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Review

Prenatal sex hormone effects on child and adult sex-typed behavior: methods and findings

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Abstract

There is now good evidence that human sex-typed behavior is influenced by sex hormones that are present during prenatal development, confirming studies in other mammalian species. Most of the evidence comes from clinical populations, in which prenatal hormone exposure is atypical for a person's sex, but there is increasing evidence from the normal population for the importance of prenatal hormones. In this paper, we briefly review the evidence, focusing attention on the methods used to study behavioral effects of prenatal hormones. We discuss the promises and pitfalls of various types of studies, including those using clinical populations (concentrating on those most commonly studied, congenital adrenal hyperplasia, androgen insensitivity syndrome, ablatio penis, and cloacal exstrophy), direct measures of hormones in the general population (assayed through umbilical cord blood, amniotic fluid, and maternal serum during pregnancy), and indirect measures of hormones in the general population (inferred from intrauterine position and biomarkers such as otoacoustic emissions, finger length ratios, and dermatoglyphic asymmetries). We conclude with suggestions for interpreting and conducting studies of the behavioral effects of prenatal hormones.
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There is now good evidence from human and nonhuman species that events occurring during prenatal development can have life-long effects on an organism. These effects are not limited to physical characteristics, but extend to a variety of behavioral traits. Thus, as described in several papers in this special issue and elsewhere, physical and emotional stressors experienced by pregnant rodent, monkey, and human females are associated with behavioral problems in offspring throughout life [1–3]. The physical and behavioral effects of prenatal stress appear to be mediated by hormone-induced changes to the developing hypothalamic–pituitary–adrenal axis [3,4].

Long-term effects of prenatal events extend beyond exposure to stress hormones. There are also marked physical and behavioral consequences of prenatal exposure to another category of hormones, those produced by the gonads ('sex hormones'). In all mammalian species studied, sexual differentiation of the reproductive system depends largely on the amount of androgens present during critical periods of prenatal life. In human beings, this critical period begins at about 7–8 weeks of gestation when the testes develop and begin to secrete testosterone [5]. The external genitalia are undifferentiated until then, and the amount of testosterone (or other androgens) determine whether they differentiate into male-typical or female-typical genitalia. With high levels of testosterone, the genitalia become the penis, scrotum, and urogenital sinus, whereas low or absent testosterone results in the development of the clitoris, labia majora, and separate vaginal and urethral canals. Intermediate levels of testosterone result in ambiguous genitalia, e.g. an enlarged clitoris with fused labia, a small penis. Sex hormones also affect the development of the internal reproductive structures.

Parallel behavioral studies in nonhuman mammals clearly show that the same prenatal hormones responsible for sexual differentiation of the body are also involved in sexual differentiation of behavior [for reviews, see 6–8]. In rodents, females injected with high doses of androgens in the newborn period show behavior more typical of males than of other females, and males that are castrated or given anti-androgens show behavior more typical of females than of other males. The behaviors involved include adult sexual behavior, juvenile rough play, adult aggression and maze performance. These effects are also found in rodents naturally exposed to atypical hormone levels from gestating next to opposite-sex littermates, as described below.

Behavioral effects of early hormones are also found in nonhuman primates: female monkeys exposed to androgens early in development are masculinized with respect to sexual behavior, rough play, grooming [9], and some learning abilities [10,11]. Studies in monkeys confirm and extend studies in rodents in two important ways. First, they illustrate the complexity of timing effects and show that there may be several distinct sensitive periods for androgen effects on behavior, even within the prenatal period, so that some behaviors are masculinized by exposure early (but not late) in gestation, whereas other behaviors are masculinized by exposure late (but not early) in gestation [9]. For example, monkeys that received androgens early in development and had masculinized genitals showed increased mounting of peers and mothers and less grooming behavior, whereas those exposed later in development had normal-looking genitals but showed increased rough play and mounting of peers, but not mounting of mothers. Second, studies in monkeys show the importance of environmental context in modifying the behavioral effects of hormones. For example, the social environment of juvenile monkeys modifies the expression of behavior that is influenced by hormones [12]. The behaviors that are the least variable across social contexts are the most affected by prenatal hormones.

The purpose of this paper is to describe behavioral effects of sex hormones in human beings, reviewing evidence that increasingly shows findings and principles derived from rodent and primate studies to apply to people as well. We focus on the methods used to obtain this evidence, given the increasing interest in this topic and the increasing availability of methods that can be used, even in typical populations. Most of the early studies exploring behavioral effects of sex hormones were conducted in clinical samples, i.e. those whose hormones were unusual because of disease or accident. Such 'experiments of nature' have limitations related to alternative explanations and generalizability, so it is important to examine convergence of evidence across methods with different limitations. It is even more important to extend this work to studies of typical populations to

examine generalizability, and this has become possible as a result of methodological advances from clever researchers. This paper is organized around the methods used to study behavioral effects of hormones, beginning with clinical samples, moving to studies in typical populations involving direct measures of prenatal hormone exposure, and then to studies in typical populations using indirect measures of hormone exposure. For each, we describe the background of the method, provide an overview of findings of studies using the methods and an interpretation of the findings in the context of the strengths and weaknesses of the method, and conclude with recommendations for researchers reading studies or using the method themselves. We end with a summary of the evidence regarding the long-term behavioral consequences of exposure to sex hormones during prenatal development and suggestions for future research.

1. General considerations and caveats

Before moving to the specific methods, we highlight a number of theoretical and methodological issues relevant to evaluating all studies of human hormone–behavior relations.

1.1. Theoretical issues

1.1.1. Timing of effects

1.1.1.1. Organizational vs. activational effects. Two types of hormonal effects have generally been distinguished: organizational and activational [8]. Organizational effects produce permanent changes in the wiring and sensitivity of the brain [13] and are thus largely irreversible; they are most likely to occur during early development when most neural structures are established. Activational effects occur later and are associated with concurrent changes in circulating hormone levels, for example, those associated with menstrual cycle variations [14,15]; here, hormones activate neural systems that were organized early in life. The distinction between organizational and activational hormones is not as clear as once believed [16]. For example, the human brain continues to develop into adolescence, so hormones that increase at puberty may change brain structure. Organizational hormones may prime the brain by changing its responsivity to hormones that are present later in life [17]. Nevertheless, most work is based on the distinction between organizational and activational effects, so we maintain it here. Because circulating sex hormones are low before puberty, any hormones affecting behavioral sex differences in childhood are likely to be due to organizational effects, i.e. hormones producing changes in the structure of the brain. Behavioral sex differences in adolescents and adults may reflect effects of organizational hormones or activational effects, given the large sex differences in the levels of circulating sex hormones after

puberty. The focus of this paper is on organizational effects occurring during the prenatal period, although there is good evidence for behavioral effects of activational hormones as well [see e.g., 6,15,18–20].

