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A role for ovarian hormones in sexual differentiation of the brain

Roslyn Holly Fitch
Victor H. Denenberg
Biobehavioral Sciences Graduate Degree Program
University of Connecticut, Storrs, CT 06269–4154
Electronic mail: *hfitch@psych.psy.uconn.edu; dberg@uconnvm.uconn.edu*

Abstract: Historically, studies of the role of endogenous hormones in developmental differentiation of the sexes have suggested that mammalian sexual differentiation is mediated primarily by testicular androgens, and that exposure to androgens in early life leads to a male brain as defined by neuroanatomy and behavior. The female brain has been assumed to develop via a hormonal default mechanism, in the absence of androgen or other hormones. Ovarian hormones have significant effects on the development of a sexually dimorphic cortical structure, the corpus callosum, which is larger in male than in female rats. In the females, removal of the ovaries as late as Day 16 increases the cross-sectional area of the adult corpus callosum. Treatment with low-dose estradiol starting on Day 25 inhibits this effect. Female callosa are also enlarged by a combination of daily postnatal handling and exogenous testosterone administered prior to Day 8. The effects of androgen treatment are expressed early in development, with males and testosterone-treated females having larger callosa than control females as early as Day 30. The effects of ovariectomy do not appear until after Day 55. These findings are more consistent with other evidence of a later sensitive period for ovarian feminization as compared to androgenic masculinization.

Keywords: activational effects; androgens; corpus callosum; estrogen; female default hypothesis; feminization; masculinization; organizational effects; sensitive periods; sexual differentiation; testosterone

1. Introduction

Reviews on the role of hormones in mammalian sexual differentiation traditionally focus on the effects of neonatal exposure to testicular androgens (e.g., testosterone, the predominant androgen) in males. This emphasis derives from overwhelming data consistently showing demasculinizing effects of postnatal castration in males, and masculinizing effects of perinatal androgens on females. These effects are seen for neuroanatomy, neurochemistry, and behavior, and include reports of cognitive differences among human populations with abnormal androgenic exposure (Collaer & Hines 1995; Masica et al. 1969; Resnick & Berenbaum 1982) as well as alterations in hypothalamic anatomy following perinatal androgenic manipulations in rats (Gorski 1984).

The role of ovarian hormones has not been as extensively or rigorously investigated. For example, some researchers have reported that ovarian manipulations had no effect on receptive sexual behavior in rats (Lisk & Suydam 1967; Whalen & Edwards 1967), but later studies that included evaluation of proceptive behavior did find significant effects (Gerall et al. 1973). Despite the prevailing assumption that the ovaries are not a critical factor in sexual differentiation, a number of reports showing behavioral and neuroanatomical consequences of ovariectomy, as well as low-dose estrogen exposure following removal of the testes, have accumulated during the past 20 years. Based on this accumulating evidence, during the past two decades a handful of researchers have suggested that estrogen plays an active role in differentiation of the female brain (e.g., Döhler 1991; Döhler et al. 1984b; Gerall et al. 1973; Stewart & Cygan 1980; Toran-Allerand 1976; 1992). Yet these hypotheses and findings have not been assimilated



Holly Fitch is Assistant Research Professor in the Biobehavioral Sciences Degree Program at the University of Connecticut. Her research is on hormonal exposure and the development of sexual dimorphism in the rat corpus callosum, sex differences in behavioral response to early focal lesions of cortex in the rat, and parallels with auditory processing defects in language impaired children. She has received Young Investigator Awards from the National Dyslexia Research Foundation, the McDonnell-Pew Foundation for Cognitive Neuroscience, and the Rita Rudel Memorial Foundation.



Victor Denenberg is Professor of Biobehavioral Sciences and Psychology at the University of Connecticut and is the author of over 250 scientific publications in the area of early experience, laterality, hormones and behavior, and animal models of developmental learning difficulties.

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into the widely accepted model of androgen-mediated sexual differentiation.

In this target article we review evidence that exposure to ovarian hormones (primarily estrogen) during development is necessary for differentiation of the mammalian female brain. Initially, we discuss mechanisms of hormone action and relevant terminology (sect. 2), followed by a discussion of the traditional model of androgen-mediated differentiation, and an abbreviated overview of relevant data (sect. 3). We then present a more detailed review of evidence regarding the developmental effects of ovarian hormones and ovarian manipulations (sect. 4). Finally, we present the results from a series of studies on the role of perinatal androgens and estrogens in neuroanatomical sexual differentiation of the rat corpus callosum (sect. 5).

2. Mechanisms of steroid action

2.1. Gonadal steroids and steroid receptors

Androgens comprise a category of chemically related hormones produced primarily in the testes in the male and in the adrenal cortex of both sexes; the ovaries also produce some small amounts. Testicular androgens are the primary catalyst for masculine sexual differentiation in mammals (see Breedlove 1992 or Toran-Allerand 1986 for review). Consequently, the term "androgen" is used in this paper to refer to the class of hormones secreted endogenously by the testes, or to exogenous androgen manipulations intended to assess hormonal mechanisms in normal males.

There are a number of different androgens that exert masculinizing effects in different regions and at different times in development, and that may be metabolized from one form to another before acting at the cellular level. For example, testosterone may be converted by the enzyme aromatase into the estrogen, estradiol. Thus in the presence of aromatase, testosterone can ultimately bind to estrogen as well as to testosterone receptors. Testosterone may also be converted into dihydrotestosterone, a nonaromatizable form of testosterone that acts only on testosterone receptors. In contrast, the primary steroids produced by the ovaries (estrogen and progesterone) generally act directly on estrogen and progesterone receptors.

2.2. Steroids and neural growth

The primary mechanism by which steroids appear to influence neuroanatomy, neurophysiology, and behavior is through binding to intraneuronal nuclear receptors in target brain areas and altering neuronal genomic expression (see McCarthy 1994 or Toran-Allerand 1986 for review). Steroid effects are largely modulated by receptor topography, density of receptor populations, and receptor affinity for steroid binding, all of which may differ at any given point in development and as a function of sex. The effects of steroid binding may be expressed as alterations in regional cell growth, proliferation, or death, which may then influence cell number, size, or packing density. Early migrational patterns, dendritic growth, and neuronal myelination may also be altered. Evidence suggests that sexual differentiation is the result of interacting steroid effects on multiple neural parameters. The result of these processes is a complex pattern of sex differences in neural circuitry and function that is not completely understood. (For reviews see Breedlove 1992; McCarthy 1994; Tobet & Fox 1992; Toran-Allerand 1986.)

2.3. Organizational versus activational effects of steroid hormones

Traditionally, mechanisms of hormone action have been divided into effects that occur early in development and are permanent (i.e., organizational) versus those that occur later in development and are transitory because they depend on the presence of circulating hormones (i.e., they are activational). Within this framework, sex differences in neuroanatomy were assumed to reflect the permanent organizing effects of steroids. Some behavioral effects were interpreted as organizational (e.g., reduced rough and tumble play following early testosterone removal in the male: Meaney 1988; Ward & Stehm 1991). Other behaviors, however, particularly those that could be mimicked by experimental manipulation of circulating steroids (e.g., the "priming" of female rodents for sexual receptivity via exogenous estrogen and progesterone), were considered activational.

Accumulated evidence has muddled the organizational/activational dichotomy. Specifically, the temporal distinction that categorized hormonal effects in development as *organizational* and hormonal effects in adulthood as *activational* apparently is not valid. Data that counter the traditional definitions include estrogenic activation of lordosis behavior in female rat pups as young as 6 days of age (Williams 1986), and changes in hypothalamic anatomy following post-pubertal hormone manipulations (Bloch & Gorski 1988). Some researchers have suggested that the primary organizational/activational distinction now depends on whether induced changes represent permanent or transient effects, whenever in life they occur (e.g., see Arnold & Breedlove 1985; Stewart 1988; Williams 1986 for discussion). Classification of hormonal effects is also complicated by increasing evidence of ongoing physiological plasticity in the adult brain. Recent evidence shows, for example, that for some neuroanatomical and neurochemical systems the adult female brain is "permanently transient" (e.g., Becker 1990; Becker & Cha 1989; Frankfurt et al. 1990; Woolley et al. 1990; Wolley & McEwen 1992). In other words, fluctuations in specific neurophysiologic measures occur in response to female hormonal cyclicity. Although these effects could reasonably be excluded from a review on sexual differentiation because they are transient, it nevertheless seems that they constitute part of what makes the female brain distinct (or differentiated) from the male brain. As such, we include these findings in our discussion.

2.4. Masculinization, demasculinization, feminization, and defeminization

Researchers studying sexual differentiation of complex systems (e.g., behavior) have noted a distinction between the suppression of male or female attributes (demasculinization and defeminization, respectively) versus the enhancement of male and female attributes (masculinization and feminization, respectively; discussed in Toran-Allerand

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1986). For example, certain hormonal manipulations can suppress female-typical behavior without inducing male-typical behavior, an outcome that would be described as defeminizing but not masculinizing (e.g., see Yahr & Greene 1992). The application of these terms to sexual differentiation of neuroanatomical structure, in which male-female differences tend to be measured on a single axis (e.g., larger/smaller, more cells/fewer cells, etc.), can sometimes complicate the use of these definitions. As an example, the sexually dimorphic nucleus of the preoptic area (SDN-POA) is larger in male rats, and increasing the size of the structure in females via early androgen treatment is interpreted as masculinizing. Such enlargement might also be interpreted as defeminizing, because it represents a deviation from the normal female pattern. However, blocking the early action of estrogen in female rats (by an estrogen receptor blocker or estrogen mRNA antisense) apparently *decreases* the size of this structure (Döhler et al. 1984c; McCarthy et al. 1993). Because these manipulations appear to interfere with the normal process of SDN-POA development in females, their effects are interpreted as defeminizing. Thus for this structure, it appears that increasing size in females reflects masculinization, whereas decreasing size reflects defeminization.

3. Androgens and sexual differentiation

3.1. Aromatization (estrogen biosynthesis)

Testosterone can be intraneuronally converted (aromatized) to estradiol in a variety of species (Beyer et al. 1994; Hutchison & Beyer 1994; Hutchison et al. 1994; 1995; Roselli & Resko 1993; see Toran-Allerand 1986 for review). This locally biosynthesized estrogen may in turn act on estrogen receptors within neuronal nuclei. It appears that this mechanism plays a critical role in the masculinization process for many mammalian species; androgens such as testosterone are secreted by the testes, but are then converted to estrogen within individual neurons before exerting developmental effects.

Aromatase enzyme has been found subcortically in a variety of species and is known to be involved in sexual differentiation of subcortical structures (see Breedlove 1992 or Toran-Allerand 1984, 1986 for review). The role of aromatase at the cortical level has, historically, been less clear. Although aromatase enzyme has been localized in fetal and newborn monkey cortex (MacLusky et al. 1986; Sholl et al. 1989), researchers reported difficulty demonstrating estrogen biosynthesis in perinatal rat cortex (MacLusky et al. 1985). More recently, aromatase has been localized in perinatal mouse and rat cingulate cortex (MacLusky et al. 1987; 1994), parietal cortex of fetal guinea pigs (Connolly et al. 1994), and cerebral cortex of young opossums (Fadem et al. 1993). These findings, coupled with evidence of transient, high-density populations of estrogen receptors in neonatal rat cortex (MacLusky et al. 1979a; 1979b) and mouse cortex (Shugrue et al. 1990), point to a significant role for biosynthesis of estrogen in sexual differentiation of the cerebral cortex in a variety of species (see MacLusky et al. 1987). These conclusions are in agreement with (1) behavioral research showing that systemic administration of an aromatase-inhibitor to intact male rat pups produced female-like patterns of maze learning, whereas implantation of estradiol into the cortex of neonatally castrated males reinstated male-like maze learning patterns in adulthood (Williams & Meck 1991); and (2) evidence that biosynthesized estrogen plays a role in sexual differentiation of catecholamine systems in rat frontal cortex (Stewart & Rajabi 1994).

Paradoxically, although female rat pups are exposed to high circulating levels of maternal estrogen in the perinatal period, they are not masculinized. Rather, they are protected from estrogen-based masculinization via a blood-born protein called alpha-fetoprotein (AFP; Raynaud et al. 1971). AFP, which is present in the early perinatal period, binds to circulating estrogen and apparently prevents it from entering the neuron as freely as unbound estrogen (although small amounts of estrogen may pass into the neuron while bound to AFP; see Toran-Allerand 1986). From an evolutionary perspective, AFP appears to protect the female brain from perinatal estrogenic masculinization. Evidence suggests that maximal levels of AFP are seen in the rat brain on gestational Day 18, and decline to low levels by postnatal Day 7, when AFP synthesis is apparently "switched off" (Ali et al. 1981; Ali & Sahib 1983). This is the same perinatal time frame during which fetal testicular androgens exert masculinizing effects in rats (sect. 3.2; see discussion below). Unlike the testes, evidence suggests that the ovaries become active in rats and mice around postnatal Day 7 (Mannan & O'Shaughnessy 1991; Sokka & Huhtaniemi 1995; Weniger et al. 1993). Thus an active role for the ovaries in development is not at odds with the early presence of AFP.

3.2. Androgens and reproductive behavior

The earliest reports of an androgenic role in differentiation showed that manipulations of neonatal androgens affected adult sexual behavior. Female guinea pigs exposed to testosterone by various regimes during the prenatal period increased male-typical sexual behavior (mounting). These subjects also decreased female-typical behavior (lordosis) when, as adults, they were gonadectomized, primed with estrogen and progesterone, and tested for sexual receptivity (Phoenix et al. 1959). Similarly, male rats castrated at birth reduced male-typical sexual behaviors and increased feminine behaviors in adulthood (Beach 1974; Whalen & Edwards 1967; Young 1961). These same behavioral patterns were seen in adult male rodents exposed prenatally to stress or alcohol, which disrupts the prenatal testosterone surge in male fetuses (McGivern et al. 1984; 1988; Ward 1972; 1983). These effects appear to be mediated by aromatization of testosterone to estrogen, because sexual behavior can be masculinized in females and reinstated in neonatally castrated males with early administration of a synthetic estrogen (e.g., diethylstilbestrol) or high doses of estradiol (Doughty et al. 1975; Hendricks & Gerall 1970).

Estrogen has also been shown to act asymmetrically in the hypothalamus to modify the reproductive behavior of the female rat. Estradiol pellets were placed in the left or right ventromedial nucleus during the first two days of life. In adulthood, subjects were ovariectomized and primed with estradiol benzoate and progesterone. Subjects with left-sided implants showed reduced lordosis as compared to right-sided implants and cholesterol controls (Nordeen & Yahr 1982; Yahr & Greene 1992). Nordeen and Yahr also

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found masculinizing effects of estradiol, with local implantation in the right preoptic area leading to increased mounting behavior in adulthood.

3.3. Androgens and nonreproductive behavior

Male and female rats differ on a large number of nonreproductive behaviors, including aggressiveness and rough-and-tumble play, and many of these behaviors are influenced by neonatal exposure to testosterone (for reviews, see Beatty 1979; 1984; 1992). With respect to cognitive behavior, female rats normally learn an active avoidance response more quickly than males, whereas males tend to outperform females on passive avoidance. Moreover, female rats treated neonatally with testosterone and primed with testosterone prior to testing appear to be indistinguishable from males in avoidance learning behavior (see Van Haaren et al. 1990 for review). Circulating testosterone may not be critical to this sexual dimorphism, however, because others have reported that early neonatal exposure to androgen alone is sufficient to induce a male-like pattern of active avoidance learning in female rats (Denti & Negroni 1975). Avoidance behavior in male rats can also be feminized. For example, prenatal exposure to the androgen receptor-blocker cyproterone acetate, followed by postnatal castration, produced males with female-like avoidance behavior in adulthood (Scouten et al. 1975).

With respect to spatial learning, male rats typically do better than females. In general, neonatal castration of males or exposure of females to androgens reverses this adult sexually dimorphic pattern (Dawson et al. 1975; Joseph et al. 1978; Stewart et al. 1975). More recently, Roof (1993a) reported that male rats performed significantly better than females on both the radial arm and Morris water maze. Roof also found that neonatal treatment with testosterone improved spatial ability in female rats to male levels, and this effect was seen as early as 21 days of age. Roof and Havens (1992) reported that neonatal treatment with testosterone led to a male-like pattern of hippocampal anatomy (as measured by size and asymmetry of granule cell layers), and improved maze learning in female rats. Moreover, maze performance correlated significantly with the size of hippocampal granule cell layers.

In a related series of studies, Williams and colleagues manipulated specific components of the extra-maze testing environment and showed that male and female rats use different strategies and rely on different cues in maze learning (Williams & Meck 1987; 1991; Williams et al. 1990a). Williams also showed that estrogen plays a major role in influencing the development of spatial ability in rats by implanting estradiol into the hippocampus or cortex of neonatally castrated males. This reinstated male-like maze learning behavior (Williams et al. 1990b; Williams & Meck 1991). These findings support a developmental role for estrogen biosynthesis (from testosterone) in sexual differentiation of the rat cerebral cortex.

Sex differences in cerebral organization are also seen in nonhuman primates. Clark and Goldman-Rakic (1989) reported that intact male monkeys made fewer errors than intact females in learning a visual object discrimination reversal task. Lesions to the orbital prefrontal cortex disrupted the ability of males, but not females, to perform the task. Furthermore, females given androgen in early perinatal life performed like normal males, and were similarly disrupted by the lesion.

This male advantage does not generalize across ages and visual learning tasks; 3-month-old male monkeys were slower than age-matched females to learn a set of visual discriminations (Bachevalier et al. 1989). In this study, testosterone levels were obtained from males and estradiol levels from females, and these were correlated against the learning scores. Within the 3-month-old male monkey group, the rank-order correlation was .95; the higher the testosterone, the slower the learning. There was no significant correlation in any other group. The authors' interpretation was that high testosterone levels temporarily slowed the maturation of the neural systems underlying visual discrimination, because by 6 months of age no sex differences or hormone-behavior correlations were found. A later study showed that ablation of inferior temporal cortex depressed visual discrimination scores in 3-month-old female monkeys, but did not affect age-matched males (Bachevalier et al. 1990), an effect that is apparently mediated by testosterone exposure (Hagger & Bachevalier 1991). These results agree with the prior interpretation that testosterone delayed the maturation of neural systems underlying the visual discrimination task (see also Bachevalier & Hagger 1991).

Finally, developmental androgen effects have been reported for human cognition, as well as sexual orientation and aggression (see Collaer & Hines 1995; Gouchie & Kimura 1991; Swerdloff et al. 1992 for reviews). Correlations between androgen level and behavior have been reported with respect to language disabilities (Kirkpatrick et al. 1993) and musical ability (Hassler 1991), and prenatal androgen levels have been correlated with mental rotation skill in girls (Grimshaw et al. 1995a; 1995b). Salivary testosterone levels have been positively correlated with spatial ability in women and negatively correlated with spatial ability in men (Gouchie & Kimura 1991).

3.4. Androgens and subcortical anatomy

Neuroanatomical sex differences are present in the rat hypothalamus, most notably in the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is approximately 8 times larger in adult males than females (Gorski 1984; Gorski et al. 1978). Castration of males on Day 1 reduces the size of this nucleus in adulthood, whereas the postnatal administration of testosterone to females enlarges the nucleus (Döhler et al. 1982; 1984a; Gorski 1984; Gorski et al. 1978). The SDN-POA can also be enlarged in female rats through the administration of a synthetic estrogen (diethylstilbestrol), which does not bind to AFP (Döhler et al. 1984a; Gorski 1984), indicating that masculinization of this structure is dependent on the intracellular conversion of testosterone to estrogen. Estrogen may mediate this sexual dimorphism by preventing a developmental loss of neurons within the medial preoptic nucleus (Dodson & Gorski 1993). Hormonal mediation of neuronal loss also affects sexual dimorphism in the size of the spinal bulbocavernosus nucleus in rats, although differentiation of this nucleus apparently depends on the direct action of androgen, rather than estrogen (see Breedlove 1992 for review). Finally, SDN-POA volume is reduced in male rats exposed prenatally to ethanol, which depresses fetal androgen production (Ahmed et al. 1991).

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Holman and Hutchison (1991) showed that neonatal ovariectomy of female gerbils followed by silastic testosterone implant enlarged the size of the sexually dimorphic preoptic area of the pars compacta (SDApc), as well as the sexually dimorphic suprachiasmatic nucleus, to the size of males. These researchers also found that the volume of the left (but not right) SDApc correlated with ultrasonic courtship vocalizations, a structural-behavioral asymmetry consistent with lateralized hormonal effects on behavior reported by Yahr and Greene (1992). Research has also shown that the volume of the posterodorsal region of the medial nucleus of the amygdala (MApd), and the volume of the encapsulated region of the bed nucleus of the stria terminalis (BNSTenc) are approximately twice as large in male as compared to female rats (Hines et al. 1992), although the hormonal mechanisms underlying these effects were not investigated.

Finally, sex differences are present in the number and pattern of synapses on dendritic spines in the preoptic area of the rat hypothalamus (Raisman & Field 1973), dendritic patterns in the preoptic area of hamsters (Greenough et al. 1977), and dendritic anatomy in the preoptic area of juvenile macaques (Ayoub et al. 1983). These differences have been attributed to the effects of neonatal androgen exposure in the male (e.g., Greenough et al. 1977), an assertion consistent with in vitro evidence that testosterone influences neuronal survival and neural outgrowth of cultured rat preoptic cells (Kawashima & Takagi 1994).

3.5. Androgens and cortical and hippocampal anatomy

Neonatal testosterone also appears to be involved in the sexual differentiation of the cerebral cortex. Diamond (1984) and her colleagues (Diamond et al. 1981) reported that certain regions of the cortex were significantly thicker in the right hemisphere than in the left in male rats, whereas females showed a nonsignificant trend toward asymmetry in the opposite direction (see also Kolb et al. 1982; Stewart & Kolb 1988). This effect appears to be mediated at least partly by androgen exposure, because neonatally gonadectomized male rats fail to show the R-L pattern of cortical asymmetry seen in intact males (Diamond 1984; Stewart & Kolb 1988). The male cortical thickness pattern is also reversed by prenatal stress (Fleming et al. 1986; Stewart & Kolb 1988), which depresses and shifts the gestational Day 18 testosterone surge in fetal male rats (Ward & Weisz 1980). Finally, the masculinizing effect of androgens on cortical asymmetry in rats appears to be mediated by conversion to estrogen, because perinatal exposure to the aromatase blocker ATD (1,4,6 androstatriene-3,17-dione) reversed the adult cortical thickness pattern in males (Diamond 1991).

More recently, sex differences have been demonstrated in the dendritic branching patterns of prefrontal cortical cells in rats, and these patterns appear to be influenced by gonadal hormonal exposure during development (Kolb & Stewart 1991). In addition, the cortical thickness of the binocular subfield of occipital cortex (Oc1B) is significantly greater in male rats than in females (Seymoure & Juraska 1992). There were also sex differences in the apical branching of Oc1B and Oc1M (monocular subfield) neurons, wherein females have longer dendrites, longer terminal branches, and longer bifurcating branches. Reid and Juraska (1992) confirmed the sex difference in binocular cortical thickness, and further reported that this effect reflects higher numbers of neurons and glial cells in males, with no sex differences in soma size or neuronal density. More recently, Reid and Juraska (1995) found sex differences in synaptic junctions in this region, with the higher number of neurons in male cortex relating to higher numbers of synaptic junctions. To our knowledge, the hormonal mechanisms underlying these anatomical sex differences have not yet been determined.

With respect to the hippocampus, Juraska (1991) reported sex differences in hippocampal dendritic anatomy that vary in direction as a function of rearing environment, and are influenced by early androgen exposure. Roof (1993a; 1993b) also reported a sex difference in the granule cell layers of the hippocampus in rats, and found that this effect is modulated by early exposure to testosterone.

Finally, neonatal androgen affects the development of cortical neurotransmitter systems. Monoamine systems innervating the anterior cortex in intact female rats develop earlier than in males or androgen-treated females (Stewart et al. 1991), and evidence suggests that sex differences in the development of frontal cortical catecholamine systems may derive from prenatal biosynthesis of estrogen from testosterone (Stewart & Rajabi 1994).

4. Female development

In mammalian sexual differentiation, each critical stage appears to rely on the production of a substance by the male – Testis Determination Factor, Mullerian Regression Factor, and testosterone. In contrast, the terms "hormonally neutral" and "default" have been used to describe differentiation of the female. Thus, Svare and Kinsley (1987) write: "it is important to note that the ovaries and their secretory products do not have a role in the differentiation process. Instead, the critical determinant of internal and external sexual differentiation is the presence or absence of testosterone secreted by the testes" (p. 16). Lisk and Suydam (1967) likewise conclude that "feminization appears to be the neutral condition" (p. 182). Yet researchers have previously suggested that estrogen (presumably of ovarian origin) may play an active role in feminization of the brain (e.g., Döhler 1991; Döhler et al. 1984b; Hendricks 1992; Toran-Allerand 1976; 1992). Toran-Allerand, for example, performed a series of in vitro studies in 1976 and concluded that "these . . . experiments suggest . . . that no pattern of sexual differentiation need necessarily be intrinsic to nervous tissue but that male and female patterns may both require active induction by steroid" (p. 411). Evidence to support this view has accumulated during the ensuing 20 years, and the following sections will review this evidence. The main findings are summarized in Table 1.

