

Research report

Neonatal estrogen blockade prevents normal callosal responsiveness to estradiol in adulthood

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Accepted 23 May 2000

Abstract

The rat corpus callosum (CC) is larger in males than females, and is responsive to hormone manipulations during development. We previously demonstrated that P25 ovariectomy (Ovx) enlarged (defeminized) adult CC, while P70 ovary transfer (OvT) counteracted this enlarging effect, resulting in smaller (feminized) CC. Since OvT females were not Ovx'd until P25, they received some neonatal estrogen (E) exposure. Behavioral data suggest that adult responsiveness to ovarian hormones depends upon prior organization by neonatal E. It has not been determined whether a similar phenomenon occurs for the feminization of brain morphology. The current experiment examined whether our previous finding of adult CC responsiveness to ovarian hormones depended upon neonatal E exposure. We investigated this by assessing the effects of P70 ovarian hormone replacement (via ovary transfer or E pellet) in females that received either (1) normal ovarian hormone exposure until P25 Ovx, or (2) the E receptor blocker tamoxifen from birth to P25 Ovx. Females receiving normal neonatal hormone exposure responded to P70 E in the female-typical manner: E reduced CC size. In contrast, females receiving neonatal E blockade responded to adult E in the opposite manner: E increased CC size. As far as we are aware, this is the first report suggesting that neonatal E exposure organizes the female brain so that it responds normally to the organizing actions of E when later exposure occurs. These findings further challenge the traditional model of female brain development, which asserts that normal female brain organization occurs by default, in the absence of gonadal hormone exposure. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Development and regeneration

Topic: Hormones and development

Keywords: Corpus callosum; Development; Feminization; Ovarian hormone; Estrogen; Neonatal

1. Introduction

The traditional model of mammalian brain sexual differentiation asserts that masculinization is a result of neonatal androgen exposure, and feminization occurs in the absence of gonadal hormones. However, accumulating evidence reveals the importance of estrogen exposure for normal female brain development, thereby demonstrating that feminization is an active process [13,14]. Consistent with this, there is ample evidence of ovarian activity by P8 in rats, at which time the estradiol-binding protein, alpha-

fetoprotein, declines to undetectable levels [1,20,21,23,29,32–35].

We have used the rat corpus callosum as an endpoint to assess gonadal hormone contributions to brain sexual differentiation. Nearly two decades of research has determined that both the larger male CC, and the smaller female CC, require gonadal hormone exposure to develop normally [14]. There are sex differences in the developmental parameters guiding these effects. The male CC is permanently masculinized before the postnatal day 1 (P1) testosterone surge [11,12,25]. In contrast, the female CC is feminized sometime between P25 and P70, since P25 ovariectomy (Ovx) enlarges (defeminizes) the adult callosum, while P70 Ovx has no effect, yielding a normal female CC [2].

Although callosal feminization is not reversed by P70

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ovarian hormone removal, a not-yet feminized CC is still affected by P70 ovarian hormone replacement. Indeed, P70 ovary transfer (OvT) counteracts the enlarging effect of P25 Ovx, resulting in feminized CC [2]. This late sensitivity to ovarian hormones is intriguing, as the tenet of hormone action on brain organization is that it occurs early in development (traditionally referred to as ‘organizational’ effects). This supposition is based upon studies demonstrating a limited neonatal sensitive period to permanent androgenic effects, combined with findings that many neural systems and behaviors respond to adult ovarian hormone exposure in a transient manner (i.e., ‘activational’ effects; e.g. [3,4,15,28,30,36]).

Some data suggest that adult responsiveness to ovarian hormones depends upon prior exposure to ovarian hormones during neonatal life. For some behaviors, females deprived of neonatal estrogen do not respond normally to estrogen later in life [16,22]. It is not known whether a similar phenomenon occurs for organization of brain morphology. Hence, the purpose of the present study was to test the hypothesis that normal female neural responsiveness to adult estrogen requires neonatal estrogen exposure.