1.1.1.2. Sensitive periods for organizational effects. It is widely accepted that organizational effects are maximal during circumscribed sensitive periods when the brain is developing, but the exact sensitive periods for human behavioral effects of sex hormones are not known. Weeks 8–24 of gestation have long been considered the key period [e.g., 21,22], given data showing a testosterone surge in male fetuses then [23]. Nevertheless, there is increasing recognition that there may be multiple sensitive periods, and that different brain regions (and thus different behaviors) may be affected by hormones at different times. There are only limited data on this issue in human beings [24,25], but ample data from other species, including primates, illustrate this point [8]. For example, as mentioned above, data from rhesus macaques show that androgen exposure early in gestation masculinizes different behaviors than exposure late in gestation [9].

There may be another sensitive period shortly after birth, associated with another peak in testosterone in male infants during postnatal months 1–5. Its significance for human behavior is not well studied, but some information can be gleaned from studies in infant male monkeys who exhibit the same early postnatal peak. Those studies show that neonatal testosterone is important for genital development [26,27], but there is not clear evidence for its role in behavioral development: neonatal testosterone has been found to affect mother-offspring interaction in one study of juveniles [28], but not in another study of infants [29], and not to affect sex-dimorphic play or sexual behavior [28–30]. Given continuing postnatal brain development, it would not be surprising to find that sex hormones continue to affect the brain and behavior after birth.

1.1.2. Feminization as an active or passive process?

Much of the evidence about both physical and behavioral sexual differentiation focuses on the masculinizing effects of androgens. For a long time, it was believed that feminization is a passive process, occurring in the absence of high levels of androgens. There is increasing recognition of the importance of other hormones for complete feminization, but much is still unknown about this process [5]. The ovary develops at about 3 months of gestation, but does not produce estradiol until later in embryogenesis [31] and even then does not produce significant amounts [5,32]. Fetuses of both sexes are exposed to high levels of estrogens from the placenta, perhaps explaining why estrogen does not play a large role in prenatal development. Very little is known about the human behavioral effects of ovarian estrogens during early development (organizational effects) [21], although much is known about their effects during adulthood (activational effects) [e.g., 6,15,19,33,34–35].

It has been suggested that estrogen's organizational effects occur during early postnatal rather than prenatal development [36,37], but there is no relevant evidence. Progestins from the ovary are thought to have anti-androgenic effects, but there are also reports that they act as androgens [21], and it may be important to consider that effects vary by dose [38].

1.1.3. Subtleties of hormone action

1.1.3.1. Hormone levels. In nonhuman species, hormonal effects on behavior are dose-dependent (animals exposed to high doses change more than animals exposed to low doses), and the amount of hormone necessary to masculinize or defeminize behavior varies across behaviors (a given dose changes some behaviors more than others) [8,39]. Some behaviors may be affected only by extreme variations in hormones, whereas others may be affected by relatively minor variations. Effects may be nonlinear. For example, there may be threshold effects, such that behavior is affected only when hormone levels exceed a specific point, with no additional effect with increasing levels.

1.1.3.2. Specific hormones. Masculinizing hormones come in many forms, and each affects different aspects of physical and behavioral sexual differentiation. For example, dihydrotestosterone is the metabolite responsible for differentiation of the external genitalia [40]; dihydrotestosterone and another metabolite of testosterone, testosterone propionate, have different effects on learning abilities in monkeys [10]. Some metabolites are more potent masculinizing agents than others, e.g. dihydrotestosterone is more potent than testosterone in masculinizing the external genitalia, but this has not been well studied with respect to human behavior. In some species, it is estradiol metabolized from androgen in the brain that is responsible for the masculine-typical development of some behaviors. For example, in rodents, estradiol masculinizes sexual behavior and learning [7,8,41], but testosterone itself or dihydrotestosterone masculinizes rough play [42].

Further, the effects of specific hormones depend on other hormones present, so that the presence of one hormone may promote or prevent the effect of another hormone [8]. For example, as mentioned above, progesterone may provide protection against masculinizing effects of androgens [43–46].

1.1.3.3. Hormone responsivity. Individuals vary not only in the levels of hormones to which they are exposed, but also in their sensitivity to those hormones. For example, variations in androgen responsivity caused by mutations in the human androgen receptor gene result in physical variations in men ranging from complete insensitivity to androgens (and thus female differentiation, as discussed below) to infertility and minor undervirilization [47,48]. There has been very little study of the behavioral effects of

variations in androgen sensitivity [49], but clearly this is something worth exploring.

1.1.4. Cross-species comparisons

Human studies are largely motivated by studies in other species, and largely confirm those studies in the general outline, that is, in revealing the behavioral importance of sex hormones present during early development. But it is crucial to recognize that the details of hormonal influences on human behavior do not always parallel those effects in other species given species differences in physiology. Key examples concern the sensitive periods for hormone effects and the specific hormones responsible for masculinization.

The sensitive periods differ dramatically across species, in direct relation to the timetable of brain development. In rodents, brain development occurs during both prenatal and postnatal life, so the early postnatal period continues to be a sensitive period for the effects of sex hormones. In primates, however, much of structural brain development takes place prenatally, so this represents the most important sensitive period for brain and behavioral effects of sex hormones. Nevertheless, as noted above, the postnatal period may turn out to be another sensitive period for effects of hormones on some aspects of human behavior.

The specific hormones responsible for masculinization and defeminization of the brain and behavior may also differ across species. In rodents, these processes are largely dependent on estradiol as it is converted from testosterone in the brain via the action of the aromatase enzyme ('aromatization'). Female rodents are protected from the masculinizing effects of estrogen by a protein which binds circulating estrogen and prevents it from entering the brain [for reviews, see 8,37]. It is unclear whether aromatization is important for human behavior, although the limited evidence suggests that it is not, with masculinization and defeminization resulting directly from the effects of testosterone or other metabolites (see the discussions of Complete Androgen Insensitivity and exposure to diethylstilbestrol, below).

1.2. Methodological issues

1.2.1. Measures

Studies in other species suggest—not surprisingly—that sex hormones affect behaviors that show sex differences. Accordingly, human studies examining behavioral effects of sex hormones have been considered valid only when the measures used differentiate between typical males and females [e.g., 21,24,50–52]. Further, it is generally believed that the same factors that produce between-sex differences produce within-sex variation. For example, sex differences in early androgens are hypothesized to contribute to sex differences in spatial ability, and natural variations in levels or availability of androgens among normal males and females to within-sex variability in spatial ability. But, some have advocated

the use of a broad range of behavioral measures, arguing against simple extrapolation of findings in animals to humans [53], or that hormones may produce sex differences in brain organization and behavioral processes without necessarily producing average differences in performance [36]. Studies from this perspective have been exploratory in approach.