4.1. Onset of ovarian activity

We noted earlier that AFP synthesis appears to "switch off" around postnatal Day 7 in rodents, and AFP levels in both brain and plasma are low by postnatal Day 7–10 (Ali et al. 1981; Ali & Sahib 1983; Raynaud 1973; Raynaud et al. 1971). It is at this time, or later, that estrogen of ovarian origin could freely enter neurons to exert organizational effects. Consistent with this view, Döhler and Wuttke (1975) measured serum levels of follicle stimulating hor-

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Table 1. *An overview of behavioral and neuromorphological features in the rat that are influenced by ovarian manipulations, with sex differences.[1] See text for details and references.*

Measure	Characteristic	Significant effects of ovarian steroids
Female Reproductive Behavior		
Receptive (lordosis)	F more than M	Neonatal Ovx + T lowered scores more than neonatal T alone Neonatal Ovx reduced scores more than postpubertal Ovx
Proceptive (hopping, darting ear wiggling)	F more than M	Neonatal Ovx reduced scores more than postpubertal Ovx Neonatal Gdx combined with E or ovarian transplant raised scores more then neonatal Gdx alone
Nonreproductive Behavior		
Open-field activity	F higher than M	Neonatal Ovx reduced activity Above restored by prepubertal low-dose E Neonatal Ovx + T lowered scores more than neonatal T alone
Plus maze	F higher than M	Neonatal TX or pubertal Ovx decreased time in open arms; neonatal TX + Ovx decreased time the most
Maze learning	F more errors than M	F errors increased after puberty Neonatal low-dose E increased M errors
Active avoidance learning	F fewer errors than M	Sex difference appeared after puberty Neonatal Ovx increased errors Postpubertal Ovx decreased errors Above reversed after 3 days of E Performance varied across estrus cycle; high E levels increased errors
AMPH-induced locomotion	F more active than M	Prepubertal Ovx decreases activity as compared to adult Ovx
Morphology		
SDN-POA	F smaller than M	Neonatal TX to F reduced size, relative to control F Neonatal E mRNA antisense reduced size, relative to control F Postpubertal Gdx + E & P increased size, relative to Gdx M Gdx alone had no effect on M
AVPv	F larger than M	Sex difference emerged at puberty with increase in F Postpubertal Gdx + E & P increased size, relative to Gdx M Gdx alone had no effect on M
Dendritic spine density		
Ventromedial hypothalamus	Varied across estrus cycle	Ovx reduced density; restored by low-dose E or E+P
Visual cortical pyramidal	After Day 20 M increased and F decreased	Day 30 Ovx prevented F-typical decrease in spines
Hippocampal pyramidal	Varied across estrus cycle	Ovx reduced density; restored by low-dose E&P
Dendritic branching		
Parietal cortical pyramidal	TP F more arbor than F	Day 150 Ovx increased arbor in oil-treated F
Cortical thickness	M, R > L; F no asymmetry or trend to L > R	Neonatal Ovx increased cortical thickness Above reversed by E, Days 40–90 Above enhanced by P, Days 40–90

1. Exclusive of corpus callosum

F: females; M: males; Ovx: ovariectomy; Gdx: male castration; T: testosterone; E: estrogen; P: progesterone; TX: tamoxifen

mone (FSH), lutenizing hormone (LH), and estrogen taken from pups of various ages, and postulated that the feedback regulation of estrogen on phasic gonadotropin release becomes functional between Days 9 and 21 in female rats. More recent serum and tissue analyses from newborn rats and mice have revealed increases in ovarian estrogen in response to hCG (human chorionic gonadotropin), LH, FSH, and cAMP (cyclic adenosine 3′,5′-monophosphate) around 1 week after parturition (Mannan & O'Shaughnessy 1991; Sokka & Huhtaniemi 1995; Weniger et al. 1993). Ovarian hypertrophy in response to unilateral gonadectomy has been observed as early as Day 10 in the rat (Gerall & Dunlap 1971), and estrogen synthesis has been observed in neonatal ovarian tissue cultured in vitro (Funkenstein et al. 1980; Levina et al. 1975). These findings support the view that ovarian estrogen could exert developmental effects in the neonatal period following the decline of AFP.

4.2. Ovarian hormones and reproductive behavior

Evidence regarding the effects of neonatal ovariectomy (OVX) on female-typical sexual behavior are mixed. Al-

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though some researchers have reported that removing the ovaries caused no significant effect on sexual behavior in female rats (e.g., Lisk & Suydam 1967; Whalen & Edwards 1967), others (e.g., Blizard & Denef 1973) have found that the inhibitory effect of neonatal testosterone exposure on female sexual behavior (lordosis) is suppressed if the ovaries are present during development. Sodersten (1976) similarly reported that intact female rats exposed neonatally to testosterone showed a greater adult lordosis response (when primed with estrogen and progesterone) than females receiving neonatal OVX and testosterone treatment. Furthermore, Sodersten found that postpubertally ovariectomized (OVXd) female rats showed more lordosis after priming than neonatally OVXd females. He concluded that "although the nature and mechanism of the action of these ovarian secretions remain to be determined we feel that the fact should be recognized that they do exert a modifying influence on psychosexual differentiation" (p. 419).

Other researchers have found that when proceptive or soliciting components of female sexual behavior – hopping, darting, and ear-wiggling – are examined, ovarian effects become even more evident. Gerall and his colleagues (Gerall et al. 1973) reported higher proceptive behavior in estrogen-primed female rats that received OVX postpubertally as compared to those OVXd neonatally. Similarly, neonatally gonadectomized males that received prepubertal ovarian transplants or low-dose estrogen treatment were more proceptive in adulthood than males that were gonadectomized only. Indeed, the former males exhibited as much proceptive darting behavior as normal females. These findings suggest that lordosis and proceptive behavior are under different hormonal control, and that proceptive behavior may be particularly sensitive to ovarian effects. This conclusion is supported by Ward's (1983) finding that prenatally stressed male rats exhibited lower mounting and increased lordosis in adulthood, but did not exhibit any of the proceptive components of female sexual behavior.

4.3. Ovarian hormones and nonreproductive behavior

4.3.1. Open-field behavior. Female rats are normally more active in the open field than males. OVX on Day 1 or 8 of life reduced open-field behavior in adult female rats to male levels (Denti & Negroni 1975; Stewart & Cygan 1980). This agrees with Blizard and Denef (1973), who found that the presence of the ovaries during development suppressed the masculinizing effects of neonatal testosterone treatment on open-field behavior in rats. Stewart and Cygan (1980) studied low- and high-dose replacement of estradiol and found that low doses increased (feminized) open-field activity, whereas high doses suppressed (masculinized) activity. They concluded that "estrogens given during the period prior to weaning can have a feminizing effect on adult open-field behavior, and that the sex difference normally observed in adult rats is dependent in part on the presence of the ovaries during a period after birth" (p. 20; see also Stewart et al. 1979).

4.3.2. Plus maze behavior. Zimmerberg and Farley (1993) found that intact adult female rats spend significantly more time in the open arms of a plus maze than males. When females were either exposed neonatally to the estrogen receptor-blocker tamoxifen or OVXd at puberty, they spent less time in the open arms as adults. Females that received both treatments spent the least time in the open. In contrast, neonatal and pubertal manipulations of androgens in males (by administration of the androgen receptor-blocker flutamide and castration) had little effect on plus maze behavior. The authors conclude that "these experiments indicate that female gonadal hormones play an important role both organizationally and activationally in plus maze behavior" (p. 1119). The Zimmerberg and Farley findings were confirmed by Leret et al. (1994), who also reported that neonatally OVXd female rats behaved like males when tested in a plus maze paradigm.

4.3.3. Spatial behavior. Krasnoff and Weston (1976) found that sex differences in maze learning emerge around the time of puberty in rats, with females making more errors after puberty, whereas male behavior remained essentially unchanged. Intact or neonatally castrated male rats, given low doses of estrogen neonatally, had lower spatial learning scores in adulthood than untreated males (Dawson et al. 1975). In humans, sex differences in spatial ability (e.g., for mental rotation) have also been reported to appear at puberty (see Halpern 1992 for review), although the hormonal basis for this gender difference is not fully understood. Fluctuations in spatial ability are seen across the menstrual cycle, however, and appear to correlate with changes in estrogen level (Hampson 1990; Hampson & Kimura 1988).

4.3.4. Avoidance learning. Sex differences in two-way shuttlebox learning also appear after puberty in rats (Bauer 1978), but are influenced by ovarian hormones at various times in development. Denti and Negroni (1975) found that neonatal OVX decreased active avoidance performance of adult female rats to male-typical levels. In contrast, postpubertal OVX led to a significant increase in shuttlebox learning in female rats, an effect that was reversed with three days of estrogen replacement (Diaz-Veliz et al. 1989). Diaz-Veliz and colleagues also reported that avoidance learning varied across the estrus cycle, with high estrogen levels associated with increased errors. These latter findings likely reflect activational hormonal effects (given their transient nature), whereas the neonatal ovariectomy appears to have had a permanent (organizational) effect on avoidance learning. It is noteworthy that, in the above example, the activational effects of estrogen on avoidance behavior are opposite to developmental (organizational) estrogen effects.

The mechanisms underlying the emergence of cognitive sex differences at or after puberty are unclear. One possibility is that neural reorganization of some sort occurs at puberty (e.g., see Jernigan et al. 1991). This would be consistent with evidence that age at puberty is related to spatial abilities in humans (Newcombe & Bandura 1983; Sanders & Soares 1986; Waber 1976; 1977).

4.3.5. Rotation and locomotor behavior. Camp et al. (1984) demonstrated sexual dimorphism of the nigrostriatal system in rats as measured by various tests of amphetamine-induced rotation (with females showing more asymmetry on some tasks and less on others). In addition, Becker and colleagues (Becker & Cha 1989; Castner & Becker 1990) reported that endogenous, or exogenously administered, pulsatile estrogen potentiated the dopaminergic and behavioral locomotor response to amphetamine (AMPH) in female – but not male – rats. This difference appears to underlie higher behavioral responsiveness to AMPH in

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females than in males. OVX depressed striatal dopaminergic release and turnover in females, whereas physiological concentrations of estrogen stimulated dopaminergic release. Castration or estrogen exposure had no similar effect on male striatal tissue. This sex difference appears to emerge at puberty through changes in the response of female striatal tissue to estrogen (Becker & Ramirez 1981). More recently, Forgie and Stewart (1994) reported that in the absence of early testosterone exposure, female rats OVXd on postnatal Days 1 or 25–26 showed less behavioral activation to AMPH than females OVXd in adulthood. This effect was seen for both estradiol-primed and nonprimed animals, although activity was highest in primed subjects. These effects were not seen for females exposed neonatally to testosterone.

4.4. Ovarian hormones and subcortical anatomy

Döhler and colleagues (Döhler et al. 1984b; 1984c) found that neonatal administration of the estrogen antagonist, tamoxifen, to female rats decreased the size of the SDN-POA in adulthood relative to controls. They proposed that whereas high levels of estrogen (derived from the intracellular conversion of testosterone) are necessary for the masculinization of this structure, feminization may require some low level of estrogen. This thesis is supported by more recent evidence that neonatal treatment with estrogen mRNA antisense significantly reduces the size of the SDN-POA in intact adult female rats (McCarthy et al. 1993). In a related study, Bloch and Gorski (1987; 1988) reported that the hypothalamic anteroventral preoptic nucleus (AVPv) is significantly larger in female rats than in males, and that postpubertal castration of males followed by treatment with low doses of estrogen and progesterone significantly enlarges this structure. These males also had a smaller SDN-POA compared to control males. Similar effects were not observed in males that were gonadectomized only. More recent work has shown that sex differences in the AVPv emerge at puberty, and are the consequence of increases in AVPv size in females (Davis et al. 1993).

Frankfurt et al. (1990) reported that dendritic spine density of ventromedial hypothalamic neurons varies across the estrus cycle in rats. They found that adult OVX reduced dendritic density, whereas estrogen or estrogen plus progesterone replacement increased density. Although such effects are properly described as activational, they demonstrate that circulating hormones can temporarily change neural structure. Furthermore, these variations apparently occur in a periodic fashion, as a consequence of hormonal cyclicity, in adult female brains.

4.5. Ovarian hormones and cortical and hippocampal anatomy

4.5.1. Cortical and hippocampal thickness. Ovariectomy on Day 1 was found to increase cortical thickness in 90-day-old female rats as compared to sham-operated littermates (Diamond et al. 1979). In this study, females were also OVXd at 90 or 300 days of age while littermate controls received sham surgery; in all cases cortical thickness measurements were obtained 90 days after surgery. No significant differences in cortical thickness were found for the 90- or 300-Day OVX groups as compared to respective shams. The increase in cortical thickness for the Day-1 OVX group reflected – at least in part – an increase in neuronal soma size. These findings support the view that ovarian hormones act specifically during prepubertal development to affect the thickness of the cerebral cortex.

Pappas et al. (1979) sought to determine the relative contributions of estrogen and progesterone. In their first experiment they replicated the neonatal ovariectomy effect on cortical thickness measured at 90 days of age. In a second experiment, a group of female rats were OVXd on Day 1 while littermates received sham surgery. From 40 to 90 days of age, the OVXd females received daily injections of ethinylestradiol while shams received an equal volume of sesame oil. At 90 days of age the OVX-plus-estrogen group had significantly thinner cortices than controls, in contrast to the thicker cortices of untreated OVXd groups from prior studies. A third experiment followed the same procedure as experiment two except that OVXd females received daily injections of progesterone from Days 40–90 and shams received oil. In this case, the progesterone-treated OVX subjects had significantly thicker cortices than sham controls. These findings suggest that estrogen and progesterone exert different developmental effects on cortical thickness in the female brain.

In later studies Diamond and colleagues found the right cerebral cortex of the male rat to be significantly thicker than the left throughout life, whereas the cerebral cortex of females showed no significant asymmetry (but a trend to L > R; Diamond et al. 1983). To investigate the role of ovarian hormones in this sexual dimorphism, females received OVX on Day 1 and female littermates received sham surgery. Cortical thickness measurements at 90 days of age showed that shams exhibited no significant asymmetry, whereas the OVXd females exhibited a male pattern of right-significantly-greater-than-left in the visual cortex (Diamond et al. 1981). Stewart and Kolb (1988) later replicated the described sex difference, but did not find that ovariectomy reversed the cortical asymmetry pattern in females.

Finally, despite evidence of ovarian effects on cortical anatomy as reviewed above, Diamond et al. (1982) failed to find any difference in hippocampal thickness between OVX and sham control female rats.

4.5.2. Dendritic spine density. Stewart and Kolb (1994) reported that OVX of intact adult female rats on Day 150 increased the dendritic arbor of pyramidal neurons of the parietal cortex as compared to controls. Similar effects were seen on dendritic arbor of pyramidal neurons of females treated neonatally with testosterone, although adult OVX of these subjects exerted no further effects. These findings may reflect a transient response to hormonal change, similar to changes observed in hypothalamic (Frankfurt et al. 1990) and hippocampal dendritic anatomy, following adult OVX of untreated females. Gould et al. (1990), for example, found that OVX in adulthood decreased dendritic spine density on CA1 pyramidal cells in the hippocampus of female rats, and that this effect was blocked by the concurrent administration of estrogen and progesterone. They suggested that variations in the density of these spines may accompany the estrus cycle, which was confirmed by Woolley et al. (1990).

Evidence also supports developmental ovarian effects on cortical dendritic anatomy. Munoz-Cueto et al. (1990) found that the development of dendritic spines in visual cortex occurs later in intact male as compared to intact

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female rats. After Day 20, dendritic spine numbers continue to increase for males, whereas females show a significant decrease. The female-typical loss of dendritic spines was prevented by OVX on Day 30, leading to a higher number of cortical dendritic spines among ovariectomized females as compared to intact females by Day 60. Munoz-Cueto et al. postulated that estrogen exerted inhibitory effects on cortical dendritic spine growth in both sexes, with aromatized testosterone delaying development in males during the early period, and ovarian estrogen promoting a loss of spines during the later time period (Days 20–60).

4.6. Why isn't ovarian estrogen masculinizing?

Given the long-standing observation that masculinization of many systems is dependent on the biosynthesis of estrogen, one might question how estrogen could exert masculinizing effects in males and concomitant feminizing effects in females. We suggest three inter-related mechanisms that could account for these dimorphic effects: (1) sex differences in estrogen levels (high for aromatized estrogen, relatively low for estrogen of ovarian origin); (2) sex differences in critical periods of estrogen action; and (3) variation in topographic distribution and density of target estrogen receptor populations as a function of sex (Brown et al. 1990; DonCarlos & Handa 1994; Kuhnemann et al. 1994) and postnatal age (MacLusky et al. 1979a; 1979b; Miranda & Toran-Allerand 1992; O'Keefe & Handa 1990; Shugrue et al. 1990). Evidence for a temporal distinction in sensitive periods for testicular and ovarian effects is discussed below.

The concept of critical, or sensitive, periods in development has played a central role in theories of sexual differentiation. Thus, it has been generally agreed that testosterone exerts masculinizing effects on the CNS of male rats during the period between about gestational Day 17 and postnatal Days 8–10, depending on the system being studied (Rhees et al. 1990a; 1990b; but see Bloch & Mills 1995). This perinatal period of sensitivity to the masculinizing effects of testosterone appears to be similar in mice (e.g., see Wagner & Clemens 1989). However, a different set of temporal parameters appears to apply to female brain development. The sensitive period for permanent structural and behavioral ovarian effects does not end by Day 10 in rodents, as generally appears to be true in males, but (depending on the system under study) appears to extend quite late in life. Support for a later sensitive period in females includes evidence that: (1) the critical period for feminization of sexual behavior apparently extends up to puberty in female rats (Gerall et al. 1973); (2) exposure to low doses of estrogen as late as Day 30–40 results in feminized open-field behavior in OVXd rats (Stewart & Cygan 1980); (3) OVX on Days 25–26 decreases the locomotor response to AMPH in female rats (Forgie & Stewart 1994); (4) ethinylestradiol exposure from Days 40–90 leads to a thinner cortex in OVXd female rats (Pappas et al. 1979); (5) postpubertal castration of male rats followed by low-dose estrogen and progesterone treatment increases the size of the AVPv and decreases the size of other sexually dimorphic nuclei (Bloch & Gorski 1988); and (6) OVX of female rats on Day 30 prevents the female-typical decrease in cortical pyramidal dendritic spines (Munoz-Cueto et al. 1990). These findings all suggest a sensitive period for ovarian feminization that extends up to or around puberty in rodents.

Therefore, it may be that early (< Day 10) high levels of intracellular estrogen interact with sex- and age-specific estrogen receptor populations to "masculinize," whereas later (> Day 10) and lower levels of estrogen interact with age- and sex-specific estrogen receptor populations to "feminize." Work by Stewart and Cygan (1980; see also Stewart et al. 1979) nicely illustrates this distinction by showing masculinizing effects on OVXd female rats with early high-dose estrogen treatment (25 ug estradiol benzoate on P2 and 3) and feminizing effects on OVXd female rats with later low-dose estrogen replacement (silastic implants of estradiol 17B on P30–40, delivering physiological levels of about 108 pg/ml serum) on the same variable – open-field behavior.

4.7. Summary

The literature reviewed here provides strong evidence that ovarian hormones influence the development of the female brain. The findings do not refute or contradict the profound evidence of androgen-mediated masculinization, but suggest that ovarian hormones may exert parallel influences on the development of brain and behavior in the female, in the absence of early androgens. This, in turn, compels us to broaden the concept of sexual differentiation, by recognizing that both testicular and ovarian hormones are active participants. This idea was anticipated more than 15 years ago by Stewart and Cygan who wrote in 1980 that "while both testicular and ovarian hormones contribute to normal male and female behavioral development, their actions are not merely reciprocal and probably occur at different times in development" (p. 24).

In the next section, we review data showing that both ovarian and testicular hormones play a critical role in neuroanatomical differentiation of the corpus callosum (CC).

5. The corpus callosum and sexual dimorphism

5.1. The original findings

We have systematically investigated the role of neonatal gonadal hormones on callosal development in the rat, prompted by the finding that the corpus callosum is significantly larger in adult male than female Purdue-Wistar rats (Berrebi et al. 1988). In our initial study, entire litters of male and female pups received handling stimulation between birth and weaning, or were nonhandled controls. Handling was included because of prior data showing that this procedure affects the development of cerebral laterality and may influence callosal size as well (Denenberg 1981). Handling consisted of removing the newborn pups from the maternity cage, leaving the mother in the cage, placing each pup into a 1-gallon can containing wood shavings, leaving the pups for 3 minutes, and returning them to the home cage (Denenberg 1977). This was done daily from Day 1 through 20, with weaning on Day 21. Subjects were then group housed with same-sexed littermates. At 110 days of age they were perfused, the brains were removed, and a mid-sagittal section of the callosum was obtained. Using a projection microscope the callosum was magnified and drawn.

Males were found to have a larger absolute cross-sectional callosal area than females. Furthermore, there

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was a Sex x Handling interaction, with handled (H) males having the largest callosum, followed by nonhandled (NH) males, then NH females, with H females having the smallest callosa. H males differed significantly from H females, and NH males differed significantly from NH females. The magnitude of the sex difference was greater within the H animals than within the NH rats, however, and this was the cause of the significant interaction (see Table 2).

Because males and females also differed in brain weight, the data were recalculated as relative values (Berrebi et al. 1988). The callosal area for each subject was divided by that animal's brain weight taken to the ⅔ power. (The ⅔ correction was used to convert the index of brain size from a 3-dimensional to a 2-dimensional measure.) However, the same significant effects were obtained as for the absolute CC values.

We then developed a software program, Stereology, to expand and simplify our data analyses (Denenberg et al. 1991a). The outline of the callosum was traced onto a digitizing tablet, and the computer calculated the following callosal parameters: area, perimeter, length, and 99 widths measured along the longitudinal axis of the callosum. The Berrebi et al. data were reanalyzed using the Stereology program (Denenberg et al. 1989). A comparison of computer-generated callosal values with those from Berrebi et al. found a mean error of 0.27%. (The Berrebi et al. values in Table 2 are derived from the computer program to maintain comparability with the other data in that table.)

Next, the measures for callosal area, perimeter, length, the 99 widths, and brain weight were entered into a factor analysis (Denenberg et al. 1989). The 99 widths fell into 7 oblique factors. An eighth factor had significant loadings on brain weight, callosal length, and callosal perimeter. Callosal area did not load on any factor. It is interesting that a highly similar pattern of 7 oblique width factors was obtained from independent analyses of human CC width measures (Denenberg et al. 1991a). This parallel was somewhat startling, given the profound shape differences be-

Table 2. *Callosal cross-sectional area means ± SE at 110 days of age. All rats are Wistars except Mack et al. 1995b, which are Sprague-Dawleys*

Reference	Treatment	Male	Female
Berrebi et al. 1988; & Denenberg et al.	NH	3.544±0.106	3.202±0.106
1989	H	3.722±0.125	3.098±0.125
Fitch et al. 1990a	H	3.568±0.062	3.257±0.063
	H+Gdx(1)	3.506±0.079	
	H+TP(4)		3.474±0.070
	H+DES(4)		3.380±0.118
	H+TX(4)	3.778±0.126	3.627±0.117
Fitch et al. 1991b	H	3.377±0.067	3.123±0.053
	H+TP(8, 12, 16)		3.189±0.049
	H+Ovx(8, 12, 16)		3.342±0.065
Fitch 1990	V(E17–21)+ShamGdx(1) +H+Oil(4)	3.253±0.060	2.857±0.043
	FLT(E17–21)+Gdx(1) +H+Oil(4)	2.971±0.094	
Fitch et al. 1991a	NH, V(E17–P2), ShamGdx(3)	3.603±0.108	3.120±0.121
	NH, FLT(E17–P2), Gdx (3)	3.272±0.084	
Denenberg et al. 1991b	NH	3.630±0.134	3.301±0.067
Experiment 1	NH+Gdx (2)	3.376±0.062	
	NH+TP(4)		3.362±0.126
Experiment 2	NH	3.544±0.093	3.298±0.070
	NH±Gdx(2)	3.686±0.082	
	NH+TP(4)		3.228±0.073
	H	3.626±0.098	3.282±0.067
	H+Gdx(2)	3.627±0.089	
	H+TP(4)		3.484±0.070
Mack et al. 1992	NH	3.800±0.117	3.468±0.066
	NH+Ovx(12)		3.721±0.117
	H	3.652±0.071	3.385±0.053
	H+Ovx(12)		3.509±0.039
Mack et al. 1993	H	3.274±0.062	2.934±0.078
	H+Ovx(12)		3.190±0.074
	H+Ovx(12)+Es(25)		2.733±0.072
Mack et al. 1996b	NH CS (E22)	3.014±0.118	2.828±0.114
	NH CS (E22)+Gdx(E22)	3.084±0.102	

NH = nonhandled; H = handled; Gdx = male castration; Ovx = ovariectomy; DES = diethylstilbestrol; TX = tamoxifen; V = vehicle; FLT = flutamide; Es = estrogen; CS = Caesarian section; E = embryonic day; P = postnatal day (E and P used for clarification; in all others, the number in parentheses refers to postnatal day of treatment); TP = testosterone propionate; SHAM = sham surgery; SE = standard error

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tween rat and human CC, and could possibly suggest that common structural factors in the CC (e.g., discrete fiber bundles) underlie the observation of statistical loading on discrete factors. The seven width factors obtained in the Denenberg et al. (1989) analysis of the rat CC have subsequently been used in all rat CC analyses.