In previous callosum studies, females given P70 OvT had received P25 Ovx. Therefore, they were exposed to some estrogen before Ovx. We hypothesize that this neonatal exposure organized the CC and/or cortex to respond in a normal female manner to P70 ovarian hormone replacement. To test this hypothesis, we assessed P70 ovarian hormone replacement effects in females that received normal ovarian hormone exposure through P25 Ovx as compared to females that received the estrogen receptor blocker tamoxifen (TX) from birth through P25 Ovx.

2. Materials and methods

Subjects were Purdue–Wistar rats bred in our closed colony. The day after birth (P1) 12 litters were culled to 7 females and 1 male. Following weaning on P21, animals were housed in like-treated pairs until sacrifice.

There were eight treatment groups, seven female and one male, all assigned within litters (Fig. 1). On P1, three females from each litter were given a TX pellet (Innovative Research of America, Sarasota, FL) designed to release 10 µg TX/day for 21 days [dose based on nine]. The pellet was inserted under light cryogenic anesthesia (maximum time on ice was 90 s). For insertion, a small skin incision was made in the scruff of the neck, a subcutaneous pocket was created, and the pellet was inserted about 1 inch from the incision site toward the pup’s posterior. The incision was closed with one silk suture. Sham (Sh) pups not receiving a TX pellet received cryogenic anesthesia, an incision, a pocket, and a suture.

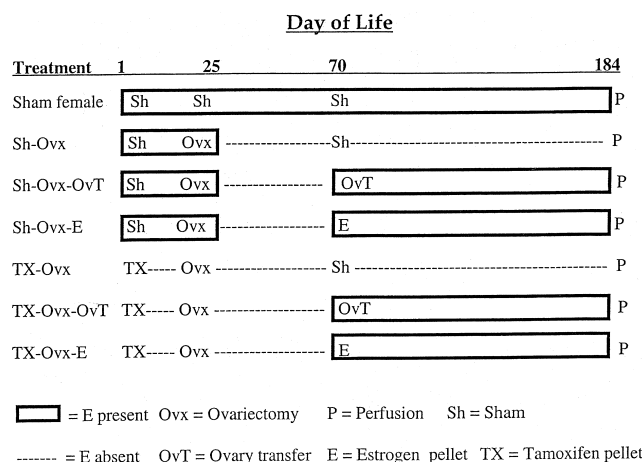


Fig. 1. Schematic representation of the estrogenic milieu for each female treatment group across the lifespan.

Animals were warmed at room temperature and then placed back with their mothers.

The remaining surgeries were performed under Ketaset (J.A. Webster, Sterling, MA) -Xylazine (Butler Company, Columbus, OH) anesthesia. On P25 all animals, except the sham female and the male, received Ovx. Ovx consisted of two dorsolateral incisions in the skin and peritoneum, removal of the ovaries and tips of the uterine horns, and muscle and skin suture. Next, the ovaries were weighed. The sham female received skin incision and suture only.

On P70, one TX-Ovx and one Sh-Ovx female received an estrogen (E) pellet (Innovative Research of America, Sarasota, FL) designed to result in circulating E levels of approximately 60 pg/ml. The pellet was inserted subcutaneously into the scruff of the neck. One TX-Ovx and one Sh-Ovx received an ovary transfer (OvT) [31]. For OvT, one-half of an ovary (from a non-experimental Purdue–Wistar rat) was inserted through a skin and muscle incision into a small pocket in the kidney capsule. Next, the ovary was probed until it was secure between the kidney capsule and cortex, and the muscle and skin were sutured. The remaining TX-Ovx, Sh-Ovx and Sham female received skin incision and suture only. Before and after P70 treatment, females were vaginally smeared for several cycles to confirm proper hormonal status.

The resulting treatment groups were: Sham female, Sh-Ovx, Sh-Ovx-OvT, Sh-Ovx-E, TX-Ovx, TX-Ovx-OvT, TX-Ovx-E, and Male.

On P184±3, rats were perfused and brains were prepared and sagittally sectioned as described previously [24]. The software package Stereology [5] yielded callosal parameters of area and 99 equidistant widths perpendicular to the longitudinal axis. Callosal widths were divided into seven region-specific factors based on previous factor analysis [5]. Beginning in the anterior, the seven callosal width factors were: widths 1–5 (W1–5), W6–17, W24–38, W46–57, W62–72, W79–95 and W96–99. Since we have consistently shown P70 OvT effects in midbody/posterior

regions [2], we also assessed W58–61 and W73–78. The final measures were callosal area, the nine width factors, and brain weight.