1.2.2. Effect sizes

The ability to detect behavioral effects of hormones, as any effect in science, requires that the study has sufficient statistical power. Effect sizes in this field vary considerably by behavior, and the design and interpretation of all studies require attention to the size of effect expected. Behaviors that show large sex differences are the best candidates for studying effects of hormones. Table 1 lists a representative sample of sex-related behaviors, along with the direction and general size of the sex difference in standard deviation units, d [54], estimated from the studies reviewed below and others in the literature [e.g., 55–62]. The size of the sex difference should be considered in interpreting the studies described in the sections below, because a measure that does not show a large sex difference is unlikely to be strongly influenced by sex hormones. (Despite appeals to use other measures, as mentioned above, it is difficult to interpret findings when measures do not show sex

differences.) It is also valuable to consider the magnitude of hormone effects in relation to the magnitude of the sex difference, because this provides an indication of the extent to which hormones are likely to be responsible for the sex difference.

2. Studies in clinical populations

The early studies in animals showing behavioral effects of early hormones prompted studies of people with atypical hormone exposure, i.e. in which the sex hormones are higher or lower than expected for a person's sex [63]. Early studies involving such 'experiments of nature' suggested an important role for sex hormones, confirming studies in other species, but they were criticized for methodological limitations, especially subjective measures and insufficient controls. Recent studies with improved methodology generally confirm the early studies, but also show the complexity of hormonal influences on behavior.

There are a variety of conditions in which there is a mismatch between a person's sex hormones and other aspects of sexual differentiation [5], but most conditions are very rare and have not been well-studied, especially with respect to behavior. We focus here on a few conditions for which there are some data and which illustrate the promise and the pitfalls for investigating behavioral effects of sex hormones.

2.1. Congenital adrenal hyperplasia

2.1.1. Background

The best-studied clinical condition is congenital adrenal hyperplasia due to 21-hydroxylase deficiency (CAH), probably because it is one of the most common problems of sexual differentiation, with an incidence of 1 in 10,000 to 1 in 15,000 live births [5,64]. Because of an enzymatic defect caused by a single gene, individuals with CAH produce high levels of adrenal androgens beginning very early in gestation. Postnatal treatment with corticosteroids (and mineralocorticoids for the 75% who are salt-wasters) reduces hormone levels, generally to normal or subnormal levels [65]. Both sexes are affected by CAH, and both have been studied behaviorally, but studies in nonhuman species enable clearer hypotheses for females than for males. Studies in male rodents and primates show that excess androgens may masculinize or demasculinize behavior, but generally has no effect. Studies in female rodents and primates, however, clearly show the masculinizing and defeminizing effects of excess androgens. Therefore, if human behavioral sex differences are affected by the levels of androgens present during sensitive periods of development, as occurs in other species, then females with CAH should show more 'male-typical' and less 'female-typical' behavior than control females. And they do in many, but not all, ways, with the size of the effects varying across

Table 1
Representative sex differences in behavior

Trait	Direction of sex difference	d, Size of sex difference[a]
Cognitive abilities		
Spatial ability: mental rotation	M > F	Large
Spatial ability: targeting	M > F	Large
Verbal ability: fluency	F > M	Small to medium
Verbal ability: memory	F > M	Medium
Perceptual speed and accuracy	F > M	Small to medium
Personality traits		
Sensation-seeking	M > F	Medium to large
Aggression	M > F	Large
Nurturance	F > M	Medium
Interest in babies	F > M	Medium to large
Gender-role behaviors		
Interest in male-typical activities	M > F	Very large
Interest in female-typical activities	F > M	Very large
Preference for boys as playmates	M > F	Very large
Preference for girls as playmates	F > M	Very large
Sexual orientation		
Arousal to females	M > F	Very large
Arousal to males	F > M	Very large

[a] d (Mean difference/standard deviation) as reported in adults.

behavior. Because these studies have recently been reviewed elsewhere [21,66–68], we focus here on the general picture of the studies and findings from CAH and the issues important for interpretation and future research.

2.1.2. Findings in females with CAH

Females with CAH differ from unaffected females (their siblings or age-matched comparisons) in a number of domains, including activity interests, personality, cognitive abilities, handedness, and sexuality. Thus, compared to controls, CAH females are more interested in male-typical activities and less interested in female-typical activities in childhood, adolescence, and adulthood, as measured by observation, self-report, and parent-report [55,69–72]. The differences are large and, when multiple measures are used, there is very little overlap between females with CAH and control females. Thus, it is reasonable to say that it is characteristic of females with CAH to prefer male-typical activities.

There are personality-related differences between females with CAH and controls, but the differences appear to be smaller than the differences in interests, although they have not been as well-studied. Thus, compared to controls, females with CAH report themselves to be more aggressive [73], are reported by their parents to be less interested in infants [60], and report themselves to be less empathic and maternal [74].

Androgens also appear to affect cognition in females with CAH. They have higher spatial ability than controls, including spatial orientation and visualization, and targeting [24,52,75]. Some studies have failed to find spatial differences between CAH and control females, but this may reflect the fact that the sex difference in spatial ability is smaller than the sex difference in other traits, e.g. interests, so differences between CAH and control females require larger samples than typically studied. Females with CAH are also more likely than controls to be left-handed [76], although the difference is small and not always found.

Sexual orientation also appears to be altered in some women with CAH. As a group, they are more likely to be sexually attracted to women than are their unaffected sisters [72,77]. Nevertheless, a majority of women with CAH report themselves to be exclusively heterosexual, and it is unclear what differentiates them from the approximately one-third who are bisexual or homosexual.

In contrast to the differences in interests, aspects of personality, spatial ability, and sexuality, gender identity is typical in the vast majority of CAH females [72,77–80]. It is unclear what accounts for gender dysphoria in a small minority of females with CAH, but androgens appear not to play a significant role: there is not a relation between gender identity and indicators of prenatal androgen levels.

2.1.3. Findings in males with CAH

Consistent with studies in other species, males with CAH have generally been found to be similar to their unaffected brothers in most aspects of behavior studied. Thus, they do not differ in sex-typed play in childhood or activities in adolescence, studied at the time [55,69,70] or reported retrospectively [72], nor in aggression [73], interest in babies [60], gender identity or sexual orientation [72]. The one domain in which males with CAH may differ from controls is spatial ability: there is some suggestion that they have lower spatial ability than control males [24,75].

2.1.4. Interpretation of findings in CAH

The masculinized and defeminized behavior of females with CAH has been confirmed across multiple studies with multiple methods and sampling strategies. It is important to note, however, that not all studies have found differences between females with CAH and control females. Many of these 'failures' can be attributed to methodological limitations, particularly to the use of measures that do not show sex differences or to relatively small samples (for further discussion, see [66]). The issue of sample size is particularly salient when the behaviors studied do not show large sex differences, because differences between CAH and control females will be smaller than the sex difference and there are considerable constraints on obtaining large samples of patients with CAH, as discussed below.