5.2. Callosal measurement: Absolute or relative?

In the Denenberg et al. (1989) analysis, the variables of brain weight, callosal length, and callosal perimeter were in a factor by themselves, not associated with any of the seven width factors or with callosal area. Statistically, this means that brain weight is independent of callosal area and width. To verify this, we correlated each animal's brain weight against its seven factor width scores and against its callosal area. Out of 64 correlations, only 6 were significant at the .05 level, and 1 of those had a negative sign (Denenberg et al. 1989). This is essentially a chance distribution ($p > .10$). In subsequent studies we have confirmed that brain weight is uncorrelated with corpus callosum (CC) area or any of the width factors (Fitch et al. 1991b; Mack et al. 1993).

The correlation pattern is directly relevant to the question of whether one should use absolute or relative CC values in assessing experimental treatments. Simply finding that there are significant differences in group means on two or more variables is not sufficient evidence to cause one to use relative measures (e.g., males have a larger CC area than females and also have brains that weigh more). It is also necessary that there be a significant association between two variables within a group before one needs to make an adjustment. (The same is true for a covariance analysis.) As an example, women weigh on average less than men, and women score lower on average than men on certain tests of spatial ability (Halpern 1992). One cannot draw any conclusion concerning an association between these two variables from such data. That can only be done if a significant correlation exists between weight and spatial scores within each gender.

In three independent studies we have failed to find any evidence that CC area and brain weight are related. In addition, when we adjusted for brain weight in the Berrebi et al. (1988) study, we still obtained the same significant effects we had obtained using absolute CC values. These findings allow us to conclude that cross-sectional callosal area and the seven callosal width factors can be evaluated in absolute terms and do not need to be corrected for brain weight. This issue also has relevance for the field of human callosal research, in which some studies use absolute measures and others use corrected ones. We discuss this issue further in the context of reviewing the literature on human callosal sex differences.

5.3. Callosal measures and hormone manipulations

The Berrebi et al. (1988) findings demonstrated a clear sexual dimorphism in callosal size in the Wistar rat, whether handled in infancy or not. This result has since been independently replicated in Long-Evans rats (Nunez et al. 1995; Zimmerberg & Mickus 1990; Zimmerberg & Scalzi 1989), and in Sprague-Dawley rats (Mack et al. 1996b).

Because many sexual dimorphisms in the brain are influenced by hormones, we set out to study the role of hormonal exposure in the development of the sexually dimorphic rat CC. The cumulative results from this series of studies are displayed in Table 2, and Figures 1 and 2. Table 2 presents the CC area means listed by reference and treatment. Figures 1 and 2 summarize the effects of various hormonal manipulations on callosal size in males and females as compared to control female values. The means and SE's in these figures were taken from the data in Table 2, and weighted as a function of N before pooling. Unless otherwise noted, all experiments described below were conducted with animals handled in infancy, because we wished to maximize the baseline sex differences. In all our CC analyses we have looked at callosal area, perimeter, length, and the seven regional width factors (that were derived from the factor analysis procedure described above). Typically, when a manipulation alters callosal size there are significant effects on callosal area, one or both of the two anterior width factors, and one or both of the two posterior width factors. For the purposes of this review only callosal area data are reported because, with one exception to be discussed below, these accurately reflect the findings from the complete data set.

5.4. Testosterone and callosal masculinization

5.4.1. Testosterone administration to females. In our first study we found that a single sc (subcutaneous) injection of 1 mg testosterone propionate (TP) administered to handled 4-day-old female pups was sufficient to increase significantly their adult CC area in comparison to oil-treated female littermates (Fitch et al. 1990a; Fig. 2). Indeed, the increase was so large that the TP female CC values did not differ significantly from those of male littermates. We then repeated the Fitch et al. experiment with nonhandled rats, fully expecting to find the same effect (Denenberg et al. 1991b). To our surprise, TP did not increase callosal size in NH females. We then did a second experiment with both H and NH animals. We replicated the finding that H

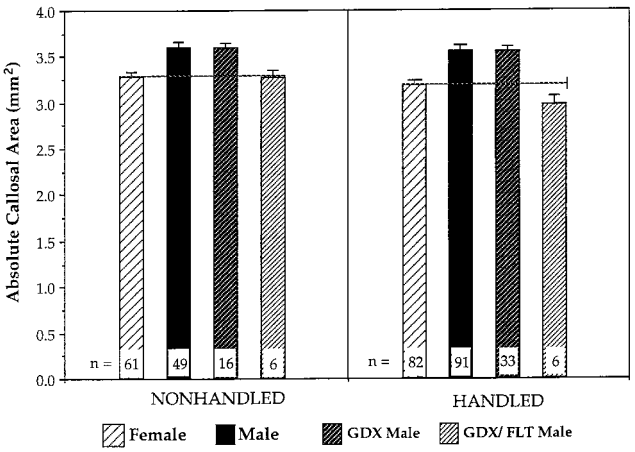


Figure 1. Effects of handling and hormonal manipulations on mid-sagittal callosal area in adult male rats as compared to control females (indicated by dotted line). Mean absolute values are given in mm² (Table 2) and averaged where multiple studies contained the given condition. Total *n* are shown for each condition, and in the case of multiple studies SEs were weighted as a function of *n* before pooling. GDX: gonadectomized; FLT: prenatal flutamide treatment.

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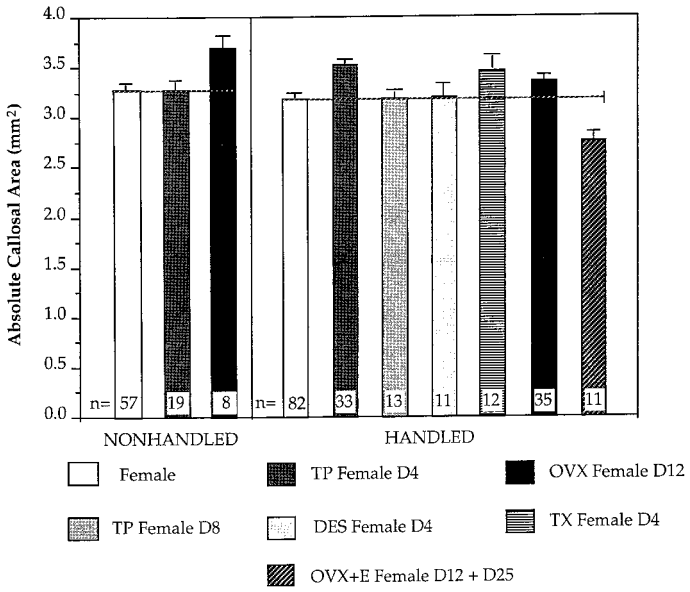


Figure 2. Effects of handling and hormonal manipulations on mid-sagittal callosal area in adult female rats as compared to control females (indicated by dotted line). Mean absolute values are given in mm² (Table 2) and averaged where multiple studies contained the given condition. Total *n* are shown for each condition, and in the case of multiple studies SEs were weighted as a function of *n* before pooling. TP: testosterone propionate; DES: diethylstilbestrol; TX: tamoxifen; OVX: ovariectomy; E: estradiol benzoate. Age of treatment given in days. Note that handled Ovx females (Day 12) included one study in which Ovx was performed on Day 8, 12, or 16, and two studies using Day 12 only. Note also that DES and TX values were corrected as a function of the difference between their control female values and the pooled control female value, because all values in the DES/TX study ran large.

females given TP had significantly larger callosa, and we also replicated the finding that NH females given TP were unaffected with respect to callosal size. We interpreted these data as suggesting a synergy between the presence of testosterone and the effects of handling on adrenal corticosteroids (Denenberg et al. 1967; Meaney et al. 1988). This hypothesis is supported by evidence that handling alters glucocorticoid receptor levels in the cortex and hippocampus (Meaney et al. 1988), and that adrenalectomy alters myelination of the cerebral cortex (Meyer & Fairman 1985). These findings support the notion that handling (and subsequent adrenal changes) might interact with gonadal steroids to influence differentiation of the cerebral cortex, particularly the CC, which is a myelinated structure.

The finding that TP treatment must be associated with handling to significantly enlarge the female's callosum suggests that the mechanism underlying this effect is more complex than simple exposure of the female to androgen. Because evidence supports the relative importance of estrogen biosynthesis to sexual differentiation of the rodent cerebral cortex (reviewed above), we examined the relative influence of testosterone's aromatized metabolite, estrogen, on the development of callosal size (Fitch et al. 1990a). We did not see any significant effects on adult callosal size when handled female pups were exposed to a synthetic estrogen, diethylstilbestrol (10 ug DES on Day 4; dose derived from Döhler et al. 1984a), although DES females did have significantly higher body weights than oil controls (thus establishing its effectiveness; Fitch et al. 1990a). This failure to find an effect of DES on callosal size suggests that masculinization of this structure may not depend exclusively on aromatization, although clearly this issue will require further study.

5.4.2. Developmental effects of TP treatment. Handled females were given TP or oil on Day 4, and littermates from each condition (as well as male controls) were sacrificed at 30, 55, or 90 days of age (Fitch et al. 1990b). Callosal size for both TP-treated females and males was significantly greater than for intact (control) females by 30 days of age, and there were no significant differences between TP females and males. This pattern was also seen at 55 and 90 days of age (Fig. 3).

5.4.3. Castration of males. To investigate the role of endogenous testicular androgens in callosal development, we castrated handled male pups on Day 1 of life (Fitch et al. 1990a; Fig. 1). Contrary to our expectations, the callosal size of these males was not affected in adulthood. Denenberg et al. (1991b) also failed, in two experiments, to see an effect of Day 1 castration on CC size.

These findings suggested that testosterone exposure in the prenatal and early (<24 hour) postnatal period had already exerted organizing effects in males. Therefore, we conducted another experiment in which an androgen receptor blocker, flutamide, was administered to pregnant dams (25 mg/kg; Fitch et al. 1991a). Based on Ward and Weisz's (1980) report that the prenatal surge of testosterone occurs on prenatal Day 18, and work by Neri et al. (1972) showing demasculinizing effects of flutamide on male reproductive structure and behavior, we chose gestational Day 17 to start the flutamide treatment. Control dams received the vehicle (polyethylene glycol) only. Prenatal flutamide exposure was followed by neonatal castration of male pups. Surgery consisted of placing a small horizontal incision in abdominal skin and muscle, and visualizing and removing the testes. All other pups (flutamide-treated

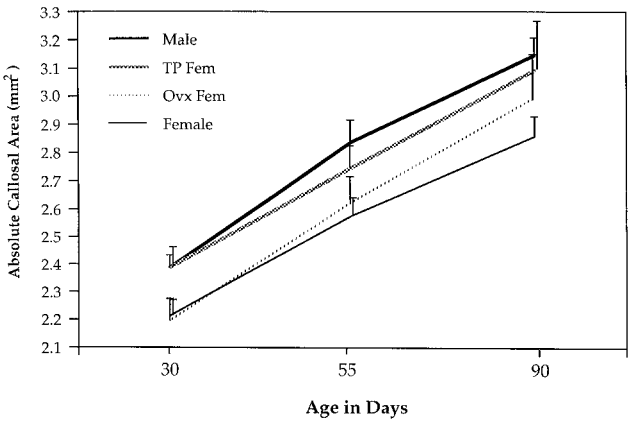


Figure 3. Effects of sex and hormonal manipulation on the development of mid-sagittal callosal area. TP was given on Day 4 and OVX was performed on Day 12. Values represent mean callosal area for each group at 30, 55, and 90 days of age, with standard errors. Note that the control male and female groups include surgical shams (for the comparison to OVXd females) and oil-treated controls (for comparison to TP females). There were no differences between these subgroups for each sex.

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females and controls of both sexes) received sham surgery, which consisted of a skin and muscle incision only. All surgeries were performed under cryogenic anaesthesia. In this study, unlike those described above, nonhandled animals were used. Results showed that the callosal area was significantly smaller among treated males than among control males, and did not differ from that of female littermates (Fitch et al. 1991a; see Fig. 1). These results have since been replicated with handled animals (Fitch 1990; Fitch et al., in preparation, Fig. 1).

5.4.4. The sensitive period for testosterone effects. Because the results described above were seen in the absence of both the pre- and postnatal testosterone surge, it was impossible to delineate the exact sensitive period for CC masculinization in males. To further assess this window, nonhandled Sprague-Dawley pups were delivered by Caesarean section on Day 22 of gestation and, within 20 minutes thereafter, males were castrated or received sham surgery (Mack et al. 1996b). Female pups received no treatment. This procedure eliminated the postnatal androgen surge in castrated males, while leaving the prenatal surge intact. At 110 days of age the CCs of castrated and sham males did not differ, but both groups had larger CCs than their female littermates. This finding indicates that the sensitive period for testosterone-mediated sexual differentiation of the male CC begins prenatally, presumably in conjunction with the prenatal testosterone surge. This assertion is supported by the fact that a sexual dimorphism in callosal size can be seen as early as postnatal Day 3 in rats (Zimmerberg & Scalzi 1989), as well as evidence that this callosal sex difference is eliminated by prenatal exposure to alcohol (which is thought to suppress the prenatal surge of testosterone in the male fetus; McGivern et al. 1988).

Our results showed that the prenatal period marks the beginning of CC sensitivity to testosterone, but it was not clear when the window "closes." That is, in the absence of prenatal testosterone, would the window of CC sensitivity to testosterone extend postnatally? Prior results showed that Day 4 TP administration to handled female rats increased their callosal size to that of males (Fitch et al. 1990a), suggesting that the sensitivity window extends to at least postnatal Day 4. Moreover, additional studies showed that the end of the sensitive period apparently falls somewhere between postnatal Days 4 and 8 because TP administered to females on Day 4 significantly increased callosal size, whereas TP administered on Days 8, 12, or 16 did not (Fitch et al. 1991b; Fig. 2). The window of CC sensitivity to testosterone appears, therefore, to begin prenatally, and end between postnatal Days 4 and 8. This sensitive period is consistent with the reports of others. Breedlove and Arnold (1983) found that castration on Day 1 did not demasculinize the size of the bulbocavernosus spinal nucleus in male rats, whereas prenatal treatment with flutamide, combined with castration, did. Wagner and Clemens (1989) found that TP treatment of female mice on postnatal Days 1, 3, and 5 – but not Days 7, 9, and 11 – significantly increased the size of this nucleus to that of males.

As an aside, the argument can be made that the developmental parameters we have derived from the effects of testosterone on intact female rats cannot be generalized to mechanisms of endogenous androgen exposure in males. To address fully the validity of this assumption it will be necessary to repeat the above experiments by administering TP to prenatally flutamide-treated, postnatally castrated males rather than intact females.

5.5. Ovarian hormones and callosal feminization

5.5.1. Tamoxifen effects. The first hint that callosal size is affected by ovarian hormones derived from a study in which tamoxifen (10 ug TX; dose based on Döhler et al. 1984c) was given to 4-day-old male and female pups. This compound is an estrogen antagonist, and our primary reason for using it was to block the action of aromatized estrogen in males (as a test of the aromatization hypothesis for CC differentiation). TX was given to female littermates primarily to provide a matched control. To our surprise, TX resulted in a near-significant ($p < .06$) increase in callosal area in females (Fig. 2), but did not affect callosal size in males (Fitch et al. 1990a). In addition, TX significantly increased two of the females' callosal width regions at a significance level of $p < .01$. In the same experiment, the synthetic estrogen diethylstilbestrol (DES) was administered to males and females on Day 4. However, DES did not significantly affect callosal area in either sex (Fig. 2), suggesting that the mechanism of tamoxifen action was not through incidental activation of estrogen receptors (which can occur when receptors are bound by tamoxifen), but rather through blocking the binding of endogenous estrogen to receptors. Another possibility is that tamoxifen interfered with ovarian development and consequent function, but this interpretation also points to a critical role for the ovaries in CC development.

5.5.2. Ovariectomy effects. The findings described above suggested (but did not prove) that the increase in the size of the female callosum was a consequence of temporary estrogen "removal" during development. One way to test this hypothesis directly was to remove estrogen via ovariectomy, and measure callosal size in adulthood. We did this experiment: handled females received ovariectomy on postnatal Day 8, 12, or 16. Surgery was performed under inhalation anaesthesia, and consisted of a dorsal horizontal incision in skin and muscle, followed by visualization and removal of the ovaries, along with the tips of the uterine horn. (This facilitated complete removal of ovarian tissue.) Sham surgery consisted of skin and muscle incision only. Results from this study showed that all three OVXd groups had significantly larger callosa than sham-operated female controls in adulthood (Fitch et al. 1991b; Fig. 2). Further, the three groups did not differ among themselves in callosal width or area. These results suggest that ovarian hormones are acting at and beyond Day 16, much later than for TP, which was effective in increasing callosal size when given to females on Day 4 but not on Day 8.

The above results have been replicated twice, for both handled and nonhandled females (Mack et al. 1993; 1992; Fig. 2). Thus, whereas ovariectomy altered callosal size regardless of the presence or absence of handling, TP effects on the female callosum were observed only in combination with handling. This distinction may relate to the later sensitive period for ovarian as compared to TP manipulations, but the mechanisms underlying this interaction will clearly require further study.

5.5.3. Effects of ovariectomy and estrogen replacement. Because OVX removes the primary source of both estrogen and progesterone, it was impossible to determine whether

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the former or the latter hormone (or both) influences callosal size in females based on the above results. The finding that TX treatment increased callosal size in female rats pointed to estrogen as the causal agent. To test this idea directly we did an experiment in which the ovaries were removed on Day 12 (as above), and females were provided with estrogen replacement via a low-dose silastic estradiol (E) implant inserted under the skin on Day 25 (Mack et al. 1993). A subgroup of OVXd females received blank controls. In adulthood, those OVXd females with the E implant had significantly smaller callosa than littermates receiving ovariectomy only. In fact they were smaller than intact female littermates as well (see Fig. 2). Our results agree with those of Pappas et al. (1979), who found that ethinylestradiol given on Days 40 to 90 decreased cortical thickness in OVXd female rats. These findings emphasize that the sensitive period for ovarian effects extends much later than that of testosterone, in this case as late as Day 25.

5.5.4. Developmental OVX effects. In a related study, handled females were given TP or oil on Day 4, and littermates from each condition (as well as male controls) were sacrificed at 30, 55, or 90 days of age (described above; Fitch et al. 1990b). In addition, other female littermates received sham or OVX surgery on Day 12 (as above), and were also sacrificed at 30, 55, or 90 days of age. The effects of TP on callosal size were significant by 30 days of age (paralleling early sex effects), whereas the effects of OVX were not evident until 90 days of age, well after puberty in female rats (Fitch et al. 1990b; Fig. 3).

5.5.5. What about the adrenals? To further support the assertion that ovarian hormones have direct effects on cortical differentiation, it was necessary to demonstrate that the removal of the ovaries did not lead to an increase in adrenal androgen output and hence indirect "masculinization." In fact, the opposite effect was found. Female rats were OVXd on Day 12 or received sham surgery (as above), and were sacrificed at 70 days of age by rapid decapitation (trunk blood samples obtained between 8:15 and 10:15 am; see Fitch et al. 1992 for further details). OVXd females were found to secrete half as much androstenedione, the primary adrenal androgen, as sham-operated female littermates. Moreover, OVXd females exposed to a novel environment prior to sacrifice did not show the stress-mediated rise in androstenedione observed for intact females.

5.5.6. Evidence that estrogen exerts organizing effects. A key question is whether anatomical changes in the callosum reflect permanent organizational steroid effects, or activational effects as a function of estrogen levels at the time of sacrifice. Three sets of data support the organizational hypothesis. First, the effects of Day 12 OVX were observed at 90 – but not 55 or 30 – days of age (Fig. 3). This argues against an activational mechanism because the ovaries are certainly active in intact females by Day 30. Second, we examined intact adult female rats for phase of estrus at the time of sacrifice, and then measured ovarian weight and uterine weight in addition to our standard measures of callosal size. Although a significant effect of estrus on uterine weight was observed, no relationship between callosal size and estrus was found (Mack et al. 1996a). Third, we OVXd a group of females at 78 days of age, well after puberty, and did sham surgery on female littermate controls. At 110 days of age we found no significant difference between the CC of the two groups ($F < 1.0$; Mack et al. 1996a). These three sets of findings strongly favor the hypothesis that ovarian hormones, primarily estrogen, exert permanent anatomical (organizational) effects on the callosum.

5.5.7 Summary. These combined findings establish that (1) the removal of ovarian hormones in early life leads to callosal enlargement; (2) these effects can be countered by the administration of estrogen; (3) the sensitive period for this phenomenon extends at least through Day 25 of life, considerably later than for testosterone effects; (4) these findings do not reflect secondary effects on adrenal androgen output; (5) ovarian effects on the callosum do not interact with handling in the same manner as androgenic manipulations; (6) developmentally, the expression of this effect begins considerably later than that of testosterone; and (7) these are permanent organizational effects, not transitory (activational) ones.

6. Discussion

The accumulated findings from multiple laboratories lead to the conclusion that ovarian hormones act during a sensitive period that extends at least through puberty, and perhaps beyond, to organize the brain of the female. Therefore, the actions of testicular and ovarian hormones contribute to the existence of anatomical differences that characterize the male and female brain. Many issues are raised by this conclusion, some of which are discussed below.

6.1. Parallel processes

The results reviewed here prompt the consideration of feminization as a process that occurs in parallel with masculinization. The two processes are qualitatively different and operate during different developmental periods. For the brain to become sexually differentiated, males need exposure to testicular androgens during the perinatal period (roughly from embryonic Day 17 through postnatal Days 8–10 in rodents), and females need exposure to ovarian secretions including, but not necessarily limited to, estrogen, during a later period that may extend to or even beyond puberty. (For a discussion of ovarian factors other than estrogen that may also be found to influence female development, see McCarthy 1995.)

Given the presence of two processes, one must consider the extent to which they interact in vivo, particularly in an experimental condition in which an intact female is treated with androgen. For example, testosterone exposure in infancy combined with handling stimulation is sufficient to enlarge the female callosum to the size of a male, yet the extent to which TP affects or redirects the feminization process that normally occurs in the female is unclear. Some findings suggest that the presence of the ovaries may modify the developmental actions of androgens (e.g., Blizard & Denef 1973). Alternatively, exposure to androgens may alter the activity of the ovaries themselves, greatly confounding the interpretation of endogenous processes (e.g., Barraclough 1961). Consequently, one must consider the implications of these findings for the common research practice of using intact females as "controls" in hormonal manipulation experiments. Although we origi-

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nally based our conclusions about the actions of androgen on the sexual differentiation of the callosum on comparisons between TP-treated intact and oil-treated intact females, it is possible that this comparison has limited validity. It is unclear to what extent endogenous ovarian hormones interact with exogenous hormonal manipulations. Certainly, in future research ovariectomized females should be used as a base-line control to assess developmental hormonal effects, just as gonadectomized males are the common control for hormonal manipulations in the male.

This leads to a consideration of the comparison between androgen-deprived males (prenatal flutamide followed by gonadectomy) and neonatally ovariectomized females. These two experimental conditions represent gonadally "ahormonal" states. If hormonal status were the only determinant of callosal size, they should be identical. However, this is not the case. Androgen-deprived males have callosa the size of intact females, whereas ovariectomized females have callosa the size of males. This implies that there are nonhormonal factors, possibly genetic ones, that are also involved in callosal development and sexual differentiation in general. In support of this position, anatomical sexual dimorphisms have been found in marsupial embryos before the onset of gonadal hormone production in either sex (Wai-Sum et al. 1988; also see discussion by Pilgrim & Hutchison 1994).