3. Results

3.1. Ovarian weights and vaginal smears

Ovarian weights were taken from P25 Ovx subjects immediately after Ovx. In accordance with the findings of others [7,9], a *t*-test of ovarian weights revealed that females given neonatal TX had lighter ovaries than females given neonatal sham treatment [mean±S.E.=0.011±0.001 and 0.025±0.001, respectively; *t*(52)=9.62; *P*<0.001].

Prior to P70 treatment, all females receiving P25 Ovx exhibited consistent leukocytic vaginal smears while Sham females demonstrated 4–5 day cycles. After P70 treatment, TX-Ovx and Sh-Ovx females exhibited leukocytic smears; TX-Ovx-E and Sh-Ovx-E females showed cornified smears; and Sham, TX-Ovx-OvT and Sh-Ovx-OvT females demonstrated cyclic smears. The OvT groups had more variable smears than Shams, occasionally exhibiting a 2–3 day estrous phase. This has been seen before in females receiving OvT around P70 [31]. Together, these data confirm complete removal of ovarian tissue in Ovx females, estradiol release in females receiving an E pellet, and cyclic ovarian hormone release in females receiving OvT.

3.2. Callosal parameters

The main objective of the present study was to determine whether callosal responsiveness to P70 ovarian hormones depended upon neonatal estrogen exposure. However, before addressing this question, it was first necessary to replicate the Sex, neonatal Ovx, and adult ovarian hormone replacement effects previously reported in our laboratory. For all group comparisons, littermate correlations revealed no significant litter effects for CC area. Thus, subjects were treated as individual observations.

3.3. Analyses to confirm previous findings

3.3.1. Sex differences

Replicating previous findings, males had larger callosa than Sham females for overall area (male mean±S.E.=3.08±0.09; female mean±S.E.=2.81±0.082) [*F*(1,17)=4.94; *P*<0.025, one-tail test].

3.3.2. P25 OVX effects

Fig. 2 shows CC widths as a function of region. As expected, Sh-Ovx females had larger CC than Shams in area, W6–17, W62–72, W73–78 and W79–95 [*F*'s(1,17)=

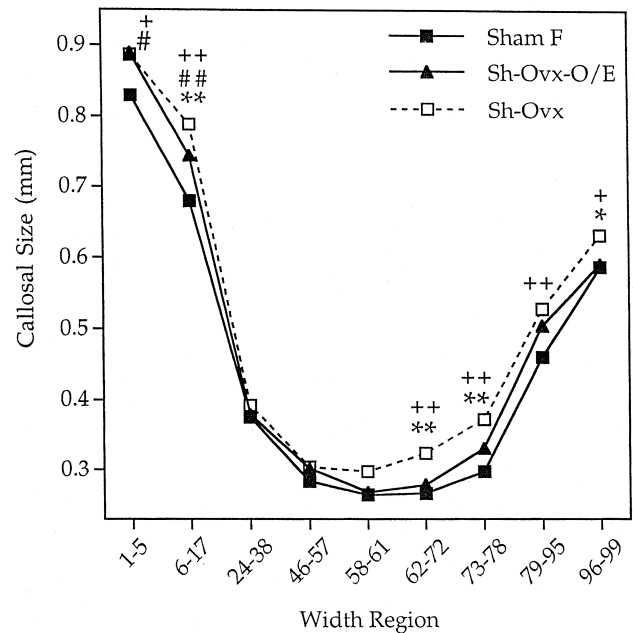


Fig. 2. Mean callosal size for each width factor region for Sham, Sh-Ovx, and Sh-Ovx-O/E females. Significance legend is as follows: Sham F vs. Sh-Ovx (+*P*<0.05; +*P*<0.10); Sham F vs. Sh-Ovx-O/E (##*P*<0.05; #*P*<0.10); Sh-Ovx-O/E vs. Sh-Ovx (***P*<0.05; **P*<0.10).

3.92, 15.07, 4.58, 7.74, 6.28; *P*'s<0.05, 0.001, 0.025, 0.01, 0.025, one-tail tests]. There were marginal effects in W1–5 and W96–99 [*F*'s (1,17)=2.77 and 2.58; *P*'s<0.10].