Nevertheless, for several reasons, females with CAH do not provide a perfect experiment to study behavioral effects of prenatal androgens. First, their genitalia are masculinized (as a result of exposure to androgens early in gestation when the genitalia develop), and this may elicit social responses, especially from parents. These responses, especially if they result in different treatment of CAH girls, might then cause the behavioral changes [81]. Second, behavioral changes in CAH may reflect effects of increased androgens continuing into postnatal life. Third, females with CAH have abnormalities beyond prenatal androgens, and some of these may be responsible for behavioral changes. Fourth, CAH is an illness, and it is possible that behavioral changes reflect the consequences of living with a chronic illness.

Evidence suggests that these alternative explanations are unlikely. Further, consistency of evidence across methods, as described below, demonstrates the relevance of the findings in CAH. Parents report that they do not treat girls with CAH differently than they treat unaffected daughters (although, of course, parents' reports may not necessarily reflect their behavior) [70,71], and they wish that their daughters with CAH were *less* masculine than they are (whereas they wish that their daughters without CAH were more masculine than they are) [82]. Compelling evidence against parent effects comes from an observational study: CAH girls did *not* play more with boys' toys when a parent was present than when they played alone [82,83].

With respect to timing of androgen effects, interest in male-typical activities in females with CAH has been found to relate to indicators of *prenatal* androgen excess, inferred from disease characteristics (e.g. clinical features indicating severity) and from genetic mutation (which is strongly

associated with clinical features reflecting severity) [82–86], and not to indicators of *postnatal* androgen excess, such as advanced bone age or hormones measured close in time to behavioral assessment [84]. In fact, females with CAH are likely to have subnormal androgen levels postnatally as a result of aggressive treatment [84,87]. There is not much information about timing of androgen effects in relation to other behaviors, although there are speculations that the early postnatal period is important for masculinization of spatial ability [24,75].

The other hormones that are abnormal in CAH, such as progesterone and corticosteroids, have smaller and less consistent behavioral effects than androgens, and may actually prevent masculinization [45]. These hormones would also be expected to affect behavior in males, and, as described above, a number of studies show no differences between CAH and control males, except perhaps in spatial ability (lower in CAH than in control males).

The behavioral effects of illness in other chronic conditions are relatively limited [88–90], and, in any event, there is no reason to expect illness to affect sex-typed behavior only. And again, behavioral similarities between CAH and control males make it unlikely that behavioral changes in CAH females reflect illness per se.

There is also some question about the extent to which findings from CAH females can be generalized to the general population given that their androgen levels are considerably higher than those of any female without CAH. Thus, the data from CAH females can be interpreted to suggest that differences between males and females are due, in part, to androgens, but they are less informative about the role of androgens in producing typical variations within females. This issue will be reconsidered below when findings from typical samples are considered.

The origin of the reduced spatial ability in CAH males is unclear. It may result from *lowered* testosterone in the prenatal or early postnatal period due to exogenous androgens from the adrenal gland reducing endogenous production from the testes [91,92]. Alternatively, it might result from hypoglycemia or salt-wasting episodes in the early postnatal period; these signs of CAH are more common in males than in females because diagnosis is usually earlier in females than males as a result of their ambiguous genitalia.

2.2. Complete androgen insensitivity syndrome

2.2.1. Background

This is another clinical condition that provides a natural experiment regarding the behavioral effects of androgens. Individuals with complete androgen insensitivity syndrome (CAIS) have a male karyotype, and the *SRY* gene initiates the cascade for male sexual differentiation, including the development of the testes. Although the testes produce normal male levels of testosterone, individuals with CAIS lack functioning androgen receptors, so the tissues do not

respond to testosterone, and the external genitalia differentiate in a female-typical direction. Individuals with CAIS are reared as females and most are not diagnosed until they fail to menstruate in adolescence. They provide an opportunity to study the behavioral effects of genes on the Y chromosome and the nature of the hormone responsible for human behavioral sexual differentiation. As noted above, behavioral masculinization and defeminization in rodent species appears to result from estrogen as it is converted from androgen in the brain via aromatase activity. If aromatized estrogen is also involved in human behavior, then individuals with CAIS should be *male*-typical because their normal male levels of androgens should be converted in the brain to normal male levels of estrogen and their estrogen receptors are normal. If, however, human behavior is directly masculinized by androgens (and not by aromatized estrogen), individuals with CAIS should be *female*-typical because they do not have functioning receptors to respond to the high (normal male) levels present. (It is important to note that other influences on behavior are confounded with hormones in CAIS. Behavioral effects of genes on the Y chromosome are indistinguishable from aromatized estrogen effects. Behavioral effects of the social environment are similarly confounded with androgen effects. But CAIS provides a straightforward test of the relative importance of androgen vs. aromatized estrogen.)

2.2.2. Findings in androgen insensitivity

CAIS is relatively rare (estimated to occur in 1 in 100,000 to 5 in 100,000 births) and usually diagnosed in mid- to late-adolescence, so there are not many behavioral studies of CAIS and they are generally restricted to adults. The evidence to date shows clearly that individuals with CAIS are female-typical with respect to all aspects of behavior studied, including gender identity, sexual orientation, and masculinity and femininity of interests (current or retrospective childhood) [93,94]. Cognitive abilities have not been well-studied, but individuals with CAIS have not been shown to have enhanced spatial abilities compared to relative controls and may, in fact, have lower abilities [95].

2.2.3. Interpretation of findings in androgen insensitivity

CAIS is not a perfect experiment because of the confounds cited above. Nevertheless, it is possible to interpret the data to suggest a negligible behavioral role for aromatized estrogens: if they were involved in behavioral sexual differentiation, individuals with CAIS should show behavioral masculinization or defeminization. These null results should be considered in light of the small samples studied and the limited assessment of cognitive abilities and personality traits that show sex differences. But, in light of data on CAH and typical populations (described below), the studies of CAIS are consistent with

the idea that androgens themselves play an important role in the development of sex-typed behavior.

2.3. Exposure to masculinizing hormones via maternal ingestion: androgenizing progestins and diethystilbestrol (DES)

2.3.1. Background

In the 1960s and 1970s, it was not uncommon for drugs to be given to pregnant women to prevent miscarriage. Many of these drugs contained sex hormones, so the offspring of these women provide another opportunity to study the behavioral effects of prenatal exposure to hormones. Unfortunately, many studies designed to exploit this opportunity are difficult to interpret because the hormone exposure varied widely (treatments consisted of hormones that would be expected to have both masculinizing and demasculinizing effects, and women were treated with varying doses for varying durations), samples were small and diverse with respect to age, adequate controls were often not used, and measures were generally single interview items [96]. There are, however, a few studies that do provide good information, and they involve two drugs that would be expected to have relatively specific masculinizing effects, synthetic progestins with androgenizing potential and diethystilbestrol (DES).