6.2. Cellular differences in the corpus callosum

It will be of considerable interest to determine the cellular dimensions of the gross anatomical effects reviewed here. We have examined the distribution of axon types in the genu of the rat and found major sex differences (Mack et al. 1995). Females had a higher proportion of unmyelinated axons than males in terms of number of axons and area taken up by axonal fibers. However, the total area occupied by neuronal material did not differ between the sexes, indicating that the sex difference is caused by the partitioning of axon types and not by differences in the amount of nonneuronal constitutents.

Juraska and Kopcik (1988) also reported sex differences in the ultrastructure of the splenial (bulbous anterior) portion of the rat's corpus callosum and showed that these were influenced by exposure to an enriched environment. Unmyelinated axons were found to outnumber myelinated axons in the splenium by a factor of 10:1, and females from both conditions had significantly more unmyelinated axons than males. Enrichment increased the number of myelinated axons in females, and increased the diameter of myelinated axons in males (see also Juraska 1991).

Consistent with the above findings on control animals, Kim and Juraska (1990) found that female rats had more unmyelinated axons in the splenium than males at 25, but not 15, days of age. Because a loss of unmyelinated axons occurs relatively early in development, although axons myelinate and increase in diameter later in life (Berbel & Innocenti 1988), it is possible that early androgen versus estrogen exposure differentially affects these two processes. It is also worth noting that ovarian hormones promote a female-typical loss in cortical dendritic spines in rats between Days 20 and 60 (Munoz-Cueto et al. 1990), suggesting that ovarian estrogen may exert inhibitory effects on callosal connectivity later in life, perhaps by influencing axonal withdrawal. In a recent study, Nunez et al. (1995) attempted to delineate early androgenic effects on CC ultrastructure in the rat, but obtained no significant results. This is probably because TP was given to nonhandled females and males were castrated on Day 1 – both manipulations we have previously shown to be without effect on rat CC anatomy (Denenberg et al. 1991b; Fitch et al. 1990a). A more effective procedure would be to use a prenatal testosterone blockade such as described in Fitch et al. (1991a). Although the hormonal mechanisms underlying observed sex differences in callosal ultrastructure are as yet unknown, it is important to emphasize that significant cellular differences do characterize the male and female callosum.

Cellular differences have also been examined in the human CC. Aboitiz (1992), for example, found regional differences in postmortem human callosal fiber composition, whereby thin fibers were most dense in the anterior callosum, decreased gradually to the posterior mid-body, and increased again in the posterior region. Large-diameter fibers showed a complementary pattern, with a peak density in the posterior mid-body, and decreasing density in the anterior and posterior poles. In the most posterior region, however, this pattern reversed, with a local increase in large-diameter fibers and a decrease in thin fibers. Based on these regional patterns, Aboitiz concluded that callosal regions that connect primary and secondary sensory and motor areas are characterized by more fast-conducting, large-diameter fibers, whereas callosal regions interconnecting "association" and prefrontal areas contained more small-diameter, slow-conducting, lightly-myelinated fibers. Aboitiz also reported that the vast majority of fibers in the human CC were small-diameter (or thin) fibers, and that callosal area measures were correlated with the total number of thin fibers. Aboitiz (1992) suggested that a larger callosa may reflect greater interhemispheric connectivity of association regions. Finally, he found no sex differences in callosal fiber composition or fiber patterns in the human CC.

6.3. The significance of human callosal sex differences

6.3.1. Are there sex differences? In the human literature there have been many reports of sex differences in cerebral lateralization or functional organization (e.g., Kimura 1987; Kimura & Harshman 1984; McGlone 1980; Shaywitz et al. 1995), but the primary finding of structural sexual dimorphism in human cerebral cortex has been for the corpus callosum. The initial paper by deLacoste-Utamsing and Holloway (1982), which stated that the splenium of the callosum is larger in women than men, stirred enormous public and scientific interest. One subsequent report on the human CC found sex differences in the shape and maximum width of the splenium favoring females, but no sex differences in overall size, or size of subdivisions (Allen et al. 1991). Another found more bulbous splenium in females than males in a post mortem sample, but no significant differences in maximum splenial width (Clarke et al. 1989). Many other reports, however, showed no sex differences in the human CC (e.g., Aboitiz 1992; Bell & Variend 1985; Bleier et al. 1986; Byne et al. 1986; Demeter et al. 1985; Kertesz et al. 1987; Nasrallah et al. 1986; Oppenheim et al. 1987; Parashos et al. 1995; Pozzilli et al. 1994).

Indeed, several reports have shown the CC to be larger,

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overall, in men (Clarke et al. 1989; Witelson 1989; 1991). A recent meta-analysis of 49 studies published between 1982 and 1994 found no evidence of a significant overall sex difference favoring females in total callosal size, or the size or shape of the splenium, whether or not an appropriate adjustment was made for brain size using analysis of covariance or linear regression (Bishop & Wahlsten 1997). Moreover, a small overall effect on CC size favoring males was observed. The authors concluded that "the widespread belief that women have a larger splenium than men and consequently think differently is untenable" (Bishop & Wahlsten 1996, p. 581).

At least two major reasons for the confusion characterizing the literature on sex differences in the human CC have been identified: (1) use of incorrect statistics in testing for sex differences, and (2) failure to account for age and handedness effects.

6.3.1.1 Pseudostatistics. Several reports have shown the CC to be larger in women than men, but in each case these measures were "corrected" by dividing the CC by a measure of overall brain size (e.g., Holloway 1990; Holloway et al. 1993; Johnson et al. 1994; Steinmetz et al. 1995). This raises the issue of absolute versus relative measurement. We addressed this issue in section 5.2 with respect to our rat research, and pointed out that it is necessary to have significant correlations between callosal size and some index of brain size within each group before one is justified in using relative values. A simple example can illustrate this principle. On average, there is no sex difference between men and women on IQ tests. However, female brains are smaller than male brains, and weigh less. One could obtain an estimate of brain size from cranial measurements or neuroimaging, divide this number into the person's IQ score, and obtain a score that measures "IQ per unit brain tissue." On such a measure females would be significantly superior to males. The reason we do not use such a statistic is that research has established that there is no within-group correlation between IQ and brain size.

Bishop and Wahlsten (1997) were able to find only four studies that reported separate correlations between brain weight and CC area for males and females. The average was .29 (significantly different from zero). However, other researchers have failed to find significant correlations (e.g., Clarke & Zaidel 1994; Kertesz et al. 1987; Parashos et al. 1995). As discussed earlier, we also failed to find any correlation between brain weight and CC size in our rat studies.

Even if one finds a significant correlation in a particular sample, the practice of dividing one number by another is not appropriate unless the correlation is high. The correct statistical procedure is to use a regression analysis or analysis of covariance to remove the linear effects of the second variable. Bishop and Wahlsten (1997) found seven studies that used analysis of covariance to remove the effects of brain or cortex size. In six of these no sex difference was found in CC size. In one study (Holloway 1990) females were found to have a significantly larger CC. However, in three other studies Holloway found no significant sex difference in CC size when brain weight was used as a covariate (Holloway et al. 1993).

Even though one can rationalize the procedure of adjusting CC size for brain size if there is a significant within-group correlation, there is a compelling biological argument for assessing only the absolute measure: insofar as the size of the callosum is related to its cellular constituents, the absolute number of axons and whether they are myelinated or unmyelinated is of critical importance in determining functional activity (see sect. 6.2 above). In this sense there is no logical need to correct for what the rest of the brain is doing.

In conclusion, the procedure of dividing brain size into CC area as a "correction factor" is incorrect, and, because the female brain is typically smaller, can lead to false results suggesting a larger "relative" CC in females than in males.

6.3.1.2. Age effects. Another source of confusion involves failure to control for the age of subjects. Cowell et al. (1992), for example, reanalyzed the Allen et al. (1991) data and examined how the size of seven regional CC widths varied as a function of age. Trend analyses showed higher order Sex x Age interactions in 3 of the regions, with males reaching a peak callosal size in their 20s, whereas females did not attain their maximum widths until age 41–50. Cowell et al. addressed the issue of failure to find replicable sex differences and stated that "the practice of pooling over age in adults has been responsible, in part, for inconsistent [sex difference] reports" (p. 191).

6.3.1.3. Handedness. The critical nature of handedness is nicely illustrated by Witelson's (1989; 1991) study of the isthmus, the narrow area just before the splenium. Her studies show that sex effects on the human CC are complicated when consistency of hand usage is taken into consideration. Witelson determined hand consistency by administering behavioral tests for such acts as writing, drawing, toothbrushing, throwing a ball, and using scissors. Subjects were scored as *right*, *either*, or *left* for each item. Consistent right-handers (CRH) were those subjects who used their right hand for all activities or had only right and either scores. The remainder, called nonconsistent right handers (NCRH), used their left hand for at least one activity, including writing. Witelson found the isthmus portion of the callosum to be significantly larger in NCRH men. This finding has recently been confirmed by Denenberg et al. (1991c) who used right-handed writing subjects and obtained the same results as Witelson. In a later study Cowell et al. (1993) examined consistent and nonconsistent left handers and contrasted them with consistent and nonconsistent right-handers (using the writing hand to define handedness group). Among the left-handers, those who were consistent in hand usage had the largest isthmus area regardless of sex; they ranked just below the nonconsistent right-handed males. In a related study, Habib et al. (1991) used both right- and left-handed subjects, and found the anterior body of the CC to be larger in NCRH males, compared to CRH males, with no difference between the two female groups.

6.3.2. Yes, there are sex differences. The human data indicate that callosal sex differences are influenced by several variables including age, handedness, callosal region, and probably other measures as yet unknown. Although many human CC studies have examined one or two of these variables simultaneously, few have conducted a careful analysis of specific callosal regions and accounted for critical variables such as sex, age, and hand-preference (using a more sensitive measure than simply "writing hand"). Yet

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this fine-grained analysis is apparently required to observe consistent group difference in the anatomy of the human CC.

Such a conclusion is not entirely at odds with the animal literature: evidence has shown interactions between sex and environmental exposure when callosal ultrastructure is examined in the rat (e.g., Juraska 1991), as well as interactive age and hormonal effects on callosal size (e.g., TP effects are seen early and OVX effects are seen later; see Fig. 3), and finally, interactions between sex, handling experience, and hormonal manipulations on the mean width of specific callosal regions (data not presented here, but see Berrebi et al. 1988; Fitch et al. 1990a). We did not present our regional callosal data here because, in the rat model, both sex and hormone manipulations exerted such pervasive effects that anterior and posterior callosal regions and overall callosal area were all affected. Because overall callosal area effectively characterizes sex and hormonal effects on the rat CC, we used this simplification to document the role of ovarian hormones on brain development. Nevertheless, it should be recognized that many other variables do exert significant influence on regional callosal anatomy in animal models. As both human and animal research progresses in teasing apart the relative importance of various developmental and structural factors on CC measurement, it is highly likely that more parallels between human and animal data will become evident.

7. Conclusions

In summary, according to the traditional model of sexual differentiation, mammalian sexual differentiation is primarily mediated by androgens of testicular origin and the presence of these androgens in early life produces a "male" brain as defined by neuroanatomy and behavior. In contrast, the female brain has been assumed to develop via a hormonal default mechanism, in the absence of androgen or other hormones. In the first part of this target article we reviewed literature supporting an active role for ovarian hormones in sexual differentiation. We then presented data demonstrating significant effects of ovarian hormones on a sexually dimorphic cortical structure, the corpus callosum, that is larger in male than female rats.

In the female rat, removal of the ovaries as late as Day 16 increased the area of the corpus callosum in adulthood. However, treatment with low-dose estradiol starting on Day 25 prevented this increase. Callosal size was also increased by a combination of handling female rats in infancy and administering testosterone prior to Day 8. The sensitive period for TP effects on CC size starts around prenatal Day 17 and is over by postnatal Day 8. In contrast, the sensitive period for estrogen action extends at least through postnatal Day 25. Further, the effects of androgen treatment were expressed early in development, with males and testosterone-treated females having larger callosa as early as Day 30, whereas the effects of ovariectomy did not appear until after Day 55.

These data support the view that ovarian hormones play an important role in the development of the female brain and that the temporal parameters and mechanisms of "ovarian feminization" are markedly different from those of androgenic masculinization. Such findings speak to the need to complement our current model of androgen-mediated sexual differentiation of the brain with what is now known about the parallel role of the ovaries.

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To normalize or not to normalize for overall size?

Francisco Aboitiz

Programa de Morfología, Instituto de Ciencias Biomédicas, University of Chile, Correo 7, Santiago, Chile. **faboitiz@machi.med.uchile.cl**

Abstract: I discuss Fitch & Denenberg's argument that no correction for brain size is needed when assessing callosal size. Morphometric criteria may not be sufficient to determine whether corrections are needed. Functional studies of callosal transfer will ultimately specify whether corrections for size are necessary in each case.

In their target article, Fitch & Denenberg (F&D) propose a provocative hypothesis on the role of ovarian hormones in the differentiation of the female brain; they provide convincing evidence from their own work. Nevertheless, I must take issue with the discussion of whether it is necessary to correct callosal size with respect to brain size. In a previous work (Aboitiz 1991) I proposed that corrections for overall size (e.g., brain size) are only meaningful if a significant correlation exists between the structure under observation and the overall size. This criterion was applied in subsequent publications (Aboitiz et al. 1992a; 1992c). If the variable is independent of overall size, the "correction" may end up being a source of statistically fallacious results, especially if the sample is small and there is a relatively high dispersion of data. (In a large sample, because there is no correlation between the variables, "correcting" for size should have no overall effect and is therefore trivial.)

Nevertheless, I believe that in certain cases it may be worthwhile to determine the ratio of callosal connections and brain size even if they are independent. Imagine a town that is divided in two by a river and there is only one bridge to cross the river. If the town keeps growing on the two sides of the river but no new bridges are built, there eventually will be a traffic problem between the two sides of the town. Although the bridge and the size of the town are not correlated, the function of the bridge depends on the size of the town. Going back to the corpus callosum, if within a species (within each sex) there is little or no correlation between brain size and callosal size (e.g., Jäncke et al. 1997), is this because interhemispheric transfer can still do fairly well with a relatively smaller callosum in larger brains? Or is it that no compensating mechanism increases callosal size concomitantly with brain weight? If the correct answer is the second, larger brains may have a constraint for interhemispheric transfer (recall the analogy above) and it hence makes sense to normalize callosal area by overall size.

On the other hand, if callosal area increases concomitantly with brain size (as may happen across species), this need not imply a functional relation. If a small number of fibers suffices for most interhemispheric transfer tasks and the relation between callosal and brain sizes is only developmental (the callosum may grow just because the rest of the brain increases in size, without implying a

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difference in callosal transfer; see Aboitiz 1996), it is possible that in a large brain, the excess of commissural fibers and cells implies redundancy of interhemispheric processes rather than increased transfer capacity.

In my view, the question of whether corrections for overall size are needed is largely empirical and depends ultimately on whether brain size has an effect not only on the size of the callosal channels but also on interhemispheric transmission. For that, we need to know whether a larger callosal size implies more fibers crossing through (Aboitiz et al. 1992b), but also whether differences in fiber numbers actually have functional relevance in brains of the same size and in brains of different sizes. To address this question, which I feel is fundamental for studies of callosal function, combined behavioral and imaging studies of interhemispheric transfer tasks and callosal and brain size are needed in humans. In some cases, an absolute difference in fiber numbers may have a functional significance regardless of differences in brain size. For example, several studies found a negative correlation between different measures of brain laterality and the absolute size (and number of fibers) of the callosal isthmus independent of overall brain size (especially in males; Aboitiz et al. 1992a; 1992c; Clarke & Zaidel 1994; Witelson & Goldsmith 1991). This would support F&D's contention that the relevant parameter to measure interhemispheric transfer is the absolute number and type of axons, but again it is not clear that this will be the case for all interhemispheric processes.

In this context, perhaps the studies of hormonal effects on callosal size should search for functional correlates of morphological differences. We need to determine which callosal functions actually depend on hormonal exposures (at least in primates, sensorimotor transfer and higher level or cognitive transfer seem to use different callosal channels, as determined by their respective fiber diameters; Aboitiz et al. 1992b), which would also address the issue of whether corrections for overall size are indeed required.

Sexual differentiation of callosal size: Hormonal mechanisms and the choice of an animal model

M. J. Baum[a,c] and S. A. Tobet[b,c]

[a]*Department of Biology, Boston University, Boston, MA 02215;* [b]*Department of Biomedical Sciences, The Shriver Center, Waltham, MA 02254;* [c]*Program in Neuroscience, Harvard Medical School, Boston, MA 02115.*
baum@bio.bu.edu; stobet@shriver.org

Abstract: Studies of callosal sexual differentiation have concentrated on global measures of callosal size, using the rat as a model for studies of potential hormonal mechanisms. It is time to shift the study of callosal sexual differentiation to a more cellular level. Finally, there are potential problems with using the female rat as the primary model for understanding hormonal mechanisms during postnatal life.

The primary thrust of the Fitch & Denenberg (F&D) target article concerns the possible contribution of perinatal ovarian hormone exposure (acting in female mammals) to the development of a sex dimorphism in corpus callosum (CC) size. Research conducted by F&D and their co-workers suggests that in rats the size of the CC is greater in adult males than in females. Their studies suggest that this adult sex dimorphism in callosal size reflects two types of perinatal hormone action: (1) an effect of testosterone, acting via androgen receptors at unspecified sites (neurons or glia?) in the fetal male brain to augment callosal size and (2) an effect of estradiol, acting at unspecified sites (neurons or glia?) in the neonatal (P7 or older) female brain to inhibit callosal size. Thus, F&D propose that testicular androgens somehow act prenatally in the male to augment callosal size. The resultant sex dimorphism in callosal size is then attenuated by the postnatal inhibitory action of ovarian hormones (not necessarily estradiol) in females. In contrast to most other widely studied examples of sex differences in neural structure, the cellular basis for the observed difference in CC size (reflected perhaps in the number or diameter of myelinated axons contained in this fiber bundle) is not hinted at by the studies published to date. Thus, we do not know for any perinatal age which neurons respond in either sex to androgen and estrogen so as to generate the reported sex difference in callosal size. Likewise, we do not know of the mechanism(s) whereby androgen and estrogen exert their apparently different actions on those neurons whose axons comprise the CC. Future studies should shift attention from the size of the CC to the cellular loci (neuronal or glial) of hormone action during the developmental periods when hormones are acting through "organizational mechanisms." Those studies will need to distinguish between an organizational action of androgens, whose net effect is to augment CC volume, and the actions of estradiol, which may be either to inhibit the growth and/or survival of CC neurons directly or (see below) to inhibit the stimulatory actions of earlier androgen exposure on CC development in females.

It is noteworthy that essentially all of the experimental literature on steroidal control of CC sexual differentiation has used the rat model. It is worth emphasizing that the female rat, in particular, may not be typical of mammalian species in so far as the female is normally exposed to a surprisingly high amount of testosterone for all but a couple of days during embryonic development: male and female rat fetuses have equivalent circulating levels of testosterone over the last week of gestation (Baum et al. 1991; Weiz & Ward 1980), except for two days (E18 and E19). The available evidence points to the placenta as the major source of testosterone in rat fetuses of both sexes, with the testes making an extra contribution in males on E18–E19. This contrasts with the situation in several other commonly studied mammalian species (reviewed in Baum et al. 1990; 1991) in which males consistently have higher circulating testosterone levels than females over the entire embryonic period. Thus, in the female rat, which is normally exposed to considerable testosterone prenatally, neural structures may normally respond to this steroid in ways that approximate the responses shown by fetal males to this steroid. There are several systems in which exposure to specific hormonal signals at one point in time primes neural elements to respond in particular ways at later points in time. In adulthood, this is best exemplified by the classic interaction of estrogen followed by progesterone at both behavioral (for receptive and proceptive behaviors) and molecular (e.g., progesterone receptor induction) levels.

In other systems, including development, early exposure to androgen may prime estrogen responsiveness (e.g., via upregulation of aromatase) or estrogen may prime androgen responsiveness (e.g., via receptor induction). As a result, the proposed inhibitory actions of ovarian estrogen, acting neonatally in the female rat, could serve to attenuate the masculinizing events set in motion by the unusually high level of androgen exposure that females of this species typically sustain. Fetal females of other species (e.g., ferrets and primates, including man) are not normally exposed to such comparatively high levels of androgenic stimulation. As a result, any inhibitory actions of postnatal ovarian estrogen on CC development may be less obvious, or even negligible, in species other than the rat. Given the additional review of the controversy surrounding studies of the CC in humans, it will be important for experimental studies to be extended into nonrodent species to allow the testing of specific hypotheses about the mechanism of hormone action in establishing sex differences in the formation of this fiber bundle.

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Sex-related differences in callosal morphology and specific callosal connectivity: How far can we go?

Stephanie Clarke
Institut de Physiologie, 1005 Lausanne, Switzerland. **sclarke@ulys.unil.ch**

Abstract: The precise relationship between callosal morphology and specific connectivity is not yet known. Callosal axons are often presumed to be arranged according to their origin. In humans, this is true for the genu and the splenium, which convey axons from the prefrontal and occipital cortices, respectively, but not for the body, where axons from wide parts of the cortex are intermingled.

Fitch & Denenberg (F&D) argue that ovarian hormones play an active role in shaping the female brain. In particular, the size and cellular composition of the corpus callosum may be influenced by their presence during the first postnatal months. Ovarian hormones appear to prevent male-like enlargement of the corpus callosum and to favor higher proportions of unmyelinated axons in the adult female rat. Massive axonal elimination occurs during the normal development of the corpus callosum and precedes myelination (Berbel & Innocenti 1988). This affects both homotopic and heterotopic callosal connections (Innocenti and Clarke 1984a; 1984b). The sex-related differences may result from differential axonal elimination and/or differential myelination. The human corpus callosum seems to follow a similar developmental pattern and putative sex-related differences appear after the presumed period of axonal elimination (Clarke et al. 1989).

In their discussion, F&D raise the issue of the significance of human callosal sex differences. As they rightly point out, the sexual dimorphism of the human corpus callosum is still controversial, possibly because of methodological difficulties. Interindividual comparison of callosal morphology is reputably difficult even if obvious pitfalls are avoided (for discussion see, e.g., Clarke et al. 1989). Furthermore, identifying corresponding regions in different corpora callosa often remains difficult. Putative sex-related differences in the human corpus callosum may reflect differences in callosal connectivity, in terms of either connection density, rapidity and efficiency of transmission, or functional specialization. The interpretation of differences in corpus callosum morphology is often based on the assumption that, according to their origin, a rather precise topographic arrangement of axons within the corpus callosum exists and that callosal connections are essentially homotopic. Data on human callosal connectivity show that these assumptions may be false and that both aspects of interhemispheric connectivity may be more complex than presumed.

A roughly ordered arrangement of axons in the corpus callosum, according to their origin, appears to be present in the genu and the splenium, but not in the body. Axons from the occipital cortex cross in the splenium, mostly in its lower part (Clarke & Miklossy 1990; Dejerine & Dejerine-Klumpke 1895; Van Valkenburg 1908); those from the prefrontal cortex cross in the genu (Beck et al. 1951). Several attempts have been made to trace callosal pathways from the temporal lobe (Van Buren & Yakovlev 1959; Zingerle 1912), but the callosal bundles could not be traced to the midsagittal plane, probably owing to their "dilution" with other, nonaffected fiber bundles (for discussion see Clarke et al. 1995). Axons from the posterior parietal and posterior temporal cortex have been reported to cross in the splenium and possibly also in the posterior part of the body of the corpus callosum. In addition, axons from the lower frontal and anterior parietal convexity cross in the genu, and axons from the upper frontal and anterior parietal convexity in the anterior two thirds of the body (de Lacoste et al. 1985).

It is often assumed that human callosal connections exist predominantly between homotopic regions of the cortex. Recent evidence from human tracing studies suggests that heterotopic interhemispheric connections are numerous and widespread. They were demonstrated at the level of cortical areas (between visual areas: Clarke 1994; Clarke & Miklossy 1990; from inferotemporal cortex to Wernicke's and Broca's areas: Di Virgilio & Clarke 1996; 1997) and at the level of lobes (calcarine region to posterior parts of the temporal and parietal cortex: Clarke et al. 1995). The pathway taken by heterotopic callosal axons has not been investigated. Therefore it cannot be excluded that these axons travel in very different parts of the corpus callosum compared to the homotopic axons, which originate or terminate within the same region.

More evidence for the role of estrogens in female differentiation of the brain

Klaus D. Döhler
Haemopep Pharma GmbH, D-30625 Hannover, Germany.

Abstract: Evidence accumulates that pre- and postnatally circulating estrogens play an active role in the differentiation of the female brain: the susceptible period for feminization of the brain seems to extend far beyond the period during which masculinization of the brain occurs. Thus, there is a need to reevaluate the widely accepted "concept of basic femaleness" in sexual brain differentiation.

