtained for W1-5, W6-17, W62-72 and W79-95 (F 's_{1,10} = 7.38, 6.50, 5.25, 5.95; P 's < 0.05). In addition, near significance ($P < 0.10$) was obtained for W46-57 and W96-99.

Ovariectomy effects

We also replicated our previous finding that Ovx females had larger callosa than sham females. Significant effects were obtained for CC area and for brain weight (F 's_{1,11} = 4.34, 3.81; P 's < 0.05, one-tailed tests). The callosal region \times Ovx analysis found an overall significant Ovx effect ($P < 0.05$). Further analyses found significant effects for W46-57 and W79-95 (F 's_{1,11} = 3.60, 3.49; P 's < 0.05). Marginal effects were found for W62-72 and W96-99. See Fig. 2.

Estrogen effects

Having replicated our two prior findings, we now turn to the primary purpose of this experiment which was to determine whether estrogen replacement would compensate for the effects of ovariectomy. This was

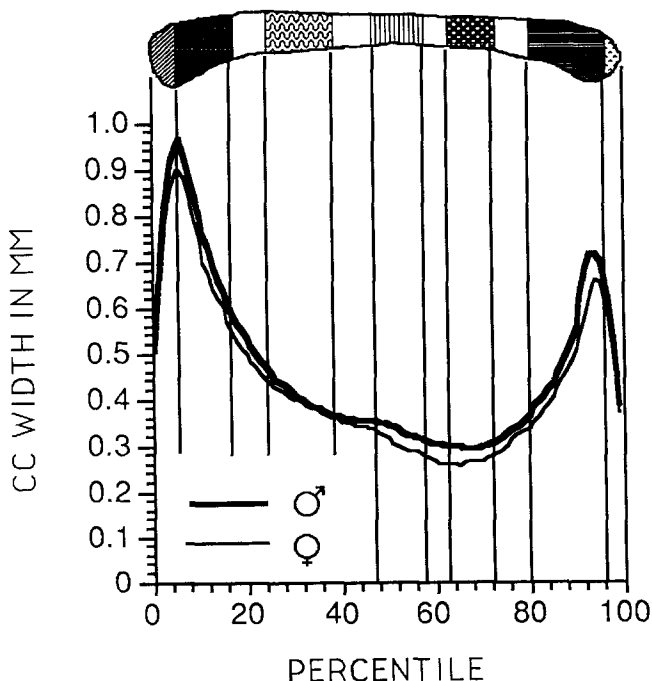


Fig. 1. Mean callosal widths of males ($n = 12$) and females ($n = 12$) as a function of percentile location along the longitudinal axis.

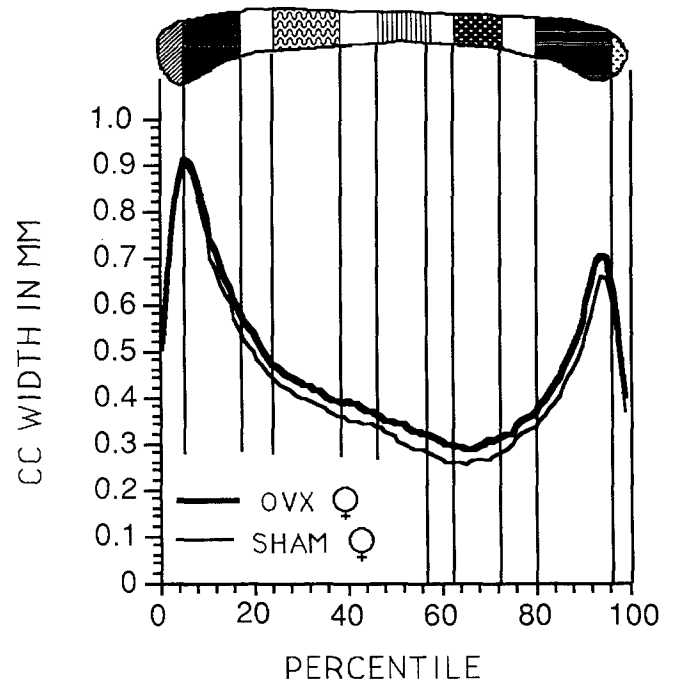


Fig. 2. Mean callosal widths of ovariectomized ($n = 13$) and sham females ($n = 12$) as a function of percentile location along the longitudinal axis.