2.3.2. Findings of behavioral studies of exposure to androgenizing progestins

An interesting study concerning the behavioral effects of exposure to this treatment used a standardized measure of aggression and sibling controls. Both boys and girls aged 6–18 years who were exposed to androgenizing progestins in utero were more likely than their unexposed siblings to report that they would use physical aggression in a conflict situation [97]. These data are consistent with those in CAH in showing a masculinizing effect on aggression of prenatal androgens, although it is important to note the complexity of androgen effects on aggression in both human and nonhuman animals. For example, in both rodents and primates, aggression in adulthood is affected by treatment with androgens during prenatal, early postnatal, and adult life [e.g., 98–101]; in humans, circulating testosterone is sometimes associated with aggression, with the effects most pronounced in adolescents and when aggression is measured as response to provocation, although the direction of causation is unclear [e.g., 101–103]. Androgens may mediate aggression through direct brain effects and through facilitation of the learning of aggression [100].

2.3.3. Findings of behavioral studies of DES exposure

Behavioral studies of women prenatally exposed to DES have been considered to provide a test of the masculinizing effects of aromatized estrogen. As indicated above, data from rodents show that many aspects of the brain and behavior are masculinized by estrogen as it is converted from androgen via aromatase in the brain. Although females are protected from this effect by factors that bind estrogen in the periphery, high doses of estrogen might exceed binding capacity and allow estrogen into the brain. The hypothesis that DES produces behavioral masculinization in females has been tested in several studies varying in sample size and comparison groups, including some describing results from controlled clinical trials designed to evaluate the medical benefits of DES (that is, whether it reduced miscarriages).

In general, the studies have found DES-exposed women to be behaviorally similar to their unexposed sisters on measures of cognition [104–106] and gender role [107, 108]. There is some suggestion that lateralization may be altered, including an increase in left-handedness, but this is not consistently found [25,104,109,110]. There is one report that DES-exposed women have higher rates of bi- or homosexuality than unexposed women [111], but this was not replicated in a very large sample, albeit with less sensitive measures [110].

2.3.4. Interpretation of findings of behavioral studies of exposure to androgenizing progestins and DES

Overall, studies of females with exposure to atypical hormones because of maternal treatment are consistent with studies in clinical conditions, showing behavioral effects of prenatal exposure to androgens, but not to estrogen. DES is not a perfect experiment, however, because estrogen exposure may not be as high as would be necessary to induce masculinization. Thus, this is probably not a good model for investigating behavioral effects of aromatized estrogen, but, given that DES is no longer used in pregnancy, it is not an opportunity that would be readily available in any event.

2.4. 46, XY individuals without a penis

2.4.1. Background

Much recent scientific and popular attention has been directed to rare clinical conditions in which boys are lacking a penis, because these cases can provide very important information about the causes of sex-typed behavior. In these cases, all aspects of sexual differentiation are male-typical except the external genitalia; thus, these individuals have a Y-chromosome, testes that produce testosterone, and receptors that respond to that hormone. For reasons described below, they have generally been reared as females, so they provide the opportunity to examine the relative behavioral contributions of prenatal hormones (male-typical) vs. sex of rearing (female-typical). If they behave and identify as girls, this suggests the importance of the social environment. If, however, they are masculinized in their behavior, this provides evidence for the importance of prenatal androgens or genes on the Y-chromosome.

There are two primary situations in which a boy might be lacking a penis. The first results from cloacal exstrophy,

a very rare congenital defect (1 in 400,000 births [112]), in which the bladder and external genitalia are not properly formed. Although it occurs in both sexes, the effect is most pronounced in males, because an affected boy is born with a malformed or absent penis but with otherwise completely normal male-typical physical development. The second situation is ablatio penis, in which a boy is missing a penis because of an accident after birth, such as a mishandled circumcision. Until recently, boys without a penis were reared as girls, because of two beliefs: (a) that development of satisfactory gender identity and overall psychological adjustment depend on having normal-looking genitalia (although some surgical correction is now possible, the penis will never look or function normally) and (b) that gender identity is determined predominantly by rearing. In contrast to the endocrine conditions described, the discordance among levels of physical sexual differentiation in these cases was caused by a change in the social environment.

The beliefs that have resulted in rearing these children as girls have been vigorously challenged in the past few years, primarily on the basis of scientific and popular reports about one child born a boy but raised as a girl and then self-reassigned to the male sex [113,114]. Although these reports have focused on the primacy of biology, the systematic evidence reveals a complex picture.

2.4.2. Findings in 46, XY individuals without a penis

The child who was the subject of much attention was reared as a girl after a mishandled circumcision and later experienced gender dysphoria and requested sex reassignment [113,114]. But, he was reared as a boy early in life (the accident happened at age 7 months, the reassignment was made in the second year, and the surgery to construct a vagina was not completed until 21 months), so there was quite a long—and probably sensitive—period when he was reared as a boy (although sex reassignment was made within the period of gender identity plasticity postulated by Money and colleagues [115]). Further, another individual with a similar history but with earlier female reassignment had a different outcome with respect to gender identity, identifying as a female [116]. Although the cases have different outcome with respect to gender identity, they are similar with respect to other behaviors, in particular masculinized interests and sexual orientation.

There has also been much attention paid to a small series of XY males with cloacal exstrophy reared as females [112]. Of 14 individuals, 6 or 8 (depending on the criteria) were stated to identify as male, which was interpreted to demonstrate the biological determination of gender identity. The study is particularly impressive in enlisting all potential participants (thus removing any concern about sample bias), but there is considerable ambiguity in presentation and interpretation of results [117,118]. First, the data come from parents' reports, but gender identity is internal. Parents may have misinterpreted behavior to reflect gender identity.

Thus, the male-typical play of these children (likely a result of prenatal androgen exposure) may have been taken to reflect male gender identity, but systematic and detailed evidence from CAH girls indicates that masculine play can comfortably co-exist with female identity [78,80]. Second, gender change may arise not just from androgen exposure, but in response to complex social conditions, e.g. a mismatch between behavior and parent expectations, peer stigmatization associated with atypical interests, internalized homophobia [79,119]. Third, follow-up methods were unsystematic and subjective, with interviewer expectations likely conveyed to participants and introduced into scoring. This might contribute to identity changes from initial to final assessment. Fourth, case reports and small-scale studies of individuals with cloacal exstrophy and other intersex conditions, including micropenis and partial androgen insensitivity syndrome produce different results, and suggest that gender identity is not simply related to prenatal androgens [119–122].