Today's prevailing hypothesis is that female sexual differentiation proceeds in the absence of gonadal hormones. This "concept of basic femaleness" is based on the misconception that gonadectomy of mammalian fetuses or neonates would clear their blood circulation of estrogens. The fact is that fetal estrogens are provided by the placenta irrespective of the presence or absence of the fetal ovaries. In animals with short gestation periods – such as the rat, mouse, and hamster – the intrauterine estrogenic milieu is carried over into the postnatal period by high levels of estrogen-binding alpha-fetoproteins (AFP) that protect circulating estrogens from metabolic clearance during the first week of postnatal life when the ovaries are still inactive (for review see Döhler 1991, pp. 23–26).

Experimental studies on hormonal influences on female differentiation of the brain have been hampered methodologically by the fact that ovarian and placental estrogens are not effectively removed from the blood circulation of fetal mammals and postnatal rats and mice. In a series of studies our group adopted the approach of inactivating the endogenously circulating estrogens by treating female rats pre- and/or postnatally with the estrogen antagonists tamoxifen or LY 117018 (for review see Döhler 1991, pp. 14–18 and 26–33). Both estrogen antagonists inhibit the biological effects of estrogens by competing for intracellular estrogen receptor binding sites.

Postnatal treatment with tamoxifen or with LY 117018 permanently inhibited the differentiation of a positive feedback mechanism for the estrogen-stimulated release of luteinizing hormone (LH); it also inhibited differentiation of the capacity to show female sexual receptivity. Our interpretation of our results was that postnatally circulating estrogens may play an active role in the differentiation of the female brain. Our interpretation opposed the prevailing concept of basic femaleness according to which female sexual differentiation proceeds in the absence of hormones and male sexual differentiation of the brain proceeds under the influence of estrogens aromatized from testicular androgens.

Critics of our interpretation suggested that the estrogen antagonists may have expressed partial estrogenic activity in the developing female brain, thereby masculinizing the treated animals. In close collaboration with Roger Gorski's group in Los Angeles (for review see Döhler 1991, pp. 14–18) we refuted this criticism by demonstrating that the development and differentiation of the sexually dimorphic nucleus of the preoptic area (SDN-POA) is stimulated by perinatal treatment with an estrogen or an aromatizable androgen, but it is inhibited by similar treatment with tamoxifen. Tamoxifen was shown to inhibit SDN-POA differentiation in male as well as female rats.

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To further support the assumption that tamoxifen expressed anti-estrogenic but not estrogenic activity in the developing rat brain we demonstrated that the disintegration of the capacity for female lordosis behavior and the induction of anovulatory sterility by daily postnatal treatment of female rats with low doses of tamoxifen was attenuated by concomitant treatment with low doses of 17β-estradiol (Döhler et al. 1984, p. 107). We further demonstrated that anovulatory sterility, induced by postnatal treatment of female rats with daily doses of 10 μg testosterone, was attenuated by concomitant treatment with daily 0.5 μg of 17β-estradiol (Döhler & Hancke 1979, p. 245).

The proposition that female sexual differentiation of the brain may need estrogenic stimulation is meanwhile supported by results from several other laboratories during the past two decades (for review see Döhler 1991, pp. 23–33). Despite its obviousness, this proposition is still not appreciated. Fitch & Denenberg's (F&D's) observation in the target article (sects. 5.5.1 through 5.5.4) that postnatal interference with ovarian hormones causes permanent enlargement of the corpus callosum – representing male-type callosal differentiation – confirms that ovarian or estrogenic hormones are needed for brain differentiation in the female. The fact that postnatal gonadectomy of male rats did not influence differentiation of callosal size (sect. 5.4.3) seems to indicate that in the case of the corpus callosum the male seems to be the "neutral sex" whereas female differentiation is actively induced by estrogens postnatally.

F&D's data (sect. 5.5.4) also indicate that the postnatal period of susceptibility for female differentiation of the corpus callosum extends far beyond the period during which masculinization of the brain occurs. These observations may provide a neuroanatomical explanation of the biological purpose of the highly elevated estradiol levels in female rats during the second and third weeks of life (Döhler & Wuttke 1975, p. 904). We had observed (Döhler & Wuttke 1975, p. 900) that the positive feedback mechanism on cyclic LH release attained a certain degree of maturity during this postnatal period of elevated estradiol levels. The available data now suggest that female sexual differentiation of the brain may not only require perinatal estrogenic stimulation for its full expression; it may further need this continuous estrogenic stimulation throughout a longer susceptible postnatal period than is known to be necessary for male sexual brain differentiation. The available data also suggest that the various components of male versus female differences in brain structures and functions may become expressed during different developmental periods and in response to different hormonal influences. The various components of male brain differentiation in particular seem to be established under the influence of androgens, estrogens, or no gonadal hormones at all – not to mention the influence of various neurotransmitters (for review see Döhler 1991, pp. 33 ff).

Recipe for a sexually dimorphic brain: Ingredients include ovarian and testicular hormones

Diane F. Halpern

Department of Psychology, California State University, San Bernardino, San Bernardino, CA 92407. **dhalpern@wiley.csusb.edu**

Abstract: New knowledge about the sexual differentiation of the brain profoundly changes our understanding of basic topics in brain development such as the false dichotomy between long-lasting and transient effects of hormones on neural activity, the importance of ovarian hormones in brain development, the plasticity of neural structures throughout the life span, and the way measurement issues affect research conclusions.

Rewrite the textbooks. Fitch & Denenberg (F&D) present a compelling case for the role of ovarian hormones in the sexual differentiation of the brain. According to earlier accounts, the female brain was the "default" template or prototype that developed in the absence of testicular hormones – an irony because it is estradiol (an estrogen converted from testosterone) that (predominantly) masculinizes developing brains. Thanks to the careful experimental sleuthing of F&D and others, there is now abundant evidence that both ovarian and testicular hormones are critical determinants of whether a developing brain will proceed in a male or female direction. The presence, absence, and proportional mix of these hormones affects the neuroanatomy of the brain, sexual behavior of the organism, and the frequency and degree of sex-typical skills and activities that develop later in life.

We infer the maleness or femaleness of brains with several qualitatively different indicators such as the size and shape of selected cerebral structures (e.g., size of the sexually dimorphic nucleus of the preoptic area), abilities (rate of learning a passive or active avoidance task), sexual behaviors (frequency of lordosis), patterns of skilled performance (spatial learning), and activity levels (rough and tumble play). Given the variety in these dependent measures, it is not surprising to find that testicular and ovarian hormones play different developmental roles that depend on the criterion being investigated. Gonadal hormones can act independently; they interact with each other and with environmental conditions such as handling, and the results depend on when in the developmental sequence they are introduced, withdrawn, or blocked. Thus, there are multiple answers to the deceptively simple question of what makes a brain sexually dimorphic; it would be naive to expect that the multiple mysteries of the brain could be solved with univariate answers.

The popular distinction between organizational effects (hormone effects that occur relatively early in development causing morphologic alterations and permanent changes in responsivity) and activational effects (hormone effects that are more transient and depend on the concentration of a gonadal hormone at the time of action) no longer reflects current understanding. As F&D argue convincingly, the dichotomy of life-long and transient effects needs to be replaced by a more continuous view of hormone actions. We now know that brains are far more plastic throughout life than earlier researchers had believed, a fact that has created renewed interest in the way experience and other environmental conditions can alter brain structures even into very old age. Thus, new knowledge of the psychobiology of the brain has brought the environment inside the skull, a change that may finally erase the false distinction between nature and nurture (Halpern 1996; in press).

Of rats and men and women. For obvious ethical reasons, experimental manipulations of hormones that are expected to alter the brain are conducted with nonhuman mammals (mostly rodents). Researchers assume that the effects in humans will be similar to those found in other mammals, but hormonal effects are not expected to be identical across species. Conclusions based on research with rodents are corroborated with data from humans – naturally occurring abnormalities such as excessive androgen exposure during the prenatal development of girls with congenital adrenal hyperplasia and genetic males with androgen insensitivity, data from individuals who undergo hormone therapies such as the administration of testosterone to elderly men, and tests of normal women in different phases of their menstrual cycles (studies reviewed in Halpern, submitted). In general, these data are in accord with predictions based on the experimental literature with rodents. F&D present an impressive body of evidence that there are sex differences in the size in the corpus callosa in rats and that gonadal hormones are implicated in these differences. The parallel conclusion about humans is less certain. The problems F&D raise in their review have broad implications for many fields of research because their reasoning transcends the question of callosal size.

The power of null results. *BBS* readers may be surprised to learn that the debate over sex differences in the human corpus callosum is more often acrimonious than scholarly. For those engaged in the politics of biology, this debate epitomizes the

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emotionally charged nature of questions about sex differences; for dispassionate researchers, the debate hinges on the question of how the corpus callosum (and, by extension, other brain structures) should be measured. What is the most meaningful way to quantify an irregularly shaped brain structure? Volume? Perimeter? Width at a particular juncture? Not surprisingly, different measures yield different answers to the question of sex differences in the largest fiber tract in the brain. The conclusion that females have larger callosa implies better connectivity between the two cerebral hemispheres, on average, for females (Innocenti 1994); thus, critical issues in politics and measurement come into play in the debate about this mass of neural fibers.

In addition, there are many reports of null results in the literature on differences in the corpus callosum; this is to be expected given that different researchers are using idiosyncratic measures of this complex structure. Null results assume a significance of their own when one end of the political spectrum is invested in the conclusion that there are no differences. There are many ways to obtain statistical nonsignificance, including sloppy research, low statistical power, and the very real possibility that there are no differences. In my own recent review of the literature, I concluded that there are sex differences in the human corpus callosum, with females having a more bulbous structure (Halpern, in press). F&D reach the same conclusion somewhat hesitantly, noting that age and laterality interactions make the main effect of sex difficult to interpret. I note here that left-sided lateralization is more prevalent in males, a fact that further confounds the relationship among brain structures, sex, and laterality.

F&D object to using a proportional measure to assess callosal size. It is at this point that we disagree. There is nothing in the mathematics that makes the use of proportional measures invalid. F&D use an analogy in which proportional measures of two individuals with the same IQs and different brain sizes would lead researchers to conclude that the individual with the smaller brain had more "brain power" per brain unit. Whether proportional measures are a meaningful or useful metric depends on the question being asked. Such a measure would rule out the notion that bigger brains are better brains, so if that were the research question, the results would be valuable. Similarly, imagine two individuals with identical callosa and large differences in the overall size of their brains. Consider an extreme example where the brain of one individual is one half corpus callosum and the other's brain is $\frac{1}{100}$ corpus callosum. The effects of these differences in relative size would be a reasonable area of inquiry. I am reminded of a lesson that I learned many years ago: one person's error variance is another person's main effect. In other words, the meaningfulness of a measure depends on the question being asked.

Advances in our understanding of brain development. We need to change how we think about the organ with which we think. Important new information includes the understanding that (1) ovarian hormones are as critical in the development of sexually dimorphic brains as testicular hormones; (2) the effects of gonadal hormones can be independent, interactive, or interactive with environmental stimulation; (3) the brain remains plastic throughout life, so the distinction between organizational and activational effects needs to be replaced with a more continuous life-long model of hormonal influence; (4) the way we answer basic questions in science often depends on fundamental issues of measurement; (5) we need the control of true experimental designs to infer causal relations between hormonal environments and the development of brain structures and functions; and (6) because our understanding of what may be the most complex organ on earth – the brain – is still in its own perinatal period, we can expect many more revisions as we coax the brain to reveal its secrets.

Is the size of the human corpus callosum influenced by sex hormones?

Elizabeth Hampson

Department of Psychology, University of Western Ontario, London, ON, Canada N6A 5C2. **ehampson@uwovax.uwo.ca**

Abstract: Fitch & Denenberg have shown that manipulations of ovarian and testicular hormones early in development can influence the adult size of the corpus callosum in the rat. The human corpus callosum is highly variable in size and shape, but data are only now beginning to emerge on whether sex steroids influence callosal differentiation in humans. I describe recent data from our own laboratory and suggest avenues for future research.

In an elegant series of studies, Fitch & Denenberg (F&D) have established that (1) the cross-sectional area of the corpus callosum is larger in the male rat than in the female rat and that (2) this sexual dimorphism is influenced by the presence of sex hormones during development. A remarkable aspect of their findings is that ovarian estrogen appears to have independent feminizing effects on callosal size. This is one of the most important pieces of evidence to date that feminization of certain brain regions is an active process that is regulated by specific hormones.

The question I will consider in this commentary is whether there are supporting data for these findings from human studies. The past 15 years have witnessed an explosion of research on sexual dimorphism in the human corpus callosum (CC). F&D cite a meta-analysis by Bishop and Wahlsten (1997) as confirming a small but significant sex difference in total callosal area in the human brain. On average, absolute callosal area is larger in adult males than in adult females. Although not cited in the target article, another recent meta-analysis of the callosal literature by Driesen and Raz (1995) reached the same conclusion. The sex difference in absolute CC area is consistent with findings in the rat. On the other hand, the meta-analysis by Driesen and Raz (1995) also found that the human CC is *proportionately* larger in the female than the male brain when investigators normalize for brain size. This would not be predicted if there is simply an allometric relation between CC area and total brain size. There is no intrinsic bias in ratio adjustments that would consistently lead to a larger female ratio. Instead, it implies a real difference in connectivity in the male and female brain, one that would almost certainly imply associated functional differences.

However exciting this possibility might be, a conservative interpretation is that at present, only the sex difference in *absolute* size of the CC has been conclusively demonstrated in humans. It is this parameter that F&D found to be influenced by early life hormone manipulations in the rat. In the rat, CC sensitivity to testosterone (T) begins prenatally and extends into the early postnatal days, with T exposure at this time resulting in a larger adult CC, independent of total brain weight. Does prenatal or early infant T have a corresponding effect in humans? Possibly. In a recent study, Scott Moffat (a graduate student in my laboratory) and I investigated the correlations between midsagittal area of the corpus callosum and individual differences in free T concentrations as measured in saliva (Moffat et al., in press). Our sample was a group of 68 healthy adult males in their mid-20s who consented to undergo magnetic resonance imaging (MRI) at University Hospital. Saliva sampling was done one week prior to scanning and was controlled for diurnal variation.

Although T did not correlate significantly with the total area of the CC, it was significantly and positively correlated with the cross-sectional area of the posterior half of the CC and especially with measurements taken from the fourth and fifth sixths of the callosum, which includes the region of the isthmus. To our knowledge, this is the first study to report a direct relationship between hormone concentrations and any feature of human neuroanatomy. Entering total brain volume as a covariate did not alter the pattern of correlations seen. Alternative corrections using total brain volume taken to the two-thirds power similarly did not alter the findings. These results are interesting in two respects: the

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correlations between T and callosal size were positive, as might be expected on the basis of F&D's studies. Second, the correlations clustered in the part of the callosum for which there is the most consistent evidence of regional sexual dimorphism in the human brain, the isthmal area. An obvious difficulty, however, in relating our study to that of F&D is that we had to measure adult T levels rather than T during early stages of development. If the prenatal or infant periods are the stages when T exerts its effects, a preferable experimental design would be to measure T at those ages and examine how the values obtained correlate with callosal measures taken at maturity in the same individuals. Practical considerations make such longitudinal studies not feasible in human beings.

The most crucial test of the generalizability of F&D's finding to humans will be the estrogen data. In theory, girls who have a developmental deficiency in estrogen might as adults have a larger CC area than control girls, if F&D's findings apply to callosal differentiation in humans. Experimental manipulation of estrogen levels is not ethically possible, but a model that could be examined is girls with Turner syndrome. In these girls, the ovaries are typically atretic by the time of birth so that estrogen production is deficient. Two recent volumetric imaging studies utilized MRI in females with Turner syndrome, but unfortunately callosal measures were not reported (Murphy et al. 1993; Reiss et al. 1995). Finding a larger CC in females with Turner syndrome than in hormonally normal control girls would provide crucial support for the generalizability of Fitch & Denenberg's findings, and would raise for the first time the possibility of an active feminization process under hormonal control in human beings.

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Activation/organization, masculinization/feminization: What are they and how are they distinguished?

Melissa Hines

Department of Psychology, City University, London, England EC1V 0HB.
m.hines@city.ac.uk

Abstract: The activational and organizational hormone effects as originally defined do not conflict with activational influences on brain structure. Ovarian hormonal influences on the rodent corpus callosum could be activational rather than organizational. The masculinization/feminization distinction in brain structure and the timing of sex differences in visuospatial abilities need to be clarified.

Theoretical models of sexual differentiation have derived largely from studies of reproductive behaviors regulated by hypothalamic structures. These models may be inadequate for understanding the sexual differentiation of other characteristics (Collaer & Hines 1995). The Fitch & Denenberg (F&D) target article describes influences of ovarian estrogen on brain and behavior and suggests that ovarian hormones exert organizational influences on sex differences in the rodent corpus callosum (CC). [See also McGlone: "Sex Differences in Human Brain Asymmetry" *BBS* 2 1980; Benbow: "Sex Differences in Mathematical Reasoning Ability in Intellectually Talented Preadolescents" *BBS* 11(2) 1988; Geary: "Sexual Selection and Sex Differences in Mathematical Abilities" *BBS* 19(2) 1996.]

If the CC data reflect organizational influences, they suggest two important conclusions. First, the ovaries may play a primary role in the sexual differentiation of this cortical structure. This would contrast with sexual differentiation of subcortical structures and their associated functions, which depend largely on testicular hormones. The second difference from sexual differentiation in subcortical regions is in timing. Influences on the CC occur later and, if organizational, their critical period is longer. Different sexually differentiated traits have different critical periods depending on when they develop. Cortical development occurs later and lasts longer than hypothalamic development, consistent with the proposed later, longer, critical period.

The target article provokes several questions. One concerns distinguishing activational hormonal effects from organizational ones. As originally proposed (Phoenix et al. 1959), activational effects are nonpermanent and occur in adulthood, whereas organizational effects are permanent and occur during development. When sex differences in brain structure were later identified, they appeared to reflect organizational hormone effects. It now appears, however, that activational influences can also involve structural changes. An example is the dendritic growth in the songbird brain that occurs with seasonal increases in testosterone (e.g., DeVoogd & Nottebohm 1981; Nottebohm et al. 1981). F&D (and other authors) suggest that adult hormonal influences on brain structure contradict the original activational/organizational distinction. However, Phoenix et al. based the activational/organizational distinction on timing and permanence, not on the existence of a structural basis. Interestingly, they did not expect even organizational effects to involve a change in "visible structure" (Phoenix et al. 1959, p. 381), making the structural nature of both organizational and activational effects surprising.

A second question concerns how to prove that a hormonal effect is organizational. The ideal way is to manipulate hormones during development in some groups but not others and then to standardize the adult hormone situation by removing the gonads and providing various identical environments via hormone replacement. When this is done, any difference between the groups can be confidently viewed as a permanent effect of early hormones (i.e., not activated by adult hormones). This has not yet been done for ovarian hormonal influences on the CC. Instead, three arguments are offered to support an organizational interpretation: (1) The effects of ovariectomy on day 12 are seen at 90 days of age, but not at 55 or 30 days of age. This is not compelling, because a separate (nonhormonal) developmental mechanism, not present at earlier ages, may be needed to reveal the effects of ovarian removal, or the ovarian influences may require a long time to become visible. (2) Estrus phase at sacrifice influences uterine weight but not the CC. One cannot assume that ovarian influences on the CC and uterus would follow the same time scale. In fact, the data from argument 1 suggest that influences on the CC require more time. (3) Females ovariectomized at 78 days of age do not differ from sham operated littermates in callosal structure at 110 days of age (i.e., 32 days later). Again, ovarian influences on the structure of the CC appear to take a long time to become visible (according to argument 1, longer than 32 days). Similarly, they may take a long time to disappear. Thus, they may reflect slow activational effects, rather than organizational influences.

A third question concerns how the concepts of masculinization and feminization apply to brain structure. Research on sexual differentiation has shown masculinization (defined as mounting) and feminization (defined as lordosis or gonadotropin regulation) to be separate dimensions (Beach 1975). F&D suggest that applying the masculinization/feminization distinction to neuroanatomy causes complications, citing the sexually dimorphic nucleus of the pre-optic area (SDN-POA) as an example. However, the SDN-POA can be conceptualized in the same way as mounting. Both are characteristic of (i.e., more common or bigger in) males, and thus reflect an aspect of masculinization. F&D point out that the reduction in SDN-POA volume seen in female rats following anti-estrogen treatment has been interpreted by some as defeminization (Döhler et al. 1984). However, this is no different in principle from viewing anti-estrogenic influences on mounting as defeminization. Female rodents treated with anti-estrogens during development have a smaller SDN-POA and show less mounting (Döhler et al. 1984; Hines et al. 1987). In both cases, endogenous estrogen moves the female animal from the extremely non-masculine end of the continuum to the point where most females

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are, roughly 20–30% of the way to the masculine end. The SDN-POA and mounting behavior are the same in this respect. Thus, the effect of estrogen on the SDN-POA reflects not feminization, but the graded effects of hormones (Collaer & Hines 1995) on a masculine trait.

Finally, an update is needed on when sex differences in visuospatial abilities appear. Although Maccoby and Jacklin (1974) suggested that they appear at puberty, this has proved incorrect. Meta-analyses (Linn & Peterson 1985; Voyer et al. 1995) indicate that only certain aspects of visuospatial ability show sex differences, but that they do so in very young children. Sex differences seemed to appear at puberty, because different visuospatial tests were used for different age groups, with younger children generally tested on measures that do not show sex differences and adolescents and adults on measures that do.

Relative size of the human corpus callosum redux: Statistical smoke and mirrors?

Ralph L. Holloway

Department of Anthropology, Columbia University, New York, NY 10027.
rlh2@columbia.edu

Abstract: Data do exist to support the fact that the corpus callosum is relatively larger in women than in men. The corpus callosum is an integral part of the brain, and contrary to Fitch & Denenberg's examples of "pseudostatistics," is not an extrinsic structure when determining its relative size.

I have no comments to offer on the findings regarding ovarian hormones and sexual differentiation of the brain, as that is out of my area of competence, and a valuable contribution by Fitch & Denenberg (F&D). However, I do wish to discuss the matter of sexual dimorphism in the human corpus callosum, and to respond in particular to the unfair characterization of our work as "pseudostatistics." My comments relate to two areas: (1) the issue of relative size of the corpus callosum in humans and (2) the meta-analysis of the corpus callosum by Bishop and Wahlsten (1997).

First, I think it is very unwise to rely on a statistical technique such as factor analysis to decide whether there are any correlations between the size of the corpus callosum and the brain of which it is a part. It is difficult to understand what is happening to a division of the corpus callosum into 99 measures, particularly in an animal as small as the rat. In all factor analytic studies that I have seen, a size component always comes out in the first factor. If I understand F&D, it does not appear until factor 8. It would seem to me most logical to simply run a Pearson correlation between brain size and corpus callosum size measures, including area and perimeter first, and then to try a factor analysis. I do not understand why this was not done, and instead the authors relied on what amounts to a very complex and relatively little used multivariate statistical technique, which most statisticians would prefer to stay away from, to "prove" a lack of correlation between brain size and the corpus callosum.

Nevertheless, whether rat brain size correlates with the corpus callosum area strongly or weakly, the size of the human brain and its corpus callosum *does* have some considerable empirical basis. We find in Tables 6 and 7 in Holloway et al. (1993, p. 488) a veritable sexual dimorphism of the correlation between brain size and corpus callosum area, in respectable sample sizes of roughly 45 each sex. Males had a correlation of 0.5031 (p = 0.0005), whereas in females the correlation was 0.1638, with a significance of 0.27. Just before these two tables, we had shown from Table 5 that when the sexes were combined, the correlation between brain size and corpus callosum area was 0.3482, with a p-value of 0.0008 (N = 90, p. 487).

I cannot understand why F&D have ignored these findings. As I recall, the Berrebi et al. (1988) paper never tested whether there was any correlation between brain size and corpus callosal measures, nor did the Denenberg et al. (1989) paper provide such a simple test, prior to their use of factor analysis. Perhaps there is no significant correlation between brain size and the corpus callosum in rats, but I doubt that factor analysis is the only way to demonstrate such a lack. In any event, humans do show such a correlation.