done by comparing Ovx and Ovx + E littermate females (two-tailed tests). The Ovx + E females were significantly smaller for CC area ($F_{1,10} = 36.57$; $P < 0.001$), perimeter ($F_{1,10} = 16.28$; $P < 0.01$), length ($F_{1,10} = 9.71$; $P < 0.05$), and brain weight ($F_{1,10} = 100.61$; $P < 0.001$). The Callosal Region \times Estrogen Implant analysis found the Implant to be significant at the 0.003 level. Further analyses found significance for W6-17 ($F_{1,10} = 15.40$; $P < 0.01$), W24-38 ($F_{1,10} = 45.83$; $P < 0.001$), W46-57 ($F_{1,10} = 18.12$; $P < 0.01$), W62-72 ($F_{1,10} = 8.02$; $P < 0.05$) and W79-95 ($F_{1,10} = 8.26$; $P < 0.05$). A marginal effect was seen in W96-99. See Fig. 3.

We were surprised to find that estrogen treatment reduced the size of the callosum of the Ovx females to values below those of sham females. Post hoc tests (two-tailed; not shown in Table I) comparing these two groups showed significant effects for CC area ($F_{1,9} = 14.17$, $P < 0.01$), W6-17, W24-38 (F 's_{1,9} = 6.69, 5.74, P 's < 0.05), in addition to length, perimeter, (F 's_{1,9} = 26.05, 22.80, P 's < 0.001) and brain weight ($F_{1,9} = 10.03$, $P < 0.05$)

Correlations of brain weight and callosal width factors

Changes in CC size were accompanied by changes in brain weight (Table I). To address the possibility that differences in callosal size were purely a function of changes in overall brain volume, Table II presents correlations between brain weight and the seven cal-

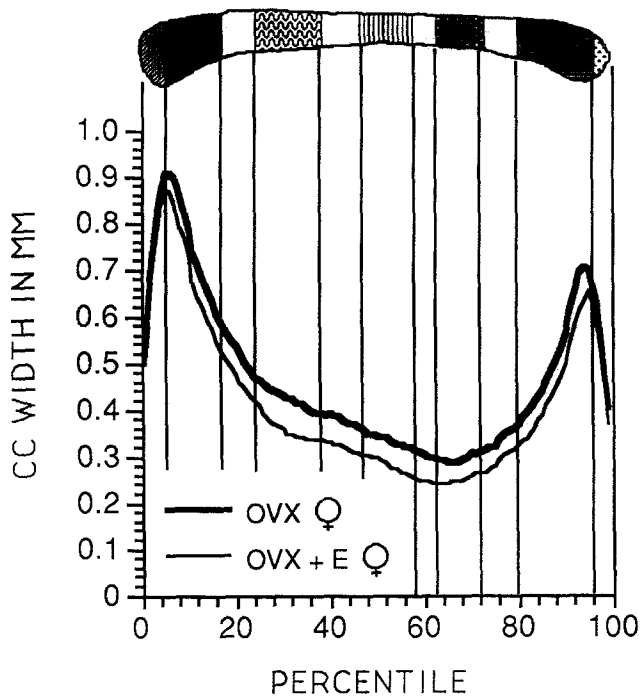


Fig. 3. Mean callosal widths of ovariectomized ($n = 13$) and ovariectomized + estrogen-treated females ($n = 11$) as a function of percentile location along the longitudinal axis.

losal width factors. Although no consistent trends were apparent within any treatment groups, several moderate correlations were found for the Ovx group, with two of these being significant.

DISCUSSION

The current experiment has confirmed two observations previously reported by our laboratory: (1) there is sexual dimorphism of the rat corpus callosum, and (2) Ovx results in increased CC size compared to intact females. Our new finding is that Ovx on day 12 followed by estrogen treatment on day 25 reduced the size of the female callosum in adulthood relative to Ovx females. Indeed, the Ovx + E group had callosal values significantly less than sham females. These data

TABLE II
Correlations of width factors and brain weight within treatment groups

Factor	Sham F	Sham M	Ovx F	Ovx + E F
W1-5	0.279	0.060	0.629 *	-0.040
W6-17	0.315	0.327	0.534	-0.407
W24-38	-0.058	0.215	0.640 *	-0.309
W46-57	-0.064	-0.071	-0.022	-0.046
W62-72	-0.027	-0.339	0.171	-0.077
W79-95	0.330	-0.419	0.546	-0.161
W96-99	0.485	-0.337	0.527	0.241
<i>n</i>	12	12	13	11

* $P < 0.05$.

lead to the conclusion that estrogen acts to inhibit callosal growth. Therefore, the enlarged CC following Ovx originally observed by Fitch et al.¹⁰ can now be attributed in large part to the absence of estrogenic inhibition.