3.3.3. Effects of P70 ovarian hormone replacement given to SH-OVX females

There were no differences in callosal size between Sh-Ovx females receiving P70 OvT or E (all *P*'s>0.14). Therefore, we combined the two groups (Sh-Ovx-O/E). Sh-Ovx-O/E females had smaller CC than Sh-Ovx females in W6–17, W62–72 and W73–78 [*F*s (1,27)=4.21, 4.79 and 3.22; *P*'s<0.05, 0.025 and 0.05, one-tailed tests; see Fig. 2]. Marginal effects were found in W96–99 [*F*(1,27)=2.21; *P*<0.10]. Sh-Ovx-O/E females had larger CC than Shams in W6–17; W1–5 was somewhat larger as well [*F*'s (1,26)=7.15 and 3.58; *P*<0.025 and 0.07, respectively].

3.4. Analyses to test the proposed hypothesis

3.4.1. Factorial analysis

The female treatment groups were designed such that we could analyze the data in a 2×2 factorial ANOVA. The first between factor was Neonatal E: present (+) or absent (–); the second between factor was Adult E: present (+) or absent (–). Table 1 shows how the seven groups were assigned to the four cells of the design. This type of experimental design allows one to assess Neonatal E and Adult E main effects as well as their interaction. Referring to the cells in Table 1 that contain more than one group,

Table 1

Treatment group designations for the 2×2 factorial analysis, and callosal size (mm) of the averaged 9 width factors ±S.E. for the combined groups within each cell

		Neonatal estrogen	
		–	+
Adult estrogen	+	TX-Ovx+E	Sham
		TX-Ovx+OvT	Sh-Ovx-E Sh-Ovx-OvT
	0.481±0.017		0.465±0.013
	–	TX-Ovx	Sh-Ovx
0.457±0.024		0.502±0.023	

repeated measures (Width factors) ANOVAs demonstrated that the groups within each cell did not differ. This confirms the validity of combining the groups in this manner.

Repeated measures (Width factors) ANOVA revealed a significant Neonatal E×Adult E interaction [$F(1,59)=4.06$; $P<0.05$]. The means are reported in Table 1. Analyzing each width region separately, there was a main effect of Neonatal E for W1–5, with females exposed to neonatal E having larger values than those not exposed [$F(1,59)=7.16$; $P<0.01$].

There were Neonatal E×Adult E interactions in W6–17, W62–72 and W96–99 [F 's (1,59)=4.73, 5.48, 4.02; $P<0.05$, 0.025, 0.05], as well as marginal interaction effects in

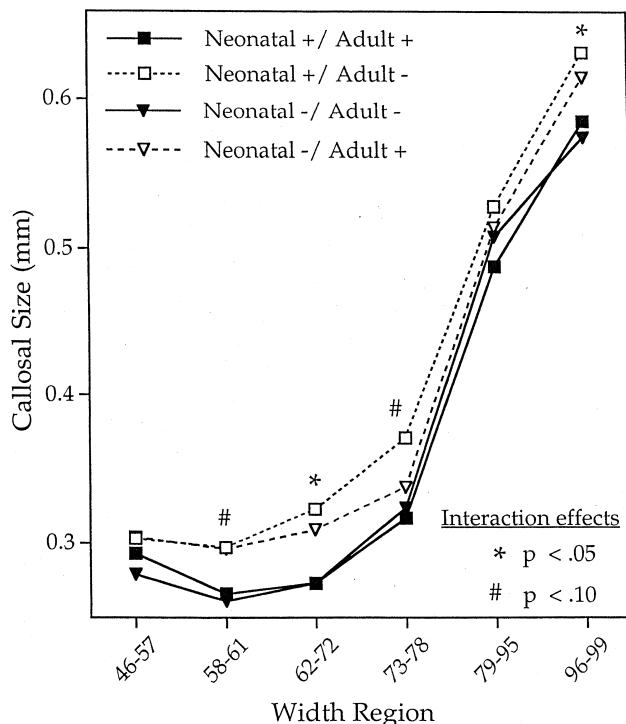


Fig. 3. Mean callosal size for the latter six width factor regions for groups in the 2×2 factorial design. There were significant interaction effects in W6–17 (not shown), W62–72, and W96–99 (*); and marginal effects in W58–61 and W73–78 (#).