We pointed out in our 1993 paper (p. 483) that both Denenberg et al. (1991) and Demeter et al. (1988) claimed there was no correlation between brain size and corpus callosum area, and thus brain size could be ignored. However, because neither ever published the full statistics on brain size (the Demeter et al. study suggested large differences of brain size between human females and males), it begs the question of how strong or weak the correlation was. Surely it is contrary to intuitive wisdom to believe that a structure such as the corpus callosum would have no correlation with the size of the cerebral hemispheres it was connecting, the cerebral hemispheres being some 76% of total brain weight in humans. In any event, as our Tables 5, 6, and 7 (Table 1 here) show, there is indeed a significant correlation within males, and certainly as would be expected given brain size dimorphism, in a combined male and female sample. Particularly strong are the correlations between corpus callosum area and posterior one-fifth area, being on the order of 0.8 for both sexes, separately and combined. Let me recall with a quote from Holloway et al. (1993, p. 495) on the range of human brain sizes from the Demeter et al. (1988) study:

> Brain weights for males cluster between 1,300 and 1,700 cc. Female brain weights cluster between 1,050 and 1,200 cc, and *do not overlap male values* [emphasis mine]. Six male values are above 1,500 cc.

The sample size comprised 22 males and 12 females, hardly enough to draw profound conclusions that brain size could safely be ignored. Those are rather extraordinary and large differences in

Table 1 (Holloway). *Pearson correlation for brain size, corpus callosum area (CCAREA), posterior one-fifth (splenium) area (POST 1/5), and dorsovental splenial distance (SPLNDV)**

Brain weight	CCAREA	POST 1/5	SPLNDV
Total sample, N = 90	0.3482 (0.0008)	0.2127 (0.0442)	−0.0516 (0.6291)
Males only, N = 44	0.5031 (0.0005)	0.3238 (0.0320)	0.0374 (0.8098)
Females only, N = 45	0.1638 (0.2767)	0.2928 (0.0483)	0.1189 (0.4313)

Based on Tables 5, 6, and 7 in Holloway et al. (1993, 487–88).
Significance level in parentheses.
Note the lack of significance and low correlations between brain size and those variables we suggested as particularly dimorphic (i.e., Post 1/5 and SPLNDV).

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brain sizes, which do overlap in most populations. It seems strange intuitively that with brain size dimorphism such as this, the mean corpus callosum area would be slightly larger in males, but that the splenial dimension would be almost identical (11.8 mm in males, 11.6 mm in females). I simply cannot understand why a study with those measures would ignore brain size and fail to consider the very simple proposition that relative to the size of the brain, females had larger splenia than males, or that given such large differences between brain sizes, there might be a relative size difference of the corpus callosum.

In that regard, let us examine F&D's statistical arguments for obviating the need for testing the very simple proposition that relative to brain size, the size of the corpus callosum shows sexual dimorphism in humans. F&D write:

> As an example, women weigh on average less than men, and women score lower on average than men on certain tests of spatial ability. . . . One cannot draw any conclusion concerning an association between these two variables from such data. That can only be done if a significant correlation exists between weight and spatial scores within each gender.

I could not agree more! However, that is *not* the hypothesis that we have been trying so hard to test; nor is it even analogous (or homologous) to the structure of our argument that relative to the total size of the brain, a brain structure, namely, the corpus callosum, is larger in females. We have not been discussing body and brain weights and spatial tests; and nowhere have we suggested that spatial ability is a part of body or brain weight, or vice versa.

Elsewhere, we discover under section 6.3.1.1, "Pseudostatistics," a similar argument:

> On average, there is no sex difference between men and women on IQ tests. However, female brains are smaller than male brains, and weigh less. One could obtain an estimate of brain size from cranial measurements or neuroimaging, divide this number into the person's IQ score, and obtain a score that measures "IQ per unit brain tissue." On such a measure females would be significantly superior to males. The reason we do not use such a statistic is that research has established that there is no within-group correlation between IQ and brain size.

Again, I would agree, but this too is rather far removed from what we have done. Nowhere did we attempt any such correlations or any dividing of the data extraneous to the brain by brain size. IQ is simply not a part of the brain; nor is body weight! On the other hand, the corpus callosum is an integral part of the brain, and, like the whole brain, it has a size, albeit an extremely difficult one to calculate, as it is the largest fiber system in the human brain. It is hard to imagine why there should be no correlation worthy of study between the corpus callosum and brain size, when in fact the corpus callosum is a part of brain size. Hence the simple hypothesis that the relative size of the structure differs between males and females is hardly "pseudostatistics." Three recent studies (Andreason et al. 1993; Reiss et al. 1996; Willerman et al. 1991) have demonstrated significant correlations between brain size and various behavioral test scores, so the issue is not quite moot.

Physical anthropologists and other comparative morphologists routinely use ratio data. If we wish to divide the weight of the brain by the weight of the body, we often do so because an extremely interesting set of facts emerges: the relative size of the brain, that is, its part of the total animal's weight, does show sexually dimorphic differences (Holloway 1980) and they vary considerably within the mammalia, and primates in particular. Encephalization quotients and a whole range of allometric analyses depend on such data. It has recently been shown by Semerdeferi et al. (1997) that the proportion of frontal lobe in humans is exactly what we would expect for a primate of our brain size, a fact shown by von Bonin back in 1948, and several others before by the simple expedient of asking how much of the brain was frontal lobe.

I would like to propose the following challenge: Let F&D (or anyone else) explain our finding in Tables 9, 10, and 11 of Holloway et al. (1993, p. 489) that whenever the sexes are compared by dividing the cerebellum, rhombencephalon, ventricles, hippocampus, amygdaloid, thalamus, cortex, or any other part of the brain (except the corpus callosum) by total brain weight, there are no significant statistical differences between human males and females, whereas for each of those absolute measures there is a significant difference. Why is it that the corpus callosum, a part of the brain just like the structures mentioned above, shows the opposite effect when divided by brain size (i.e., a statistically significant difference between human females and males, but no significant difference in absolute size)? Both the Wesseley (1970) and Zilles (1972) data were published well in advance of the simple hypothesis that relative to the size of the brain, one of the brain's structures was larger in females than in males, so we can be reasonably certain their data are unbiased. For all of the structures mentioned above there are indeed positive correlations between their size and the size of the brain.

Last, I wish to address the Bishop and Wahlsten (1997) meta-analysis cited by F&D but not yet in our library. I remember being asked by those authors to provide our original data for the meta-analysis. At the time I refused, because I simply could not understand why mixing two such different approaches in the same statistical analysis could be meaningful. Please recall that part of the controversy over the corpus callosum involves two basic approaches: (1) the study of autopsy data where brain size is available and (2) magnetic resonance imaging (MRI) studies where brain size has largely been ignored, until only recently, when algorithms for adding sections together provide a close approximation to actual brain size. F&D (like Fausto-Sterling 1992) describe how many studies supported our hypothesis and how many did not, as if all the studies were somehow of equal force or merit. Let me give but one interesting example from our 1993 paper. We found that the Byne et al. (1988) study was frequently placed in the category of not supporting our hypothesis. I quote from Holloway et al. (1993, p. 495):

> Magnetic resonance imaging, 15 males, 22 females; dimorphism reported as not significant. Mean CC area was 519 (M) 601 (F). Brain size was not studied. Both CCAREA and posterior one-fifth (splenium) were absolutely larger in females. The splenium was 160 mm^2 for men, and 168 for women in the age $>$ than 40 sample. In the total sample, posterior one-fifth was 170 in females and 160 in males. Given these findings, and the usual dimorphism of brain size being larger in males, these results are fully consistent with our findings.

Here there were absolute size differences in corpus callosum area and splenial area that were larger in females! I can certainly understand how such differences might not be statistically significant using t-tests, but it surprises me that it would not appear important to consider brain size in such analyses. In fact, in almost all the papers we reviewed, the absolute differences between males and females were seldom if ever significant and were very close. Our paper concluded by suggesting that no fewer than 16 of 25 studies reporting no significant differences actually did have results consistent with our findings, if the relative size of the corpus callosum was considered. Bishop and Wahlsten's "meta analysis" is particularly flawed given the mix of studies they combine, and the others that they avoid. Almost all of the earlier published MRI studies that claimed a lack of sexual dimorphism in the corpus callosum simply failed to include the size of the brain in their analyses, and some of the autopsied studies that similarly claimed no dimorphism never provided brain weight data for independent analysis. MRI has advanced to the point where total brain volume is now readily calculable.

Finally, for whatever it is worth, we have found no apparent sexual dimorphism in relative measures of the corpus callosum in other primate species (Holloway & Heilbroner 1992), including, most recently, the chimpanzee (Broadfield et al. 1997, which did suggest some minimal dimorphism in the abstract). We believe, and always have, that more microscopic analyses must be done with types of fibers (myelinated and nonmyelinated) and their distributions (both within the cortex and the corpus callosum) before meaningful functional statements can be made about any

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dimorphism between human male and female brains. Nevertheless, it is possible that humans have evolved some species-specific repertoire in their cognitive evolution that includes ethological differences between males and females and have some underlying neural basis. The possibility that the *relative size* of the corpus callosum is larger in females than in males remains to be fairly tested.

The corpus callosum: More than a passive "corpus"

Kenneth Hugdahl

Department of Biological and Medical Psychology, University of Bergen, N-5009 Bergen, Norway. **hugdahl@psych.uib.no**

Abstract: Fitch & Denenberg provide excellent evidence for the existence of dynamically complex interactions between the structural and functional development of the nervous system. They are to be congratulated for showing how subtle social variables (e.g., handling) may not only influence hormonal "cascade effects" on the developing nervous system, but may also alter the structure of brain tissue, such as the corpus callosum.

My commentary is focused on the sections of the target article that deal with the corpus callosum and sexual dimorphism (sects. 5 and 6). I would like to congratulate Fitch & Denenberg (F&D) for presenting a coherent discussion of several lines of research on the complex functions of the corpus callosum. Although the target article pertains mainly to the effects of gonadal hormones on callosal development, it also deals with several other important aspects of the behavioral significance of the corpus callosum.

I agree that the frequent use of relative measures (and the strong pressure from the "scientific community" to do so) can sometimes result in the typical type II error of "throwing out the baby with the bath water." Unfortunately, however, the "statistical" solution F&D advocate – that there should be "a significant association between two variables *within a group* before one needs to make an adjustment" (sect. 5.2) – may likewise lose the baby. The problem is not that there should be a significant association or correlation between the target variable and a covariate, but that the association should also have a *theoretical* foundation. The example F&D provide in section 5.2 – that women weigh less than males and have lower scores on certain spatial ability tests – does not warrant the conclusion that their spatial scores are related to their weight unless there is a "significant correlation . . . between weight and spatial scores within each gender" (sect. 5.2). The problem is that women and men differ on a variety of variables, of which some may correlate spuriously with the target variable by chance alone.

The finding that testosterone propionate treatment must be associated with handling in order to significantly enlarge the female's callosum is indeed intriguing. As F&D quite correctly state, "this effect is more complex than simple exposure of the female to androgen" (sect. 5.4.1). This finding also points to a few other possible interactions with callosal development and functioning. "Individual variation" is ignored in many neurobiological subfields. Traditionally this is treated as "error variance." However, it is quite possible that behind the differential handling effect on callosal development is a complex interaction of individual variation in susceptibility to the social environment, which in turn affects callosal morphology. Such variation could well have a hormonal basis.

The finding by Aboitiz (1992), as well as by others (e.g., Cowell et al. 1992), that there are regional differences along the callosal axis in area size and fiber density and that they interact with "general" functions such as gender and age, is an example of what I would call a "hardware/software" interaction. Aboitiz's conclusion, with which F&D seem to agree (sect. 6.2), was that the callosal regions connecting primary sensory motor areas have large-diameter myelinated fibers. The other callosal regions at the borders of the large-fiber areas consist of small-diameter fibers with a more "diffuse" spatial orientation. My argument is that optimal transfer of information across the callosum, including greater interhemispheric connectivity, may not be exclusive to the large-fiber areas (as both Aboitiz and F&D seem to believe). I would like to argue for a "two-stage" model of callosal transfer in which the small-diameter, diffusely spread fibers reflect "cognitive gating" that may dynamically enhance or inhibit primary sensory transfer depending on the cognitive "set" of the subject or patient. Some dichotic listening data from my own laboratory can be used as an example of the relationship between interhemispheric transfer and callosal size. This suggests a "two-channel" threshold model of callosal transfer that would also include transfer of attentional resources and the gating of sensory transfer (e.g., by attention).

In a recent study on hemispheric asymmetry for auditory stimuli in multiple sclerosis (MS) patients (Reinvang et al. 1994) we specifically examined callosal sector size and left ear performance. The left ear performance in dichotic listening is thought to involve transfer across the corpus callosum (see Hugdahl 1995 for further details about the dichotic listening technique). The corpus callosum often shows atrophic changes in MS patients and measures of the corpus callosum are often included in the diagnosis. In our study we had magnetic resonance imaging measures of callosal sector size. The study involved three conditions, one in which the patients were requested to report both ear inputs, as well as possible, and two conditions in which they were instructed to monitor (attend to) the left or right ear input only. The results showed the expected right ear advantage (better recall from the right than left ear) in both the MS patients and a healthy control group during the nonattention condition. When the subjects were instructed to focus their attention to the left ear, however, the correlations between left ear performance and callosal size were clearly significant, particularly for the three most posterior sectors (including the auditory sector anterior to the splenium).

It thus seems as if an "attention-gating" factor is needed in order to enhance callosal transfer of the left ear score, particularly in a subject population that already has degeneration in the primary sensory callosal pathways. This may suggest a two-channel threshold model of callosal transfer, with a sensory modality-specific channel involving the large diameter myelinated fibers and a diffuse nonspecific sensory channel involving the small diameter nonmyelinated fibers, which are responsible for the transfer of cognitive information.

In closing, I would like to congratulate F&D for their convincing demonstration of the complex, often counterintuitive interactions between structural and functional development. They should be further congratulated for showing how subtle social variables may not only cause hormonal "cascade effects" on the developing nervous system (cf. Geschwind 1984) but that such variables may also alter the structure of the brain tissue "hardware."

Updates on axons in the rat corpus callosum

Janice M. Juraska

Department of Psychology and Neuroscience Program, University of Illinois, Champaign, IL 61820. **jjuraska@s.psych.uiuc.edu**

Abstract: Developmental counts of axons in the splenium of the rat corpus callosum are compatible with the hypothesis that estrogen may be acting late in development to sculpt the female nervous system.

Our recent data expand the discussion (sect. 6.2) of sex and the axonal composition of the rat corpus callosum. Our findings may also have implications for pubertal effects in female rats that are concordant with the observations on gross size from Denenberg's laboratory and other studies discussed by Fitch and Denenberg (F&D).

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We concentrated on one portion of the corpus callosum – the splenium – that carries axons between the visual cortices, where we have documented dramatic sex differences in neuron number (18%, males > females; Reid & Juraska 1992). To define the splenium as a nonarbitrary entity, we examined the topography of axons traveling through the corpus callosum that were labelled with discrete horseradish peroxidase injections in the posterior cortex. The area under the posterior fifth of callosal length contained visual (and a small number of temporal) axons and was defined as the splenium. In an extensive sample of this area, we found no sex differences in the total number of axons in 60-day-old rats housed in standard social conditions. Males, however, had more myelinated axons than females (Kim et al. 1996). We attribute our earlier report of sex differences in rats raised in both complex and isolated environments (Juraska & Kopcik 1988) to a less thorough sampling strategy.

This leads to a caveat: even within a callosal subarea containing only fibers from the posterior cortex, there is considerable heterogeneity of axon density, and small, nonsystematic samples can be misleading. Attempts to quantify axons in the human corpus callosum are further affected by this heterogeneity because tissue degeneration prevents visualizing the smallest axons (sect. 6.2). We do not have enough cellular details to make generalizations for the human corpus callosum; this renders the controversies over its *size*, much less its shape, not very meaningful.

Of particular interest for F&D's article, we also compared the number of axons in the splenium of male and female rats at 15, 25, and 60 days of age. Both sexes lose axons between postnatal days 15 and 60 (approximately 15%), but the pattern of loss differs. Males lose the axons between 15 and 25 days, while their total number stays constant between 25 and 60 days. (Note that this refers to *number*, not density. Density decreases across these ages due to increasing myelination.) Females, on the other hand, lose most of their axons between 25 and 60 days (Kim & Juraska, in press). This late loss coincides with developmental patterns in the dendritic tree in the visual cortex such as that demonstrated by Muñoz-Cueto and colleagues (sect. 4.5.2). Indirect support also comes from our laboratory: female rats have larger pyramidal neuron apical dendrites than males in the visual cortex at day 25 (Seymoure & Juraska 1992); this difference was not found at day 55 in a separate study (Juraska 1984). Thus dendrites and spines appear to peak and regress in the female visual cortex in the same time frame as axons in the splenium.

There are direct comparisons to work from Denenberg's laboratory on the gross size of the rat corpus callosum (sects. 5.5.2 and 5.5.3). Estrogen, perhaps associated with puberty, appears to decrease the size of the corpus callosum. This could be due to an effect on the withdrawal of axons. Although we often have failed to find the corpus callosum significantly smaller in females than in males (Kim et al. 1996; Kim & Juraska, in press), the direction of the means (male > female) is congruent, and we have found a significant sex difference (male > female) in callosal size in a related experiment (Nuñez et al. 1995). Thus our work is compatible with the possibility that estrogen promotes the withdrawal of axons between postnatal days 25 and 60, but direct tests of the hypothesis need to be performed.

That being said, I am not certain that I view these late, admittedly organizational, effects as being on par with the profound early effects of testosterone on masculinization. The presence or absence of androgens early in development results in a sexually functioning male or female, while the later effects of pubertal hormones, perhaps in both sexes, build on and modulate the system that was formed earlier.

I have one final note. Denenberg and his students have done an admirable job in examining hormonal influences on the size of the rat corpus callosum and in documenting the late, unexpected influence of estrogen. We must now dig into the complex cellular basis for the changes in callosal area. For example, between days 25 and 60, female rats are losing axons while male rats are undergoing proportionally more myelination. These events cannot be readily separated by examining size (or even axon density) alone, and each may be influenced by different hormones. Understanding later hormonal effects on cellular development is the next step.

Estrogens in human psychosexual differentiation

Heino F. L. Meyer-Bahlburg

New York State Psychiatric Institute and Department of Psychiatry of Columbia University, New York, NY 10032.
meyerb@child.cpmc.columbia.edu

Abstract: There is some very limited evidence for a role of estrogens in human psychosexual masculinization; its interpretation is uncertain. Fitch & Denenberg's demonstration of a role for estrogens in the behavioral feminization of nonhuman mammals implicitly suggests an answer to a riddle posed by the syndrome of congenital adrenal hyperplasia in women.

Fitch & Denenberg (F&D) make a very convincing case for the role of estrogens in the sexual differentiation of behavior in nonhuman mammals. The few data available on the role of estrogens in human psychosexual differentiation have usually been interpreted in the context of the two-pathway model of testosterone effects in the process of masculinization/defeminization (Meyer-Bahlburg 1997). The evidence comes from the offspring of pregnancies that were treated with exogenous estrogens, or from patients with abnormalities of the prenatal hormonal milieu secondary to endogenous endocrine disorders.

An example of the first approach is our studies of several samples of women with a history of prenatal exposure to the nonsteroidal estrogen diethylstilbestrol (DES). The DES-exposed women showed increased rates of bisexual or homosexual orientation in comparison to several control samples, but very little indication of any masculinization of childhood behavior (Meyer-Bahlburg et al. 1995). Thus, these data suggest the possibility of a relatively subtle contribution of prenatal estrogens to the development of homosexuality in women. It is not certain, however, that the mechanisms involved are the same as in physiologically normal psychosexual differentiation. Possible alternative explanations are, for instance, DES-induced stimulation of androgen production or toxic effects of DES on the differentiating brain. Of the endocrine disorders, 46,XY individuals with the syndrome of complete androgen insensitivity (cAIS) are of particular relevance to this discussion (Meyer-Bahlburg, in press). Somatically, these individuals respond to their endogenous (gonadal) estrogens but not to their androgens; because of the female appearance of their external genitalia, they are assigned to the female gender, and they grow up and self-identify as women. Published data include only 46,XY cAIS women with female-typical gender role behavior of childhood and later, and with an exclusive sexual attraction toward men. This speaks against a significant masculinizing role of prenatal estrogens in these women's psychosexual development, but the small number of patients studied and other considerations do not yet permit definitive conclusions.

Two new syndromes have recently been described that offer the possibility of studying the behavioral effects of estrogen deficiency states in humans (Meyer-Bahlburg, in press): estrogen insensitivity syndrome due to estrogen-receptor deficiency and aromatase deficiency syndrome with ensuing inability to produce estradiol. The first two case reports on adult 46,XY men with these syndromes (but otherwise normal gonadal differentiation and testicular hormone production) include some preliminary psychosexual data that do not indicate an effect on gender role behavior or sexual orientation, although one had a history of very low sexual desire of unknown etiology. Thus, neither form of estrogen deficiency in males seemed to be associated with a major effect on psychosexual differentiation. Obviously, a systematic evaluation of

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a larger number of individuals is needed before we can draw more definitive conclusions.

The potential organizational role of estrogens in the process of human feminization/demasculinization has not yet been systematically explored. This is an attractive model, however, for the explanation of one of the puzzles presented by the syndrome of congenital adrenal hyperplasia (CAH; for references, see Meyer-Bahlburg et al. 1996). In 46,XX individuals, CAH is characterized by excessive prenatal androgenization from the adrenals due to enzyme defects in the cortisol synthesis pathway. At birth, 46,XX individuals with this condition typically present with genitalia that are more or less markedly masculinized. Despite the high prenatal androgen levels and often continuing slight to moderate hyperandrogenemia after birth due to difficulties in mimicking the physiologic pattern of release of adrenal glucocorticoids by replacement therapy, these women show an only modest increase in bisexuality and homosexuality, and the majority become apparently exclusively heterosexual.

This appears to contrast with the much higher rates of homosexuality reported in such syndromes as 5-alpha reductase deficiency or 17-beta-hydroxysteroid dehydrogenase deficiency in 46,XY individuals who are born with rather female-appearing external genitalia and were therefore raised as females, before the syndrome became better known in the communities where they were originally studied. It may well be the presence of ovaries and their secretions, possibly estrogens, in CAH females that prevents the full impact of androgens on the development of a female-directed sexual orientation.

Even Dörner's classical rodent model for the development of homosexual orientation in females is compromised by the presence of the ovaries (Meyer-Bahlburg 1979, p. 112): Dörner's own data on neonatally androgenized female rats clearly showed predominance of female copulatory behavior (despite an increased incidence of mounting in the presence of a receptive female), and only when the neonatally androgenized rats were later ovariectomized and treated with testosterone propionate did male copulatory behavior predominate. Since homosexual women typically have functioning ovaries, Dörner's rat data do not provide an adequate model of female homosexuality in non-intersex women. Similar data that F&D have brought together from many different sources further strengthen this argument.

For the human condition, the issues discussed here are not only of theoretical interest, but may have significant clinical implications, especially for the medical management of certain syndromes of intersexuality. For example, if it can be shown that human feminine development is co-influenced by organizational effects of estrogens before puberty, one obviously should devise an appropriate early-onset low-dose estrogen-treatment regimen in female-raised (46,XX or 46,XY) children with gonadal dysgenesis or gonadectomy.

Indirect influences of gonadal hormones on sexual differentiation

Lesley J. Rogers

Department of Physiology, University of New England, Armidale, NSW 2351, Australia. **lrogers@metz.une.edu.au**

Abstract: Indirect routes by which gonadal hormones influence sexual differentiation are considered. In rats, differentiation may depend on the way in which the mother responds to the hormonal condition of her pups, and this has implications for the interpretation of the data for humans. Interaction between gonadal hormones and light experience in chicks is compared with the mammalian systems covered in Fitch & Denenberg's review.

As Fitch & Denenberg (F&D) have demonstrated so convincingly, both estrogen and testosterone play active roles in brain differentiation, but the mechanisms by which these hormones act have yet to be clarified. Do gonadectomy or hormone administration during prenatal or neonatal development have direct effects on the differentiation of neural structures and behavior, as is usually assumed, or might the influences be indirect, via changes in parental behavior? The integral role of handling on the differentiation of the corpus callosum (CC) in rats highlights the need to investigate potential effects of the hormonal manipulations on maternal behavior.

The handling procedure is known to affect maternal behavior: the mother's licking of her pups increases after they have been returned (Smotherman et al. 1977). This may be an indirect route by which hormonal treatment influences sexual differentiation. Moore and Morelli (1979) have shown that maternal rats direct a greater amount of licking of the anogenital region to male than to female pups. Castrated male pups receive less anogenital licking than intact male pups, and testosterone-treated female pups receive a greater amount than untreated female pups (Moore 1982; 1984). Apparently, a product of testosterone excreted in the urine stimulates the maternal animal to lick the anogenital region. The outcome of receiving greater amounts of anogenital licking is masculinization of adult behavior and of the hypothalamic-pituitary axis, irrespective of genetic sex. Female pups that receive artificially elevated amounts of anogenital stimulation by using a paintbrush, display masculinized behavior. These results raise the possibility that the amount of maternal licking that accompanies the handling procedure varies with the hormonal condition of the pups. If so, the long-term effects of gonadectomy or treatment with gonadal hormones during neonatal life may occur indirectly by changing maternal behavior (changed paternal behavior might also be involved in more natural conditions).