The few studies which have examined ovarian hormone effects on morphology in the rat cerebral cortex support an inhibitory role for estrogen. Pappas et al.¹⁷ found estrogen treatment following Ovx to reduce cortical thickness. At the cellular level, this same group found Ovx to increase neuron perikaryon size in motor cortex. Gonadectomy has also been reported to prevent the decrease in dendritic spine density observed from day 20 to 60 in visual cortex of normal females¹⁵.

These reports are in contrast to the neurotrophic properties of estrogen in subcortical structures. For instance, estrogen treatment to Ovx females has been shown to have growth promoting effects on dendritic spine density in the hippocampus¹² and on soma size, synaptic contact and dendritic spine density in the ventromedial nucleus of the hypothalamus^{2,16,19}. These data show estrogen to have opposite effects on some neuromorphological parameters in cortical vs. subcortical brain regions. Increased neuritic growth in cortex following estrogen treatment has been reported using an *in vitro* preparation²⁰, but it is difficult to compare that finding to the *in vivo* data.

Estrogen was also capable of compensating for Ovx effects on the CC beginning on day 25, a time in development beyond the typical perinatal period of organizational effects of gonadal steroids. Interestingly, activational effects of estrogen on female sexual behavior in 6-day-old animals has also been found²¹, which further challenges the notion that activational and organizational effects of gonadal steroids occur during isolated periods in development. The extended postnatal sensitivity of the female CC to ovarian hormones is in contrast to the limited time frame of sensitivity to testosterone. In the male, prenatal androgen antagonism in combination with castration at birth led to a smaller callosum⁹. In the female, TP ceased to exert its effect on the CC sometime between postnatal day 4 and day 8¹⁰. It thus appears that each sex possesses a unique 'active' period when their endogenous gonadal hormones act on the developing callosum. It is of interest to note that this sexually dimorphic steroid sensitive period parallels the emergence of effects: Ovx effects on the CC are not observed until after 55 days of age⁸, whereas the effects of androgens have emerged by day 3²².

In addition to changes in gross callosal size, brain weight was concomitantly affected, and therefore it could be argued that differences in callosal size simply

reflected changes in overall brain volume. Several findings argue against this interpretation. We found no consistent relationships between brain weight and the seven width factors within any treatment group, although the Ovx group did show several moderate correlations. With respect to these females, the mean brain weight of Ovx females was intermediate between the male and female values, yet their mean CC area value was three times closer to the CC area of males than to females. Also, those width factors which showed statistical significance between group means did not exhibit significant correlations with brain weight. These findings are similar to our previous report on the effects of Ovx on brain weight and the CC¹⁰. Thus, the observed changes in CC size cannot be attributed simply to overall changes in brain size.

There are two mechanisms which could account for the smaller callosum in estrogen-treated females as compared to intact controls. First, the difference may be due to the absence of progesterone in the Ovx + E group after day 12. This explanation parallels the work of Pappas et al.¹⁷ who found slight stimulatory effects of progesterone on cortical thickness. An alternative explanation involves the chronic nature of the estrogen treatment. Estrogen cyclicity is prevalent during the active period of ovarian hormones, and this hormonal variation may influence CC development. These factors need consideration in future studies to further our understanding of ovarian steroid influence on the development of the female corpus callosum.

The current study provides strong evidence for an active role of estrogen in female callosal development. In addition, sexual dimorphism of gonadal steroid action on the CC with regard to the (1) onset of action, (2) duration of sensitivity, and (3) developmental manifestation of effects emphasize the importance of considering ovarian influence on cortical development in general.