Table 2

Mean brain weight (g)±S.E. and n for groups in the 2×2 design

Groups	Brain weight	n
Neonatal+ /Adult+	1.29±0.013	28
Neonatal+ /Adult-	1.35±0.028	10
Neonatal- /Adult+	1.25±0.026	17
Neonatal- /Adult-	1.32±0.021	8

W58–61 and W73–78 [F 's (1,59)=3.04, 3.23; P 's<0.10]. Fig. 3 shows the means for the latter width regions, where interaction effects occurred.

Analogous to the method of analysis used above, a 2×2 ANOVA (Adult E: + or -; Neonatal E: + or -) was performed to investigate group differences in brain weight (Table 2). There was an Adult E main effect [$F(1,59)=7.42$; $P<0.01$] with a lower brain weight in groups exposed to adult estrogen, and a marginally significant effect of Neonatal E [$F(1,59)=3.04$; $P<0.10$]. To ensure that group differences in callosal size were not due to group differences in brain weight, brain weight was used as a covariate in the 2×2 factorial analysis using the 9 callosal width factors as repeated measures. We found the same results as the factorial without brain weight as a covariate; there was a significant Neonatal E×Adult E interaction [$F(1,58)=4.48$; $P<0.05$]. It is noteworthy that both Neonatal+ and Neonatal- groups exhibited a decrease in brain weight in response to Adult E, given that the groups responded to E in a divergent manner for callosal size. These findings lend further support to the assertion that changes in callosal size in response to hormone manipulation are not a result of changes in brain size see [14] for discussion.

4. Discussion

The current study replicated previous findings that males and P25 Ovx females have larger CC than Sham females, and that P70 OvT counteracts the enlarging effect of P25 Ovx in midbody and posterior regions, resulting in smaller CC that did not differ from those of Sham females. This is the fourth replication showing a late, feminizing effect of ovarian hormones on CC size, see also [2]. Since an estradiol pellet given on P70 was also found to reduce callosal size, the P70 OvT effect is mediated, in part, by estrogen. This is consistent with previous data showing that P25 estradiol replacement reduced callosal size, resulting in feminized adult CC [27].

4.1. Females deprived of neonatal estrogen do not respond normally to later estrogen exposure

In this experiment we found that females receiving normal ovarian hormone exposure before P25 Ovx responded to P70 estrogen in the normal female manner —

estrogen *reduced* CC size [27]. In contrast, females that received TX before P25 Ovx, followed by adult estrogen, exhibited larger CC than TX females not receiving adult estrogen. Therefore, in TX treated females, adult estrogen *increased* callosal size. This direction of change in callosal size in response to adult estrogen is opposite that of females receiving normal neonatal estrogen exposure. These findings indicate that females deprived of neonatal estrogen do not respond in the normal female manner to estrogen in adulthood. Thus, not only can ovarian hormones alter callosal morphology in adulthood, but these developmentally later effects appear to be dependent upon the previous organizing actions of neonatal estrogen exposure [2]. Such an assertion is consistent with data showing that hormone exposure during early development modifies receptor populations e.g. [10], which may 'prime' the brain and affect its later response to the target hormone in an ongoing interactive fashion. As far as we are aware, this is the first study to suggest that developmentally later feminization of a brain structure is dependent upon the organizing properties of neonatal estrogen stimulation.

The herein reported neuroanatomical findings are consistent with data suggesting that neonatal estrogen exposure is required for normal behavioral responsiveness to estrogen in adulthood. For example, adult female rats Ovx'd within the first 10 days of life showed less sexual receptivity to males in response to hormonal priming compared to females Ovx'd between 35 and 60 days [16]. Others found that female rats Ovx'd on P1 responded in a less female-typical manner than females Ovx'd on P60 in response to estradiol priming on tests of adult anxiety [22].