In my opinion, it is now important to determine whether gonadal hormones influence neural differentiation in rats directly or indirectly, as the findings will have implications for the research on sex differences in brain morphology and behavior in humans. Indeed, to F&D's list of possible explanations for contradictory results between different studies that have investigated the influence of sex on the size of the human CC, I would add individual differences in early experience. The gonadal hormones might have affects on the development of the CC in humans, as they do in rats, but so too might early experience, and the latter might be highly variable from study to study. Indirect routes of causation cannot be eliminated from studies involving maternal-offspring interactions or other social interactions that might be modulated by gonadal hormones. Only in species that can be raised without parental care or sibling contact is it possible to isolate direct from indirect effects of hormonal manipulation.

In precocial avian species, maternal and other social interactions can be removed by incubating the eggs artificially and raising the chicks in isolation. Hormones can be administered during embryonic and posthatching stages of development and controlled stimulation can be applied. Domestic chicks exhibit sex differences in behavior even within the first week after hatching (Andrew & Brennan 1984) and males have a greater degree of asymmetry in the organization of the visual projections from thalamus to forebrain than females (Rajendra & Rogers 1993). The asymmetry in the visual projections is determined by both hormonal condition and light experience during the last stages of embryonic development (reviewed by Rogers 1996). Elevated levels of either estrogen or testosterone promote growth of the projections from both sides of the thalamus and symmetry results (Rogers & Rajendra 1993; Schwartz & Rogers 1992). In normal development, male embryos have a trough in testosterone levels just before hatching, at a stage when light experience promotes development of the neural connections fed by the right eye (Rogers et al. 1993). The visual projections fed by the left eye do not develop to the same extent as those fed by the right eye because the embryo occludes its left eye with its body. In this system, gonadal hormones interact with light stimulation but, unlike the additive effects of handling and testosterone on the size of the CC in rats, light stimulation generates asymmetry only when

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the levels of steroid hormones are low enough. Comparing factors that influence differentiation in the avian and mammalian systems may be helpful in elucidating the mechanisms involved in sexual differentiation.

Female and flexible?

Jane Stewart

Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University, Montreal, Quebec H3G 1M8, Canada.
stewart@csbn.concordia.ca

Abstract: The fact that the female mammalian brain remains responsive to estrogens throughout life may open the way for other instigators of neuronal plasticity, making the female brain different from that of the male in its response to the actions of a number of hormones, to injury and to aging.

Fitch & Denenberg (F&D) have marshaled convincing evidence of a role for ovarian hormones in the development of the brains of female mammals. As they point out, the influence of ovarian hormones in female brain development has often been overlooked in the face of the marked effects of testicular hormones in the development of both the external genitals and the brain of male mammals. The effects of ovarian hormones on brain development are subtle and often difficult to characterize. We now see that these effects occur later than those of testicular hormones, beyond the "critical period" for masculinization (and, perhaps more important, for defeminization). F&D point out that there are at least three types of influence from exposure to ovarian hormones on the brain throughout life: permanent or long-lasting structural effects that occur early and are seen even in the absence of later exposure to these hormones, recurring structural effects in response to cyclic changes in hormone levels, and activational effects probably due to changes in transmitters and receptors.

A characteristic of the brain of females is that it can be modified by ovarian hormones throughout life. This feature of the female brain appears to result largely from having had less exposure than the male brain to testicular hormones in the perinatal period. This greater modifiability probably does not imply that all areas of the brain remain more plastic in animals that have had less exposure to testicular hormones; rather, it may be that it is those areas of the brain that are targets for testicular hormones in perinatal life that remain plastic. The continued sensitivity of the female brain to hormonal and other influences may mean that those regions of the brain that are late to mature, such as the hippocampus and cerebral cortex, are especially able to respond to changes in ovarian hormones and to other influences, making the female brain subtly but significantly different from the male brain. These points, though touched on in several places in their paper, were not emphasized by F&D.

Numerous findings support these observations about the continued plasticity of the female mammalian brain. Early work showed that female guinea pigs and rats lose the ability to respond sexually to the priming effects of estradiol and progesterone when they are exposed to testosterone perinatally (Gerall & Ward 1966; Phoenix et al. 1959). Recently it was shown that perinatal exposure of females to testosterone blocked the dendritic branching response to ovariectomy of the cortical pyramidal neurons in adult female rats (Stewart & Kolb 1994). Adult male and female rats treated with testosterone at birth do not respond to the enhancing effects of circulating estradiol on amphetamine-induced locomotor activity seen in normal females and in males gonadectomized at birth (Forgie & Stewart 1993). The latter findings suggest that the female brain may react not only to variations in ovarian hormones over the estrous or menstrual cycles, but that the response of the female brain to the loss of ovarian hormones in menopause may be dramatic.

There are suggestions in the literature that cells in some brain areas of the adult female rat may remain more responsive than those of males to environmental enrichment or insult. For example, it has been found that the extent of axonal "ingrowth" of noradrenergic neurons to the dentate gyrus following cuts to the septal-hippocampal regions was greater in female rats than in male rats, and that this greater plasticity was reversed by neonatal exposure to testosterone (Loy & Milner 1980; Milner & Loy 1982). Similarly, in female rats dentate granule cells show enlarged dendritic arbors in response to an enriched rearing environment, whereas those in males do not (Juraska et al. 1988).

In conclusion, the occurrence of cyclic changes in the hypothalamic-pituitary-gonadal axis of female mammals from the time of puberty requires that the brain remain responsive to ovarian hormones throughout life. This requirement may open the way for other instigators of neuronal plasticity and thereby make the female brain different from that of the male in its response to the actions of a number of hormones, to injury, and to aging. It may be that underlying all of these effects is a differential responsiveness of the brain to neurotrophic factors (Horvath et al. 1997). It will be important to determine the extent of such differential responsiveness and whether it, in turn, is determined by differential exposure to testicular hormones in the perinatal period.

Effect sizes and meta-analysis indicate no sex dimorphism in the human or rodent corpus callosum

Douglas Wahlsten and Katherine M. Bishop

Department of Psychology, University of Alberta, Edmonton, Alberta T6G 2E9, Canada. **wahlsten@psych.ualberta.ca**

Abstract: Sex dimorphism occurs when group means differ by four or more standard deviations. However, the average size of the corpus callosum is greater in males by about one standard deviation in rats, 0.2 standard deviation in humans, and virtually zero in mice. Furthermore, variations in corpus callosum size are related to brain size and are not sex specific.

Fitch and Denenberg (F&D) present evidence that ovarian hormones play an active role in rat brain development. We agree and will instead focus on four matters where we differ.

First, F&D and many others (e.g., Constant & Ruther 1996) refer to statistically significant differences as evidence of sex "dimorphism." This word is misleading. The Oxford English Reference Dictionary (Pearsall & Trumble 1996) defines dimorphic as "exhibiting, or occurring in, two distinct forms" (p. 399), just as it defines dichotomy as "a division into two, esp. a sharply defined one" (p. 395). Examples of genuine sex dimorphism abound in nature; examples are the plumage of birds and the genitalia of mammals. The corpus callosum (CC), on the other hand, is not at all dimorphic in either rats or humans.

Effect size compares the difference between group means to the standard deviation (S) within a group. For sample data, $d = (M_1 - M_2)/S$ estimates the population effect size δ. Cohen (1992) regards d values of 0.2, 0.5, and 0.8 as small, medium, and large, respectively, in published psychological research with humans. When d is 1.0, the difference between groups accounts for only 20% of the total variance, and scores of males and females overlap considerably. The degree of overlap can be found with Guttman's (1988) discrimination coefficient *disco*, which indicates the probability of identifying group membership from an individual's score.

Genuine dichotomy or dimorphism occurs when d is 4.0 or greater, or *disco* is close to 1.0 (Fig. 1). However, sex differences in the rat CC in papers cited by F&D range from d of 0.85 to 1.35. We have found d is 1.25 for CC area of Sprague-Dawley rats. These are large effects but not at all dichotomous; many females have a larger CC than many males. Promulgating such group differences as a "dimorphism" grossly exaggerates the size of the sex difference. We propose that more accurate descriptors, especially effect size or *disco*, be employed.

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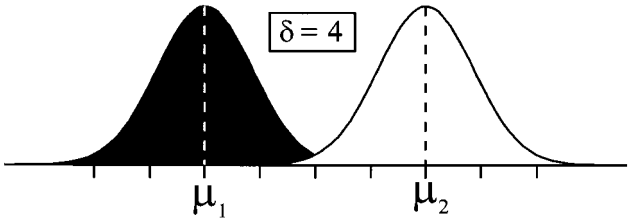


Figure 1 (Wahlsten & Bishop). Frequency distributions of two populations whose means differ by four standard deviations.

Second, a superior estimate of the magnitude of a sex difference may be obtained by combining evidence from several independent studies with meta-analysis. Bishop and Wahlsten (1997) found the following 95% confidence intervals for the sex difference δ in 49 studies of humans: brain weight, 0.95–1.46 (larger in males); CC area, 0.13–0.29; ratio of area of splenium (posterior fifth, not anterior fifth as stated by F&D in sect. 6.2) to whole CC, −0.25–0.02. Thus, males had substantially larger brains on average and slightly larger CC area but no difference in CC shape.

When another study appears, its results should be combined with all previous studies in an updated, cumulative meta-analysis. For example, adding the study by Constant and Ruther (1996) changes the estimate of δ for CC area from 0.2092 based on 42 studies to 0.2048 and narrows the 95% confidence interval slightly (0.13–0.28). Once the literature becomes sufficiently voluminous, any new report could not nudge the estimate of δ a noteworthy amount, and the case can be closed. This we believe is well justified for sex differences in the human CC. Hopefully, the onslaught of individually excellent but cumulatively uninformative research on this topic will soon cease.

Third, F&D argue that in general CC size should not be corrected for whole brain size unless the correlation with brain size is large and significant. We disagree. By the allometric growth principle, we *expect* to find a larger CC in an individual with a larger brain, regardless of sex. Whether a sex difference in CC area is *sex specific* depends on whether the difference between male and female means exceeds what is expected from allometry. For example, with our data on 44 Sprague-Dawley rats, the regression equation for predicting CC area from brain weight ($Y = -0.89 + 1.94X$) accounts for an R^2 of 0.53 of variance in the CC. Adding sex to the equation increases R^2 nonsignificantly to 0.54; hence, there is no sex-specific effect. Fortunately, applying the regression method to adjust for brain size will not change the results for CC size if no relation exists. Thus, it is both safe and wise to make the adjustment. In the rat studies cited by F&D, the CC versus brain correlation is often nonsignificant because of small samples and low power. Meta-analysis reveals a significant correlation of r of 0.38 for six values from those studies in Table 2 that provide adequate details.

We join F&D in condemning the ratio method. A ratio is justified only when the relation is isometric (straight line through the origin, Y-intercept 0). CC versus brain size is an allometric relation and the CC/brain ratio changes as a function of brain size, which means a ratio will not remove the influence of brain size from the data. In this case, the misuse of a ratio may create the appearance of a nonexistent effect or may mask a real effect. These artifactual mistakes will not be made with the regression method.

Finally, F&D suggest that data from rats are relevant for the human brain. However, mice often show no sex difference in the anterior hypothalamus (the so-called sexually "dimorphic" nucleus) or in the CC. If results with rats cannot be generalized to the genetically and ecologically closely related house mice, how can they be cited in support of arguments about the human brain?

Ovarian influences on female development: Revolutionary or evolutionary?

Kim Wallen

Department of Psychology, Emory University, Atlanta, GA 30322.
kim@emory.edu

Abstract: The Fitch & Denenberg target article focuses almost exclusively on short gestation mammals, which differ substantially from long-gestation mammals in the timing and type of hormonal contribution to their sexual differentiation. Conclusions regarding the role of ovaries in female sexual differentiation may accordingly apply to only a limited number of species. Specific criticisms of the organizational effects of hormones stem from an incomplete reading of the original literature. The mechanisms proposed in this target article reflect an extension of the principle of hormonal organization, not a revolutionary restructuring.

The notion that the ovary contributes to female sexual differentiation has been around for more than 30 years and the Fitch & Denenberg (F&D) target article could be a major contribution to the literature on sexual differentiation. The first sentence, however, which states: "Reviews on the role of hormones in mammalian sexual differentiation traditionally focus on the effects of neonatal exposure to testicular androgens . . . in males," already makes it clear that this is not the comprehensive review we need to understand the importance of the ovary. Instead F&D's review reflects a very restricted reading of the literature on sexual differentiation. Their general conclusions about processes of sexual differentiation are based almost exclusively on data from short-gestation mammals, primarily rats, in which a substantial portion of sexual differentiation occurs neonatally and is strongly influenced by estrogens. Totally ignored is evidence from long-gestation mammals, such as guinea pigs and rhesus monkeys, where all the major sexual differentiation events occur prenatally and estrogens appear to be of little or no importance.

An article presenting general conclusions about ovarian influences on sexual differentiation cannot make its case by ignoring a substantial body of literature from species in which perinatal hormonal manipulations have almost no impact on sexual differentiation. Surprisingly, F&D never explain that there are quite marked species differences in the timing, duration, and type of hormone affecting sexual differentiation. They do not even acknowledge the many studies of sexual differentiation using both guinea pigs and rhesus monkeys. Only the original paper by Phoenix et al. (1959) is cited, but without mentioning that in that paper the revolutionary notion that gonadal secretions could organize the developing nervous system was first proposed. None of the later work in guinea pigs, nor any of Goy's work with rhesus monkeys is cited or discussed. The selective reading of the literature leads one to the conclusion that the important differentiating events in mammals occur after birth and that estrogen is probably the most important hormone in both male and female sexual differentiation. Why F&D chose to ignore the literature contrary to this view is puzzling.

As a treatise on rodent development, the target article offers much and marshals a substantial body of evidence that the ovary can modulate aspects of female development. It is clear from the data presented that ovarian effects, though statistically significant, are not of similar magnitude to those produced by testicular manipulations. For example, Grady et al.'s (1965) study reported that neonatal castration of male rats dramatically altered their gentilia and adult sexual behavior in a femalelike direction. In contrast, as F&D found, neonatal ovariectomy produced much more subtle developmental changes. Ovarian contributions to sexual differentiation, though important, do not require reformulating the organizational hypothesis of hormone action. In fact, the primary weakness of F&D's paper is that the organizational hypothesis is never fully described, yet the authors attempt to revise several aspects of it that were never part of the original formulation.

Phoenix et al. (1959) stated the organizational hypothesis as follows: "The results are believed to justify the conclusion that the

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prenatal period is a time when fetal morphogenic substances have an organizing or 'differentiating' action on the neural tissues mediating mating behavior. During adulthood the hormones are activational" (p. 369). Later in the same paper they stated the notion: "that modification of behavior follows an alteration in the structure or function of the neural correlates of behavior . . . [assumes] . . . that testosterone or some metabolite acts on those central nervous system tissues in which patterns of sexual behavior are organized" (p. 381). Note that these researchers were circumspect about exactly what hormone or hormones were involved and did not limit such hormonal action to a single developmental period, concluding only that the prenatal period, but not adulthood, was a time when such neural organization could occur. The criticisms F&D raise in regard to the organizational hypothesis – for example, that it defined a single organizational period or that only androgens produce organizational changes – are incorrect. Interestingly, F&D argue against specific aspects of the organizational effect of hormones but never document who made the original claim. Thus, when they provide evidence against a single period of hormonal sensitivity, they never cite the claim that there is a single sensitive period. As in the critiques of Arnold and Breedlove (1985) and Fausto-Sterling (1995), F&D argue against aspects of hormonal organization that were never articulated by the scientists who first formulated the principle.

The claim made almost 40 years ago that exposure to androgens or their metabolites during a specific developmental period permanently alters the development of the nervous system remains a revolutionary concept that has fundamentally altered the way in which we explore sexual differentiation. That there are additional processes involving different developmental agents occurring at different developmental times and that the ovary may be involved in this process in some species, reflects a natural evolution of the insight Phoenix et al. had in 1959. Only if one fails to consider all of the work done on sexual differentiation in the intervening years can one consider the findings presented in this target article as either controversial or revolutionary.

Parallel or serial processes in sexual differentiation?

Christina L. Williams and Noah J. Sandstrom

Department of Psychology: Experimental, Duke University, Durham, NC 27708. **williams@psych.duke.edu**

Abstract: We argue that estrogen feminization of the brain is the result of a series of events initiated by differential androgen exposure. There is no need to postulate a feminizing process parallel to androgen-induced masculinization to explain the findings.

Fitch & Denenberg (F&D) argue that the current view of the process of sexual differentiation of mammalian brain and behavior proposed by Phoenix et al. (1959) needs to be amended. In their view, two parallel periods of hormone action are required for complete sexual differentiation: a perinatal period when androgens masculinize and defeminize the male brain and a later prepubertal period when estrogens act to feminize the female brain. They present evidence from their own work on the sexual differentiation of the rat corpus callosum to support their view that estrogenic feminization, which occurs during postnatal days 12–35 for the corpus callosum, is a much neglected process that is required for complete neural sex differentiation in mammals. Although this is an interesting idea, particularly in light of the fact that estrogenic feminization *is* the mechanism underlying sexual differentiation of birds and some reptilian species (Adkins-Regan 1988), we believe that there is another, broader context in which to place these data. Our alternate view is that the effects of estrogens on the female brain during this "second period" of hormone action are a result of the differentiation process, rather than a part of it. That is, because males are exposed to testicular androgens during the perinatal period, they become unresponsive (or differentially responsive) to estrogens for the remainder of their lives. Females, in contrast, do not get exposed to much androgen or estrogen during the perinatal critical period, and thus they remain responsive to estrogens. We would argue that this sexually dimorphic response to estrogen is apparent throughout the lifetime of the rat and is not just restricted to the prepubertal period.

To make a case that two parallel processes are required for full sexual differentiation, F&D would have had to provide convincing evidence that there is a critical period for estrogen action as there is for androgen action, and demonstrate that endogenous or exogenously administered estrogen can feminize both females and males. The only evidence of a time-limited period for "feminization" of the corpus callosum is that an enlargement of the corpus callosum occurs following ovariectomy (OVX) on postnatal day 12 (PD 12) but not following OVX on PD 78 (Mack et al. 1996a). The enlargement following OVX on PD 12 requires over 43 days to be expressed, yet the corpora callosi of adult rats were examined only 32 days after OVX. It may be that if F&D had waited another 2–4 weeks, a change in corpus callosum size would be detectable. This is particularly important because the feminizing effect of estrogen takes so long to influence the size of the corpus callosum. Moreover, F&D have not tried to replace estradiol following the presumed critical period to determine whether there is indeed a time-limited window for the developmental effect.

A second argument against F&D's interpretation of androgenic and estrogenic influences as parallel processes lies in the lack of effect of estrogen-feminization manipulations on males. While the manipulation of perinatal androgens has similar consequences for both males and females over a wide range of tasks and measures (see sects. 3 and 5.4), only females appear to be affected by manipulations of estrogen during the prepubertal period. Also, the fact that sex differences in callosal size are apparent at 3 days of age (Zimmerberg & Scalzi 1989) indicates that ovarian secretions are not required for the expression of this sexual dimorphism.

A more parsimonious way to account for the data is to remember that the responsivity of the female brain (e.g., corpus callosum) to estrogen during the prepubertal period may simply be the result of differentiation that occurs perinatally. This is certainly not a novel idea. Phoenix et al. (1959) proposed that "androgen administered prenatally has an organizing action on the tissues mediating mating behavior in the sense of producing a responsiveness to exogenous hormones which differs from that of normal adult females" (p. 369). For example, it is well known that male rats and female rats that have been exposed to androgens perinatally do not show a positive feedback effect of estrogen (Karsch et al. 1973). We have recently discovered that acute estradiol administration increases spine density of hippocampal dentate gyrus granule cells in aged female rats, but not in male rats (Henderson et al. 1997). As in the case of the corpus callosum, a structural change is induced in female rats by estradiol, but male rats are unresponsive. In F&D's terminology, these findings indicate that females are "feminized" by estrogen (while males are not) at numerous times during their life span.

Though one might argue that the effects reported above are "activational" while the developmental effects of estrogen reported by F&D may be "organizational" (though as indicated above, the data are not yet convincing), it is clear that the distinctions between these presumably different types of hormone action are blurring. Activational effects can be seen early in development (Williams 1986); organizational effects can be seen in adulthood (Arnold & Breedlove 1985). In addition, it is now well accepted that activational and organizational effects of hormones can alter specific neuronal structures and functions. Furthermore, the work of Toran-Allerand (1995) suggests that estrogens may work by interacting with neurotrophins and their receptors or with other locally synthesized growth factors. This might represent a universal mechanism underlying the multiple and varied actions of estrogens at different stages of life.

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We feel that the crucial event for sexual differentiation is the presence or absence of androgens during the perinatal period. As a consequence of this experience, a cascade of other events occurs that further polarizes the behavior and morphological differences between males and females. One such event is probably the sexually dimorphic response to pubertal exposure to estrogens that normally occurs only in females. Another example might be the finding that androgen exposure perinatally leads to an increase in maternal licking, which has been found to further masculinize the male rat pup (Moore 1992). The responsivity of the corpus callosum to estrogen during late development is an important and intriguing finding that can be explained without the need to amend the "organizational theory" with a second parallel process.

Authors' Response

Default is not in the female, but in the theory

Roslyn Holly Fitch and Victor H. Denenberg

Biobehavioral Sciences Graduate Degree Program, University of Connecticut, Storrs, CT 06269-4154. **hfitch@psych.psy.uconn.edu; dberg@uconnvm.uconn.edu**

Abstract: A number of commentators agree that the evidence reviewed in the target article supports a previously unrecognized role for ovarian hormones in feminization of the brain. Others question this view, suggesting that the traditional model of sexual differentiation already accounts for ovarian influence. This position is supported by various reinterpretations of the data presented (e.g., ovarian effects are secondary to the presence/absence of androgen, ovarian effects are smaller than testicular effects, ovarian effects are not organizational). We discuss these issues, and reiterate our position that evidence of neurobehavioral ovarian effects is incompatible with the currently accepted model of sexual differentiation. Other points regarding species generalizations, the direct versus indirect action of estrogen, and nonhormonal mechanisms of sexual differentiation are also discussed. Finally, we address the controversial issue of using ratio scores in the assessment of the human corpus callosum (where CC scores are divided by an index of brain size). Future applications to human research are also discussed.

R1. Support and new data

We acknowledge the support of authors who commented favorably on the ideas put forth in our target article, including **Clarke, Döhler, Halpern, Hampson, Hugdahl, Juraska, Meyer-Bahlburg, Stewart,** and **Wahlsten & Bishop.** We thank these commentators and others who offered encouragement and constructive commentaries throughout the arduous review process.

A number of commentators go on to cite additional data consistent with the hypothesis of active ovarian effects on brain development: **Döhler, Juraska,** and **Williams & Sandstrom.** Indeed, Juraska's suggestion that the later loss of axons in the CC (corpus callosum) of female as compared to male rats may (though not yet empirically tested) be the consequence of ovarian exposure in females is consistent with the comments of **Clarke,** who noted that the emergence of sex differences in the human CC follows the presumed period of axonal withdrawal.

R2. Addressing alternate interpretations of ovarian effects

A number of other commentators take the position that data reviewed in our target article are neither novel nor revolutionary, and are already accounted for in the groundbreaking work of Phoenix et al. (1959). We address some of these assertions below.

R2.1. Ovarian effects: Primary or secondary? It is suggested by **Williams & Sandstrom** that the responsiveness of the female brain to estrogen is a secondary phenomenon resulting from the absence of testosterone in early sexual differentiation. They state that "the crucial event for sexual differentiation is the presence or absence of androgens during the perinatal period" and that evidence of ovarian influence on brain and behavior can be regarded as "the [secondary] result of differentiation that occurs prenatally." Whereas we refer to testicular masculinization and ovarian feminization as "parallel" processes (sect. 6.1), **Williams & Sandstrom** argue they are more accurately described as "serial" processes.

This distinction speaks to the temporal relationship between masculinization and feminization. Masculinization does occur first (in fact, the later sensitive period for feminization is a major point in our paper) and must *not* occur in order for feminization to occur. We do not dispute the importance of this juncture in differentiation. However, the female is not normally exposed to high levels of androgens (a largely experimental condition), and it appears superfluous to define the process of feminization by the absence of something not normally there.

Instead, we discuss differentiation of the female brain as a process defined by active ovarian effects, and sexual differentiation as a process where testicular masculinization and ovarian feminization reflect sex-specific steroid exposure, differing in origin, timing, and consequence. We discuss the traditional view of sexual differentiation, specifically that the absence of androgens during the perinatal period is *necessary and sufficient* to produce a female brain. We concur that it is necessary, but we have presented compelling evidence that it is not sufficient.