2.4.3. Interpretation of findings in 46, XY individuals without a penis

The data from males with ablatio penis and cloacal exstrophy clearly show the importance of biological influences on interests. It seems most likely that this effect is specifically due to androgens and not genes on the Y chromosome, given the masculinized interests of females with CAH and the feminine interests of individuals with CAIS. Further, data from boys with cloacal exstrophy who are reared as girls provide evidence that the sensitive period for androgen effects on interests is confined to the prenatal period, given that they are castrated in the early neonatal period and thus do not experience a postnatal testosterone surge.

But, the evidence from individuals born as males but reared as females because of absent or defective penis is not as clear regarding gender identity, given the variability in outcome both within and across studies. Systematic studies of these individuals using standardized objective assessments (such as those used by [78,123]) are sorely needed, but difficult to do given the very low incidence of the conditions. It will also be very important to assess other behaviors in these individuals, to examine androgen effects on, e.g. sexual orientation and cognitive abilities.

2.5. Recommendations for behavioral studies in clinical samples

Studies in CAH, CAIS, ablatio penis, cloacal exstrophy, and other clinical populations have the potential to reveal not only whether hormones affect behavior but how they do so. For example, it is possible to examine postnatal sensitive periods by careful study of boys with prenatal masculinization who are reassigned as girls and gonadectomized in the neonatal period, and girls with CAH who are not diagnosed and treated until later in the first year.

But, there are several challenges that must be met in studying patients with sex-atypical hormones. First, it is crucial to obtain a *representative* sample. Patients are generally recruited through physicians responsible for the care of the hormone condition, usually pediatric endocrinologists in academic medical centers. It is currently unknown whether these patient samples represent the population, given the possibility (at least in the US) that patients seen in academic centers have a severe condition or require special treatment. It is now possible to study population samples of CAH obtained through newborn screening programs [124], and it will be interesting to compare those data to those obtained from clinic samples. Further, it is important to avoid bias in recruiting clinical samples, which often results from difficulty locating patients and then recruiting them. There is considerable variability in participation rate in published studies (some as low as 50%), and there is concern that patients who decline to participate may differ in meaningful ways from those who participate (although this can never be known because, by definition, nonparticipants are unwilling to allow any information about them, including medical records, to be used) [125].

Second, it is essential to ensure that the study has adequate statistical power for testing hypotheses (which is usually accomplished by controlling sample size). It is humbling to realize the number of subjects necessary to achieve acceptable power; for example, in a comparison of patients and controls, it is necessary to have 50 subjects per group to detect a single difference of moderate size (.5 standard deviations) with one-tailed *t*-test and Type I error of .05. Yet, many studies in this area do not have 50 subjects with patients and controls combined, and are studying behaviors which probably show relatively small differences between patients and controls, given that they show only moderate-sized sex differences.

Third, the ability to detect effects depends on the use of an appropriate comparison group. The best comparison group includes same-sex siblings as a control for approximate age and general genetic and environmental background (especially important for examining traits that show substantial familial resemblance, such as cognition, or parent effects, such as sex-typed attitudes) [126]. Unfortunately, many studies use age-matched controls from the general population, which decreases sensitivity beyond the generally small samples.

3. Studies in typical populations

Clinical populations have provided valuable information about hormonal contributors to behavior, but they are not perfect experiments (because of the methodological limitations and concerns about generalizability described above) and they are also difficult to study (because of their relatively low frequency and the sampling problems described above). There has thus been an increased interest in developing alternative ways to study behavioral effects of prenatal hormones, particularly within the normal range. These methods, including *direct* measurement of hormones to which the developing fetus is exposed and *inferential* (*indirect*) measures about the fetal hormone environment, are covered in the next sections.

3.1. Studies in typical populations: prospective studies with direct measures of hormones

Findings in nonhuman mammals and in clinical populations strongly suggest that sex hormones play an important role in the development of behavioral differences between the sexes, and probably in producing within-sex behavioral variations, but it is important to test the generality of these effects by studying typical populations. An ideal study would involve direct measurements of fetal hormones, taken serially at many points in gestation, to ensure reliable measures of the hormones at several likely sensitive periods, and then a follow-up behavioral study in childhood and beyond. The ideal is obviously difficult to realize because of the risks associated with collecting serum from living fetuses. But, it is possible to obtain 'snapshots' of the fetal hormone environment, from samples of peripheral hormones, which are somewhat removed from direct fetal processes.

There are three types of studies that have examined fetal hormones: (a) studies of perinatal hormones obtained from *umbilical cord blood* at birth; (b) studies of prenatal hormones obtained from *maternal serum* by venipuncture during routine prenatal medical care; (c) studies of prenatal hormones from *amniotic fluid* obtained during routine amniocentesis for diagnosis of genetic anomalies. In all types of studies, the focus was primarily on effects of testosterone, given findings in other species and in human clinical conditions for the importance of androgens in masculinizing the body and behavior. Some studies also measured other androgens, such as androstenedione, as well as 'female-typical' hormones, that is, those that are higher in females than in males, such as estradiol and progesterone. In this section we review the findings from these three types of studies and what they reveal about effects of prenatal sex hormone exposure on postnatal behavior in typical human populations, including a discussion of the advantages and disadvantages of the different methods and recommendations for future studies. In evaluating the studies, we also consider what aspects of hormone secretion are actually being measured from hormones obtained from umbilical cord blood, maternal serum, and amniotic fluid, including their origin, how the various compartments relate to each other, and what source seems to be the best for investigating effects of early fetal androgen exposure.

3.2. Studies of hormones in umbilical cord blood

3.2.1. Findings: umbilical cord hormones and behavior

The evidence relating umbilical cord hormones to behavior comes from the Stanford Longitudinal Study, which also examined other influences on sex-typed behavior [127–130]. Approximately 125 children were tested on a variety of behaviors. Hormones included those that generally show sex differences in prenatal and adult life: testosterone and androstenedione (which are higher in males than in females), and estradiol, estrone and progesterone (higher in females than in males, although less so prenatally than in adulthood). It was expected that hormones might have different effects for boys and for girls, so analyses were conducted separately by sex. The design and analyses were exploratory, although the authors did consider previous studies.

3.2.1.1. Temperament. The studies of temperament were not based on evidence of sex differences, nor on evidence of hormone effects in animal studies or clinical samples. In fact, these studies initially addressed the question of the existence of sex differences in temperament, and then the question about the relationship between temperament and umbilical cord sex hormones. The general hypothesis was that levels of sex hormones might be related to individual differences in the readiness to make emotional responses, with high levels of androgens (testosterone and androstenedione) increasing male-typical behavior. There were no hypotheses about estradiol, estrone and progesterone. A trait called 'timidity' was assessed by reactions of (6–18-month-old) infants to fear-provoking toys [127]. There were small sex differences in reaction to the toys, with girls somewhat more timid than boys. As expected, low timidity was associated with relatively high perinatal testosterone level, but not with androstenedione, and only in boys. Timidity was also related to progesterone (negatively) and estradiol (positively), again only in boys. In girls, there were no relations between timidity and sex hormones.