4.2. Callosal characterization of females deprived of postnatal estrogen exposure

Females receiving neonatal estrogen blockade and no adult estrogen supplementation exhibited smaller callosa in adulthood. This group represents a brain that is genetically female and theoretically devoid of estrogen exposure throughout postnatal life. The findings of a 'normal-sized' female CC in this group may appear paradoxical given our findings that both neonatal and adult estrogen contribute to a normal-sized female CC. However, previous research has shown that male rats receiving prenatal androgen blockade, followed by P1 castration, also exhibited smaller adult CC [12]. This group represents a brain that is genetically male and theoretically devoid of androgen exposure throughout perinatal and postnatal life. These data converge to suggest that when gonadal hormones are absent throughout development, a smaller callosum is exhibited in adulthood. This leads one to question the characterization of the smaller ahormonal female callosum as 'normal.'

Since there are sex differences in various parameters of axon myelination in the rat CC, it seems likely that the smaller CC of ahormonal females differs ultrastructurally from the smaller CC of intact females [18,19,26]. Similar-

ly, the larger male callosum may be different ultrastructurally from the larger callosum of females receiving only neonatal or only adult estrogen exposure. Investigations of the fine structure of the CC may ultimately clarify the mechanisms of enlargement or reduction in gross size as a function of gonadal hormone exposure.

4.3. Females treated with neonatal tamoxifen sustain cyclic ovarian function in adulthood

Dohler and colleagues found that neonatal TX treatment resulted in permanent anovulatory sterility (PAS) in adult female rats [7,9,17]. It is not known if PAS is a result of a tonic (defeminized) hypothalamus-pituitary (HPA) axis or ovarian dysfunction. Interestingly, Pfeifer [31] showed that females Ovx'd at birth exhibited cyclic smears after adult OvT. Dohler argued that these females cycled because their brains were exposed to neonatal estrogen released from non-ovarian substrates (e.g., adrenals), and that females receiving neonatal TX do not cycle in adulthood because their brains have been deprived of neonatal estrogen and therefore, the HPA axis has not been feminized [6,8]. In our study, using a dose comparable to Dohler's, we found that neonatal TX treated females exhibited cyclic smears after adult OvT, indicating that the HPA axis was capable of sustaining an ovary. Hence, the PAS exhibited in Dohler's studies may have been a result of ovarian dysfunction and not a tonic (i.e., defeminized) HPA release pattern. It appears then that adult callosal sensitivity to estrogen may depend upon neonatal estrogen exposure while feminization of the HPA axis does not. More information on adult ovarian function in females treated with neonatal TX is needed in order to evaluate this hypothesis.

We chose to use TX because of the many reports showing that it exerts anti-estrogenic properties resulting in defeminized, but not masculinized, effects on female brain and behavior [8,9,17]. However, TX can exert estrogenic properties in the periphery. It is unlikely that the effects reported here are due to the possibly masculinizing, estrogenic properties of TX since we have previously found that: (A) neonatal treatment with the synthetic estrogen, DES, did not affect callosal size of intact females [11]; (B) the androgen receptor blocker, flutamide, demasculinized male CC suggesting that masculinization is mediated via androgen, not estrogen, receptors [12]; and (C) females receiving neonatal TX and no adult estrogen exhibited smaller adult callosa. If neonatal TX treatment masculinized callosa, these females would have had larger CC.

In summary, females exposed to estrogen both neonatally and in adulthood exhibited smaller (feminized) callosa that did not differ in gross size from intact females. Conversely, females exposed to estrogen only neonatally, or only in adulthood, exhibited larger (defeminized) callosa. These data suggest that neither neonatal estrogen

alone, nor adult estrogen alone, is sufficient for CC feminization. Rather, neonatal and adult estrogen act together to produce a feminized adult CC. Our findings, taken with the behavioral data of others, provide strong evidence that for normal female brain morphology, the developmentally later, feminizing actions of estrogen depend upon the previous organizing effects of neonatal estrogen exposure. Thus, neonatal and adult estrogen appear to act in concert to produce a normal female phenotype.

Acknowledgements

The authors wish to thank Kathleen Galle and Michael Casler for technical assistance, and Seth Balogh and Amy Jo Stavnezer for comments on this manuscript. This research was supported, in part, by predoctoral NRSA grant # 1-F31-MH12416-01.

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