Other commentators remark that ovarian effects are of lesser magnitude than the effects produced by testicular manipulations. The issue of whether ovarian effects are "larger" or "smaller" than androgen effects, and whether they "build on and modulate the system that was formed earlier" (**Juraska**), does not seem to us to undermine the intrinsic importance of understanding what ovarian hormones are doing. That is the issue we address in our target article.

R2.2. Ovarian effects: Organizational or activational? Several commentators (**Hines, Williams & Sandstrom**) suggest that the effects of ovarian manipulations on the CC, as reviewed in our target article, could be construed as activational phenomena. Hines suggest that OVX effects on the CC may reflect "slow activational effects, rather than organizational influences," given the fact that the effects of day 12 OVX were not seen until 90 days of age (78 days later), but that females who received OVX on day 78 were sacrificed at 110 days (32 days later). She also suggests that failure to find a correlation between CC size and estrus phase reflects a slower time course for presumed activational effects of estrogen on the CC. Our interpretation was

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that the "late" divergence in CC size between day 12 OVX and sham females, which was not seen until 90 to 110 days of age, reflected the early timing of OVX and the delayed expression of changes in brain structure until after puberty, rather than a simple numeric time course for the expression of effects. Presumably this delay would not apply to females who received OVX as adults. Moreover, that morphological changes in hypothalamus and hippocampus of adult female rats in response to estrogen are seen within a matter of days (Frankfurt et al. 1990; Gould et al. 1990; Woolley et al. 1990; Woolley & McEwen 1992) would seem to undermine the premise that CC changes in adult females require over a month to be expressed. Nevertheless, the specific issue of organizational versus activational effects on the CC will only be resolved with additional empirical data.

On a larger scale, however, accumulated evidence clearly suggests an organizational role for ovarian hormones. Stewart and Cygan (1980) exposed OVX female rats to estrogen only during a delineated neonatal window and found changes in adult open-field behavior. Zimmerberg and Farley (1993) exposed neonatal female rats to an estrogen-receptor blocker but left the ovaries intact and nonetheless observed behavioral changes in adult plus-maze behavior. Denti and Negroni (1975) found effects of neonatal OVX on adult avoidance behavior that were opposite to those seen following postpubertal OVX (Diaz-Veliz et al. 1989). Forgie and Stewart (1994) found changes in behavioral activation to amphetamine following neonatal OVX in both primed and nonprimed adult females and, moreover, showed that these effects were not seen following adult OVX. Diamond and colleagues (1979) found that OVX on day 1 but not day 90 or day 300 altered cortical thickness, even though measures were taken 90 days later for each condition. These studies provide unequivocal evidence that ovarian manipulations can exert effects on brain and behavior that fall well within the traditional parameters defining "organizational" phenomena.

Williams & Sandstrom also suggest that in order for ovarian effects to be considered as a parallel, organizational process in sexual differentiation, we must provide evidence that there is closure for the critical period of sensitivity to ovarian hormones. Interestingly, other commentators noted specifically that the life-long sensitivity of the female brain to estrogen is a critical factor that permanently differentiates the female brain from the male brain.

Finally, **Williams & Sandstrom** state that for ovarian effects to be considered organizational, they must be demonstrated in prepubertal males. We disagree. A neural substrate that has already been masculinized is unlikely to respond in a female typical manner to later manipulations of estrogen. This only reflects the fact that masculinization occurs first and, having occurred, cannot be undone. Interestingly, early androgen exposure does not appear to eliminate sensitivity to ovarian hormones – at least in females – since some researchers have reported that the presence of the ovaries modified the behavioral effects of early androgen exposure. In a latter paradigm, however, it is not clear what effect high doses of androgen have on the subsequent activity of the ovaries. Since there are virtually no studies in which females are ovariectomized at birth, exposed to androgen, and compared to females who receive OVX followed by both androgen exposure and physiological estrogen replacement, we really have no idea how testicular masculinization and ovarian feminization interact in the intact system. Finally studies in which males are deprived of androgens prenatally (e.g., via treatment with androgen receptor blocker), castrated at birth, and then exposed to female-typical levels of estrogen, are also lacking. In this experimental scenario we would expect males to show female-like responsiveness to estrogen.

R3. Species generalizations

A number of researchers commented on the fact that the bulk of our conclusions derive from rat studies. One commentary questions whether our model can be applied to long-gestation species (**Wallen**), and comments on our failure to discuss evidence from long-gestation species (e.g., guinea pigs and rhesus monkeys), where differentiation occurs prenatally and estrogens appear to be "of little or no importance" (Wallen). We did, in fact, cite research demonstrating aromatase activity in the brains of fetal guinea pigs (sect. 3.1; Connolly et al. 1994). Connolly et al. concluded that "the guinea pig brain contains high levels of [aromatase] during the critical period of sexual differentiation," and that aromatase activity was measurable in parietal cortex. Such findings speak to the role of estrogen (derived from the aromatization of androgen) in masculinizing the guinea pig brain. We are not aware of comparable data that address the role of estrogen of ovarian origin in guinea pig or monkey development, and to our knowledge no such studies have been conducted. We note, however, the long-standing view that estrogens were of little or no importance in the sexual differentiation of a rather well-studied long-gestation species, humans, and the recent accumulation of evidence showing that estrogen is important to the brain and behavior of women after all (**Stewart, Halpern;** see also Fitch et al. 1998).

Wahlsten & Bishop ask how we can justify our argument (and that of many other researchers) that findings with rats can be generalized to humans, at least in principle, when results with rats cannot be generalized to "genetically and ecologically closely related house mice." A major reason (though not the only one) is that the house mouse used in research is not genetically and ecologically closely related to the rat. The mice used in biological research are inbred strains. These animals are produced by brother $\times$ sister matings for at least 20 successive generations (sometimes much, much more). This brings about three major results. First, all genetic loci are homozygous, meaning that they are either AA or aa. None of the loci will be of the form Aa, which is always found in natural breeding populations. Second, all animals of the same sex within an inbred strain are "identical twins" (or clones). Third, excepting the X and Y chromosomes, the genes of males and females are also identical. Thus, the purpose of mouse breeding is to generate genetically homozygous animals. There is no heterozygosity within an animal, and no heterozygosity among the autosomes within a strain. These restrictions are what make the inbred mouse so valuable as a research tool for genetic studies.

In contrast, the breeding protocols used with the usual laboratory rat (e.g., Long-Evans, Sprague-Dawley, Wistar) avoid brother $\times$ sister matings and try to maintain some degree of the genetic heterozygosity typical of a "natural" population. Thus, in research using inbred mouse strains, the resulting variance (whether measuring a behavioral or a biological variable) is not due to any genetic factor – all

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mice are genetically identical (except for sex). Conversely, there is genetic variability in most rat colonies, and it is this genetic variability that may allow one to generalize from rat to human but not from rat to mouse.

Finally, evidence presented by **Baum & Tobet** suggests that the female rat is exposed to unusually high prenatal levels of testosterone derived from the placenta, and as such may represent a hormonal anomaly. Hence evidence from rats demonstrating attenuating effects of ovarian hormones on sex differences cannot be readily applied to other species, where greater sexual divergence in testosterone exposure characterizes the prenatal period. In response, we must point out that sex differences in prenatal androgen exposure in the rat are apparently of sufficient magnitude to nevertheless produce a broad array of neuroanatomic and behavioral sex differences. With respect to the CC, these differences are sufficient to produce sex differences in size as early as 3 days of age (Zimmerberg & Scalzi 1989). Therefore it would appear that female rats are not exposed to "masculinizing" levels of androgen prenatally. Nevertheless, **Baum & Tobet's** point about the ability to generalize data obtained almost exclusively from rats is a concern, and we agree with them that "it will be important for experimental studies to be extended into nonrodent species to allow the testing of specific hypotheses about the mechanism of hormone action in establishing sex differences in the formation of this fiber bundle [the CC]."

R4. Routes of estrogen action: Direct or indirect

Research by **Rogers** calls into question whether estrogenic effects on the brain occur via direct or indirect routes. **Rogers** cites differential maternal licking of male and female pups and the effect of such differences on the consequent emergence of sex differences in behavior as one possible route whereby steroids can affect behavior indirectly (in this case, by altering maternal response to pup urine). We consider it unlikely that this particular mechanism could account for sex differences in the rat CC because sex effects are already seen at 3 day of age (Zimmerberg & Scalzi 1989), and since Day 1 gonadectomy in males does not alter CC size despite affecting maternal behavior (Fitch et al. 1991). Moreover, ovariectomy (OVX) effects on the CC were seen for both handled and nonhandled female pups, an intervention that markedly alters maternal behavior toward pups. It is nevertheless highly likely that future research will continue to elucidate complex interactions between environmental experience and the effects of steroids, including those of ovarian origin, on brain and behavior.

R5. It's not all hormonal

Although we have emphasized the role of ovarian estrogen in organizing the female brain, it must be noted that there are other mechanisms that are likely to bring about sex differences. In a commentary on an earlier version of the target article which appeared in *Psycoloquy*, McCarthy (1995) notes the possibility that other ovarian products, including growth factors, prostaglandins, oxytocin, progesterone (acting directly, as an antiandrogen, or as a GABAergic neurosteroid), and inhibin, as well as external factors such as afferent input, may also affect sexual differentiation. McCarthy also notes the possibility of direct genetic contributions to sexual differentiation.

Indeed, the Y chromosome contains many genes in addition to the testis-determining gene, and there is evidence that some of these genes participate in bringing about sex differences. One example is Wai-Sum et al.'s (1988) finding of anatomical differences in marsupial embryos before the onset of gonadal hormone production in either sex. We have recently been studying the behavior of mice that are phenotypic females but genotypic XY males (Seaman & Denenberg 1996; 1997). The cause of this anomaly is that the testis-determining gene on the Y chromosome is not activated in the male (Eicher et al. 1995). We found that these phenotypic female mice did not differ from genotypic control XX females on open-field activity, shuttlebox avoidance learning, or water escape learning. Moreover, the scores of both groups differed significantly from control males, indicating that androgen exposure (or more precisely, the presence or absence of the testes) was a determining factor in the sex differences (Seaman & Denenberg 1996). Interestingly, however, the two female groups did differ on the visuospatial Morris maze, with the XY "females" having the better scores. We have recently replicated this finding (Seaman & Denenberg 1997). These data imply that the often found sex difference in spatial learning is not mediated by androgen alone, but is also affected by other processes associated with the Y chromosome.

R6. Callosal measures: Absolute or relative, the debate continues

No one issue raised in the target article generated as much controversy as whether callosal measures are appropriately "corrected" by some measure of brain size in men and women. This controversy supports **Halpern**'s observation that "the debate over sex differences in the human corpus callosum is more often acrimonious than scholarly."

In our target article we suggested that studies intended to assess the existence of sex differences within the human CC are most appropriately conducted using absolute measures of size. We advanced this view based on the fact that (1) we did not obtain significant correlations between CC size and brain size in any of our rat studies, and subsequent factor analyses using a large sample failed to load brain weight and callosal area on a combined factor; and (2) most of the studies that report male-female analyses of the human CC fail to report correlations between CC size and any measure of brain size. Where correlations are found, they do not appear to be that strong and may be found in one group and not another (data reviewed by **Holloway** are a case in point). Nevertheless, we recognize the diversity of opinion on this issue and we address the various arguments and counterarguments raised below.

R6.1. Adjusting CC size: Ratio numbers, regression analysis, and pseudostatistics. It is first necessary to clarify a misunderstanding. **Wahlsten & Bishop** write: "F&D argue that in general CC size should not be corrected for whole brain size unless the correlation with brain size is large and significant." That is not what we wrote. Our words (emphasis added) were: "Even if one finds a significant

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correlation in a particular sample, *the practice of dividing one number by another is not appropriate unless the correlation is high.* The correct statistical procedure is to use a regression analysis or analysis of covariance to remove the linear effects of the second variable." The regression analysis is also what **Wahlsten & Bishop** recommend. Thus, there is no fundamental disagreement between us.

This leads to the topic of using ratio measures of CC/brain size. **Wahlsten & Bishop** agree with us that this procedure is rarely justified. Several other commentators generally agree, though offering exceptions. **Hugdahl** agrees about ratio numbers and about the need for a significant correlation before one can justify making a statistical correction. He adds the proviso, however, that the significant association must also have a theoretical foundation, otherwise there is a danger that the correlation is spurious, a happenstance. We agree that one should always make use of a strong theoretical base when it is available. Neuroscience (including behavioral neuroscience) is still basically empirical, however, and most of us would be loath to throw away a significant correlation for lack of a meaningful rationale. Nor would one necessarily embrace the correlation even if a rationale were available. The scientific reflex response in this situation is to replicate the study and determine whether the correlation holds up. If so, one can then give serious thought to the implications of the finding.

Halpern suggests that whether proportional measures are meaningful or useful depends on the question being asked. No one would disagree with this proportion as long as the question is meaningful. In this context a meaningful question would have to (1) meet the mathematical requirements of a ratio scale, including the demonstration of a significant correlation within the group being examined and, as described by **Wahlsten & Bishop,** a straight line isometric relationship between the two variables (see sect. R6.2 for further discussion); it would also have to (2) lead to the demonstration of functional validity (that is, a functional justification for the mathematical relationship between the two variables). Neither of these requirements has been met, to our knowledge, for measures of CC and whole brain (or even cortex). **Aboitiz** speaks to the issue of functional validity by presenting the metaphor of a town divided in two and connected by only one bridge. Initially, the bridge is adequate to handle all the traffic, but as the town grows and traffic increases, a traffic problem will occur. This metaphor breaks down because it assumes that all the vehicles in both towns are equally likely to want to cross the bridge; that is not the way the brain is wired.

Several commentators took the position that the use of ratio measures is acceptable because arguments against use of the procedure are not convincing. **Holloway** simply states that he and colleagues set out to test the hypothesis "that relative to the total size of the brain, a brain structure, namely, the corpus callosum, is larger in females" without giving a rationale for this hypothesis.

R6.2. The Holloway et al. data. Because **Holloway** takes serious exception to our comments concerning the inappropriateness of ratio scales, we will briefly review the Holloway et al. (1993) paper described in his commentary. In that paper there were three autopsy samples designated as a Columbia, a Mt. Sinai, and an Australian Aboriginal sample. In all three, males had significantly heavier brains than females. In none of the samples was there a significant sex difference in CC area. An ancova was done to determine whether there was a sex difference in CC area after brain weight was removed as a covariate. Apparently this was first done with all three samples pooled (no information is given in the text). No significant effect was found. Holloway et al. then did an ancova on just the two New York samples and again found no significant sex effect. For the latter analysis the correlation between brain weight and CC area for both samples and both sexes combined was $r = .3482$. As Holloway indicates in his commentary, this correlation was the result of pooling the significant correlation obtained in the male sample with the insignificant correlation obtained in the female sample (a questionable practice). Thus, when regression analyses were conducted to remove the linear effects of the brain weight differences, Holloway et al. found no evidence that the female CC area was statistically different from the male CC area.

In continuing analyses, Holloway et al. divided a subject's CC area by the $\frac{2}{3}$ root of brain weight. The resulting statistic was called RELCC (relative CC size). The Columbia sample did not differ on that statistic, but the Mt. Sinai and the Australian samples found females to have significantly larger RELCC values than males. Our position has been, and still is, that this use of this ratio is invalid and generates pseudostatistical data. What Holloway et al. have done is to create a ratio in which the numerators are not different (males and females do not differ on CC area), but the denominators are very different (males have larger brains than females). Obviously, the ratios will differ, but this only reflects the large mean difference in brain size. In essence, they are evaluating the reciprocal of brain size and are finding, to no one's surprise, that the females have a larger value. That obviously tells us nothing about the CC.

With respect to the use of ratio scores, measurement theory states that an "absolute zero" is needed to justify a ratio scale. **Wahlsten & Bishop** state specifically that the regression line relating these two variables must go through the coordinates (0,0). That is, when the CC (X) is set equal to zero, the brain value for Y (i.e., the Y-intercept) must also be 0. Moreover, these structures must grow in direct proportion to each other, else the ratio of their measures will change over time. From a developmental perspective, no one has addressed this precondition before using a CC/brain ratio. Indeed, the lag between cortical and CC development (with CC fibers crossing after embryological cortical tissue has been established) might be said to violate this principle. If so, then according to **Wahlsten & Bishop,** the relationship is not isometric but allometric, and the ratio between the sizes of these structures will change as the brain grows. This means using a ratio score will not properly remove the influence of brain size from CC data.

Although the validity of an isometric relationship has not been tested, an allometric relationship is clearly seen when the brains and CCs of animals (including humans) of differing ages are measured. These variables will be significantly correlated because of common growth processes (although if CC/brain growth is isometric, this correlation should be very high). In our research, however, all animals are sacrificed at the same age. Thus, we have eliminated all variance associated with allometric growth. This is certainly one major reason why we find low and usually insignificant correlations.

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In summary, a number of the commentators have agreed with us and none of those who disagreed have raised persuasive objections. Our position remains that the procedure of dividing brain size into CC area as a "correction factor" is incorrect. Regression (covariance) analysis is the proper procedure to use to remove the effects of an extraneous variable. It is important to reiterate that if no relationship exists between brain size and CC size, the regression analysis will not change the results of the statistical analysis. This point has also been noted by several of the commentators.

R6.3. Individual differences in CC/neurobehavioral relationships. Several research groups have found significant relationships in the human between CC measurements (obtained via neuroimaging or postmortem analyses) and other neurobehavioral measures. **Hampson** cites a recent study from her laboratory in which a significant positive correlation was found between salivary testosterone samples and midsagittal area of the posterior section of the CC in healthy men. **Hugdahl** found significant correlations between left ear performance and callosal size in a dichotic listening task, particularly for the three most posterior CC sectors. **Aboitiz** (1992) reported an inverse correlation between anatomical asymmetry in the Sylvian fissure and the size and number of fibers in specific regions of the CC in the postmortem brains of men but not women. **Hines** and colleagues (1992) reported a negative correlation between the size of the posterior callosum and language laterality scores in a sample of adult women. With respect to animal research, two studies have correlated callosal measures against behavioral scores. Zimmerberg and Mickus (1990) found a negative correlation of −.552 between callosal area and open-field activity in adult female rats and a correlation of −.511 for males. Mack and Denenberg (1993) reported a significant correlation (−.82) between the callosal width in the posterior-most region and open-field activity scores of handled female rats.

Clarke, however, sounds a cautionary note concerning the interpretation of CC behavior correlations. She summarizes research showing that there are many heterotopic connections across the callosum in humans. Fortunately, in the genu and splenium (where fibers from prefrontal cortex and occipital cortex, respectively, cross) most connections are homotopic. Thus **Hampson**'s, **Hugdahl**'s, and **Hines**'s data probably reflect differences in CC fibers that originate primarily in occipital cortex.

R6.4. Cellular analysis of the CC. Ultimately the dispute about how to measure gross size of the CC will be rendered moot, because as many commentaries point out, continuing studies must address sex differences in the specific regional cellular composition of the CC (**Aboitiz, Baum & Tobet, Clarke, Juraska**). Indeed, an anonymous reviewer of the third draft of our manuscript made the cogent observation that the number of axons within a given region of the CC, and whether or not they are myelinated, are the critical factors that modulate functional activity. From this perspective the relative amount of CC area is irrelevant. We found this such an astute comment that we unabashedly put it into our manuscript and take this opportunity to thank the reviewer. Let us once again emphasize that we have used measures of gross callosal size as a benchmark of sex differences in order to assess and delineate the parameters of hormonal influence on sex differences in this structure. We acknowledge that these efforts represent a first pass, and that it will remain for cellular anatomists such as **Aboitiz, Clarke,** and **Juraska** to delve into the specific cellular parameters that underlie sex differences in gross size of the CC, and behavioral biologists such as **Hugdahl** to further uncover the functional relevance of sex differences in gross size and cellular composition of the CC.

R6.5. What does sexual dimorphism mean? Finally, **Wahlsten & Bishop** raise the valid point that use of "dimorphism" in the literature does not agree with its dictionary definition; on that definition, the CC of neither rat nor human is dimorphic. **Wahlsten & Bishop** recommend restricting this term to situations where the effect size is 4 or greater. We agree that the word is often used inaccurately and that it is probably best to reserve the word for conditions where there is minimal overlap in the populations, though it is perhaps too much to ask to demand a minimal effect size of 4 before being permitted to utter the "d" word. (We have not used "dimorphic" in our response to the commentaries.) We also recommend, along with **Wahlsten & Bishop,** that researchers report effect sizes with their other statistical tests.

R7. The callosum as a cortical map

Though we have focused on the CC throughout this discussion, it is important to remember that no neurons originate there. Neurons originate in specific cortical areas and pass through the CC to make homotopic or heterotopic connections in the opposite hemisphere (**Clarke**). Thus, by studying the two-dimensional sagittal section of the CC, we gain insights about the structure of the three-dimensional brain, much like the relationship between a two-dimensional map and the three-dimensional world it represents. Hence, when we state that estrogen acts to organize the CC, we are really saying that estrogen is acting to organize the brain structures that project fibers through the CC. In our studies with the rat we have consistently found our major effects of estrogen in the splenium and the genu. This means that the occipital cortex and the prefrontal cortex are specific target sites for the organizing effects of estrogen.

In this regard, Mack et al. (1995) and **Juraska** (Kim et al. 1996) have intriguing findings on CC composition in the splenium and genu. Mack et al., in an electron microscopic (EM) study of the rat's genu, found females to have a greater number of unmyelinated fibers than males, with no difference in the number of myelinated fibers. Thus, for the males, a greater proportion of their fibers were myelinated. Juraska's EM study of the rat's splenium found that males had more myelinated axons than females. The general consistency across two distinctly different CC regions suggests that fast transmitting myelinated fibers are associated with males, whereas females have a greater number of slower transmitting unmyelinated fibers.

R8. Application to humans

If we map the EM data described above onto **Hugdahl**'s "two-channel" model, males would appear to be more specialized for rapid sensory information transfer (via more myelinated fibers), whereas females are transmitting a greater amount of cognitive information across the CC, but at a slower rate (via more unmyelinated fibers). Insofar as

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we can generalize to the human, these findings suggest that developmental ovarian effects may contribute to sex differences in visual perception (occipital cortex) and complex decision-making processes (prefrontal cortex). Long-term neurobehavioral research will be required to test such hypotheses.

Several commentators (**Halpern, Hampson, Meyer-Bahlburg, Stewart**) noted that data reviewed in the target article may open new avenues of study among human populations. **Stewart** notes the requirement that the female brain remain sensitive to ovarian hormones throughout life in order to support the cyclic changes in the hypothalamic-pituitary-gonadal axis, and comments that this factor "may open the way for other instigators of neuronal plasticity, making the female brain different from that of the male in its response to the actions of a number of hormones, to injury, and to aging." We wholly agree with this point, although we did not emphasize it in our target article. Supporting evidence concerning the existence of male/female differences in response to brain injury among humans includes reports of higher numbers of males than females with neurodevelopmental disorders, as well as better cognitive recovery in female as compared to male premature infants with comparable intracranial "bleeds" (Raz et al. 1995). The potential role of ovarian hormones in mediating these differences has not yet been considered (for further discussion see Fitch et al. 1998).

Moreover, accumulating evidence now points to a critical role of estrogen in the incidence and management of clinical disorders in women (e.g., Alzheimer's disease), as well as in the normal aging process (reviewed in Fitch et al.1998). We share the hope of **Halpern** and **Stewart** that evidence concerning the role of estrogen in modulating the female brain across the life span may prompt renewed interest in studying the effects of normal hormonal variation in human females (e.g., menarche, pregnancy, lactation, oral contraceptive use, menopause, and estrogen replacement therapy) on brain and behavior.

Finally, data presented in the target article may prompt a reinterpretation of neuroanatomic and behavioral data obtained from human populations with endogenous endocrine disorders, as well as exogenous hormonal exposure, as noted by **Hampson** and **Meyer-Bahlburg.**

R9. Summary

In summary, we have presented, reviewed, and defended data that we believe speak to a heretofore unrecognized role for ovarian hormones in sexual differentiation of the female brain. Data obtained by studying the rat CC suggest that this cortical structure is one developmental target of ovarian influence. Moreover, sex differences in specific regions of the callosum reflect sex differences in cortical areas of axonal origin that, in turn, must be sensitive to gonadal steroids. We hope that future research will enable us to assess the effects of ovarian hormones on the cortex itself, as well as the relationship between cortical and CC structure and, ultimately, complex behavior. The implications of these findings for humans are many. We hope that our target article and the commentaries it has elicited will influence the way in which studies investigating hormonal contributions to neurobehavioral sex differences are designed and conducted.

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Letters "a" and "r" appearing before authors' initials refer to target article and response, respectively.

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