Temperament was also assessed in children aged 6–26 months by mothers' 24 h diary reports of their children's moods, with the main scores being the proportion of time spent in one of three moods: happy/excited, quiet/calm, or negative [130]. There were small, but significant, sex differences in mood: boys were more often reported to be in a happy/excited mood, girls in a quiet/calm mood. Again, hormones were related to behavior for boys but not girls. In boys, androstenedione, estrone, and progesterone (but not testosterone) were positively related to happy/excited mood and negatively to calm/quiet mood in the first two years of life, with moderate correlations (absolute values 0.25–0.36). This might be interpreted to reflect a facilitative effect of androgens on male-typical behavior, but not in a simple way, given that mood was not related to testosterone, and there were no effects in girls.

The association between negative mood and umbilical sex hormones was studied because of the sex differences in quality of negative emotion, with boys more likely than girls to show anger and girls more likely than boys to show fear. Unfortunately, it was not possible to explore the differentiated aspects of negative emotion because of the low frequencies of anger and fear. A score of negative moods created by combining anger, fear, crying, and general unhappiness did not show a sex difference (consistent with previous studies in infants). There were no significant relationships between umbilical cord sex hormones and negative emotionality (composite).

3.2.1.2. Cognition. Although hormonal effects on cognition were not specified in the Stanford Longitudinal Study, it might have been expected for spatial ability to be positively associated with testosterone and androstenedione in 6-year-old girls. But, the associations were negative ($r = -0.34$ and -0.37, respectively, with a composite score) [129]. Unfortunately, the study did not provide a compelling test of hormone effects on cognition given that the measures did not show sex differences.

3.2.1.3. Physical traits. Adult sex differences in strength are also well-documented and related to circulating hormones. But, it is unclear whether there are childhood sex differences in strength, and whether there is an influence of early hormones. These questions were explored in another part of the Stanford Longitudinal Study [128], with complex findings. Although boys were stronger than girls, strength was not consistently associated with umbilical cord androgens for either boys or girls. It was associated with neonatal progesterone, negatively for girls and positively in boys. Effects in girls can be related to studies in several species showing progesterone to have anti-androgenic effects, but effects in boys are unexplained. It is interesting to note that neonatal progesterone was associated with masculinization in boys across traits, including strength and temperament [127,128,130].

3.2.2. Interpretation of findings: umbilical cord hormones and behavior

The results of the Stanford Longitudinal Study are difficult to interpret in light of the measures used (many did not show sex differences and would not be expected to, especially in very young children) and hypotheses and evidence about the timing of effects of organizational hormones. In essence, the sensitive period for behavioral effects of hormones probably occurs before birth, and the hormones in umbilical cord blood reflect more than just the fetus, so hormones from the umbilical cord probably do not reflect the levels that the fetus was exposed to during sensitive periods. The umbilical cord is part of the maternal-fetal unit. There are sex differences in umbilical sex hormone levels, but they are small and not always detected. Boys have higher levels of testosterone than girls [131–

133]; they also may have higher levels of androstenedione and progesterone [133], although differences are small and not consistently found. Substantial proportions of androstenedione and testosterone are produced by the fetus from the adrenal gland and testes, whereas progesterone comes from the placenta. The fetal ovary seems to be quiescent during the last weeks of gestation [133]. Further, the umbilical cord contains blood from both the mother and the fetus. Umbilical venous blood comes primarily from the mother, whereas arterial blood comes primarily from the fetus, so the best reflection of fetal or neonatal hormones would require collection of arterial blood. But, most studies collect material from the umbilical vein rather than the artery because it is easier to obtain, suggesting that umbilical cord hormones are a poor reflection of the levels in the fetus and neonate [134]. Finally, the process of labor itself may affect hormone levels. Thus, in light of the small sex differences in neonatal hormones, the unclear association between prenatal and neonatal hormones, the difficulty in collecting cord blood unique to the neonate, and the possible confounds from the labor process, it is difficult to consider umbilical cord hormones a good index of organizational effects of sex hormones.

3.3. Studies of sex hormones in mother's blood during pregnancy

3.3.1. Findings: maternal hormones and behavior

Two studies of relatively large samples examined the relationship between testosterone in mother's blood during pregnancy and offspring's later gender-role behavior, one when the offspring were adults [135–137] and the other when the offspring were preschool children [51]. Both studies found markers of high testosterone in mothers to be associated with masculinized gender-role behavior in female offspring.

In the study of adults, restricted to females, a broad measure of gender-role behavior was examined in relation to maternal serum hormones testosterone and sex-hormone binding globulin (SHBG), which binds testosterone and therefore prevents its action, and circulating hormones in the offspring herself when she was an adult. The behavioral measure was composed of single items and scales from other instruments, measuring several constructs, such as the importance of home, feminine interests, job status, and personality traits related to dominance and expressivity. Regressions were conducted to examine maternal hormones and offspring adult hormones together, but the analyses varied with respect to the specific hormones included. In one analysis, 26% of the variance in adult feminine gender-role behavior was associated with hormones (with main effects as expected): feminine behavior was predicted from maternal serum testosterone (negative), maternal serum SHBG (positive), adult androstenedione (negative), adult SHBG (positive), and the interaction between testosterone in maternal serum and the daughter's adult androstenedione

(negative) [135]. In another analysis using adult testosterone instead of androstenedione, 18% of the variance in the same behavior was attributed to maternal serum SHBG (positive), adult testosterone (negative) and the interaction of testosterone in maternal serum and the daughter's adult testosterone (positive) [136]. Consistent with suggestions about the importance of prenatal weeks 8–24 as a sensitive period, behavior was related to hormones from maternal serum only during the second trimester, and not during the first or third trimesters. This study is quite intriguing, but the results are confusing, because of differences across reports in the specific hormones analyzed and in the direction of the interaction between maternal serum and adult hormones (details of the interactions are not provided).

In the study of children, mothers' levels of testosterone in blood samples, collected between week 5 and week 36 of gestation, were examined in relation to (mother-reported) preschool activity interests of male and female offspring at age 3.5 years [51]. Consistent with expectations, based on findings in CAH girls and studies in other mammals, mothers who had high serum testosterone during pregnancy were more likely than mothers with low serum testosterone to have daughters who were interested in boy-typical toys and activities. Activity interests were not significantly related to maternal testosterone in sons, or to SHBG in either daughters or sons.