REFERENCES

- Berrebi, A.S., Fitch, R.H., Ralphe, D.L., Denenberg, J.O., Friedrich Jr., V.L. and Denenberg, V.H., Corpus callosum: region-specific effects of sex, early experience and age, *Brain Res.*, 438 (1988) 216–224.
- Carrer, H.F. and Aoki, A., Ultrastructural changes in the hypothalamic ventromedial nucleus of ovariectomized rats after estrogen treatment, *Brain Res.*, 240 (1982) 221–233.
- Denenberg, V.H., Assessing the effects of early experience. In R.D. Meyers, (Ed.), *Methods in Psychobiology*, Academic Press, New York, 1977, pp. 127–147.
- Denenberg, V.H., Berrebi, A.S. and Fitch, R.H., A factor analysis of the rat's corpus callosum, *Brain Res.*, 497 (1989) 271–279.
- Diamond, M.C., Dowling, G.A. and Johnson, R.E., Morphologic cerebral cortical asymmetry in male and female rats, *Exp. Neurol.*, 71 (1981) 261–268.
- Dohler, K.D. and Wuttke, W., Changes with age in levels of serum gonadotropins, prolactin, and gonadal steroids in prepubertal male and female rats, *Endocrinology*, 97 (1975) 898–907.
- Fitch, R.H., Berrebi, A.S., Cowell, P.E., Schrott, L.M. and Denenberg, V.H., Corpus callosum: effects of neonatal hormones on sexual dimorphism in the rat, *Brain Res.*, 515 (1990) 111–116.
- Fitch, R.H., Berrebi, A., Cowell, P., Schrott, L. and Denenberg, V., Corpus callosum: neonatal hormones and development, Paper presented at the International Society for Developmental Psychobiology Annual Meeting, Cambridge, England, 1990.
- Fitch, R.H., Cowell, P.E., Schrott, L.M. and Denenberg, V.H., Corpus callosum: demasculinization via perinatal antiandrogen, *Int. J. Dev. Neurosci.*, 9 (1991) 35–38.
- Fitch, R.H., Cowell, P.E., Schrott, L.M. and Denenberg, V.H., Corpus callosum: ovarian hormones and feminization, *Brain Res.*, 542 (1991) 313–317.
- Garcia-Segura, L.M., Olmos, G., Robbins, R.J., Hernandez, P., Meyer, J.H. and Naftolin, F., Estradiol induces rapid remodelling of plasma membranes in developing rat cerebrocortical neurons in culture, *Brain Res.*, 498 (1989) 339–343.
- Gould, E., Woolley, C.S., Frankfurt, M. and McEwen, B.S., Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood, *J. Neurosci.*, 10 (1990) 1286–1291.
- Kronibus, J. and Wuttke, W., Positive feedback action of oestradiol on gonadotropin release in 15-day-old female rats, *Acta Endocrinol.*, 86 (1977) 263–272.
- Mack, C.M., Cowell, P.E. and Denenberg, V.H., Corpus callosum: interactive effects of handling and ovariectomy in the rat, *Soc. Neurosc. Abstr.*, 1992, in press.
- Munoz-Cueto, J.A., Garcia-Segura, L.M. and Ruiz-Marcos, A., Developmental sex differences and effect of ovariectomy on the number of cortical pyramidal cell dendritic spines, *Brain Res.*, 515 (1990) 64–68.
- Nishizuka, M. and Pfaff, D.W., Intrinsic synapses in the ventromedial nucleus of the hypothalamus: an ultrastructural study, *J. Comp. Neurol.*, 286 (1989) 260–268.
- 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.
- Phillis, J.W. and O'Regan, M.H., Effects of estradiol on cerebral cortical neurons and their responses to adenosine, *Brain Res. Bull.*, 20 (1988) 151–155.
- Segarra, A.C. and McEwen, B.S., Estrogen increases spine density in ventromedial hypothalamic neurons of peripubertal rats, *Neuroendocrinology*, 54 (1991) 365–372.
- Toran-Allerand, C.D., On the genesis of sexual differentiation of the central nervous system: morphogenetic consequences of steroidal exposure and possible role of α -fetoprotein. In G. De Vries, J. De Bruin, H. Uylings and M. Corner (Eds.), *Progress in Brain Research*, Vol. 61, 1984, pp. 63–98.
- Williams, C.L., Estradiol benzoate facilitates lordosis and ear wiggling of 4- to 6-day-old rats, *Behav. Neurosci.*, 101 (1987) 718–723.
- Zimmerberg, B. and Scalzi, L.V., Commissural size in neonatal rats: effects of sex and prenatal alcohol exposure, *Int. J. Dev. Neurosci.*, 7 (1989) 81–86.
- Zimmerberg, B. and Mickus, L.A., Sex differences in corpus callosum: influence of prenatal alcohol exposure and maternal undernutrition, *Brain Res.*, 537 (1990) 115–122.