3.3.2. Interpretation of findings: maternal hormones and behavior

Although two studies suggest that testosterone levels in maternal serum during pregnancy are related to later gender-role behavior in female offspring, their interpretation is not straightforward for two reasons. First, the specific results are not consistent across the studies and there is some inconsistency even within the first study, especially regarding the importance of testosterone vs. SHBG. Second, the mechanism responsible for the association between maternal hormones and offspring behavior is not clear. The hormones measured in maternal serum are produced by the mother and the placenta. Maternal androgens increase in pregnancy [138–140], perhaps due to increased binding by SHBG and thus higher levels of bound (but not unbound) testosterone [132,141]. Maternal testosterone does not appear to come from the fetus; several studies failed to find a difference in testosterone serum levels between women carrying a male and those carrying a female fetus [142–145].

Thus, it is unlikely that hormones in maternal serum reflect the fetus's own levels. It is important, therefore, to consider the mechanisms that might account for the association between mother's testosterone and offspring behavior. First, the relation may reflect the hormones to which the fetus is exposed environmentally. Given experimental studies in other species, it is clear that behavior can be affected by exogenous exposure. But, aromatase in the placenta generally provides protection against high levels of

testosterone; for example, the daughters of women with polycystic ovary syndrome do not have virilized genitalia although maternal androgen levels are elevated [146]. Second, the relation may reflect social factors. A woman with relatively high testosterone may interact differently with her daughters than a woman with relatively low testosterone, for example, by being interested in male-typical activities herself and encouraging her daughter to play with boys' toys. This seems less likely in light of Udry's findings that the relation was specific to the second trimester, but it is consistent with the fact that women retain the same rank-order position with respect to testosterone level across pregnancy, e.g. in one study, correlations for hormones in maternal serum between the second and third trimester were 0.8–0.9 for free testosterone and androstenedione, and 0.7–0.8 for SHBG [145]. Third, the relation may reflect genetic factors: a woman with high testosterone may transmit genes for high testosterone to her daughter, and the relation between maternal testosterone and daughter behavior may be mediated by the daughter's own testosterone (which is correlated with her mother's for genetic reasons); this assumes that the same genes influence prenatal and circulating testosterone. In order to differentiate among these alternatives, it is necessary to examine testosterone in women before, during, and after pregnancy, and to examine a woman's own gender-role behavior in future studies of maternal hormones in relation to offspring behavior.

3.4. Studies of sex hormones in amniotic fluid

3.4.1. Findings: amniotic hormones and behavior

There are two studies of postnatal behavior in relation to amniotic hormones, although each has produced multiple reports. Finegan and colleagues were the first to recognize the value of this method. After showing the sex difference in amniotic testosterone [147], they examined its relation to behavior in about 60 children. Testosterone levels were measured in amniotic fluid collected during routine diagnostic amniocentesis between weeks 14 and 20 of gestation. The children were tested at ages 4 and 7, primarily on measures of cognition, and at age 10 on measures of cerebral lateralization [53,148, 149]. A second, recent study by another group of investigators in about 85 infants focused on social functioning and vocabulary, and testosterone and estradiol collected from amniotic fluid between weeks 14 and 21 of gestation [150,151].

3.4.1.1. Cognition. A broad spectrum of cognitive function was assessed at age four in children whose prenatal amniotic testosterone levels were known [53]. Many of the analyses were exploratory, because the cognitive abilities measured were not similar to ones used in other studies of hormonal influences on cognition. This partly reflects the age of the sample, because few other studies tested such young

children, and, at the time of the study, there were few good measures for young children that showed sex differences.

In girls, prenatal testosterone was related to measures of several abilities: it was related in a curvilinear (inverted U-shaped) fashion to language comprehension and classification abilities, and related negatively to counting and number facts (with large effects). It is unclear exactly what these measures reflect, and whether there is a single basic cognitive skill that underlies them. There was also an unexpected large negative relation between testosterone and spatial ability (measured here with block building), but this is consistent with results from the study of umbilical cord blood [129]. Overall, the results of this study are difficult to interpret given the lack of sex differences on the measures used, and, as the authors themselves note, the children were too young to permit reliable assessment of spatial abilities (with the instruments available at the time). In boys, no significant relations were found.

Consistent with the importance of age effects, assessments when the children were 7 years old revealed meaningful positive associations between spatial ability and prenatal testosterone [148]. Girls with higher amniotic testosterone levels had faster (but not necessarily more accurate) performance on a mental rotation task than did girls with lower levels ($r = 0.67$). Although these findings are consistent with the hypothesis that androgens masculinize spatial ability, the large picture from the study is complex. The effect of testosterone was found only in the small subgroup of 12 girls who used a rotation strategy, but, inconsistent with expectation, girls were faster at rotation than boys. There were no significant relations in boys. There were no relations between prenatal testosterone and children's spatial play activities (as reported by the parents), but this may reflect limitations in the measure used (e.g. parent-report and not observation, limited sampling of activities).

3.4.1.2. Cerebral lateralization. In the same sample, prenatal testosterone levels were found to relate to indicators of lateralization at age 10, as measured by handedness and by dichotic listening tasks (processing of language and emotion, for left and right hemisphere lateralization, respectively) [149]. Results focused on the partial correlations with testosterone, controlling for effects of gestational age, parental handedness, and birth stress. For girls, testosterone was positively correlated with degree of right-handedness ($r = 0.41$) and degree of left-hemisphere lateralization (right-ear advantage) for language ($r = 0.43$). For boys, testosterone was positively correlated with degree of right-hemisphere specialization (left-ear advantage) for the recognition of emotion ($r = 0.48$). Results were interpreted to be most consistent with the hypothesis that high androgen levels lead to more lateralization in both sexes [152].

3.4.1.3. Early infant behavior. The second study examining behavior in association with levels of testosterone in amniotic fluid focused on early development [150,151]. This study was motivated by the idea that early eye contact and vocabulary are precursors of developmental disorders that show sex differences (especially autism) and that, therefore, might be influenced by prenatal testosterone [153, 154]. Thus, high prenatal testosterone levels were hypothesized to be associated with reduced eye contact [150] and small vocabulary [151] in infancy. There were moderate-sized sex differences in these characteristics: compared to girls, boys made less eye contact ($d=0.53$) and had smaller vocabulary ($d=0.67$). But, the hypothesized relations with testosterone were generally not found, except for one between eye contact and testosterone in boys (the size of the effect could not be calculated from the information provided in the paper). Behavior was also examined in relation to estradiol, testing the hypothesis from animal studies that this hormone would have opposite effects to testosterone [155]. Results, in fact, showed estradiol to be negatively associated with vocabulary size, but only with the sexes combined and not within sex, suggesting that the effect is due to group differences in hormones and behavior. It is difficult to interpret the findings (both positive and negative) of this project: failure to find associations may reflect low statistical power with small samples (as suggested by the authors); unexpected findings might reflect Type I error associated with multiple comparisons.

3.4.2. Interpretation of findings: amniotic hormones and behavior

Results of studies examining associations between testosterone in amniotic fluid and childhood behavior have been inconsistent. Although some findings support the hypothesis that testosterone has masculinizing effects, others do not, and are even in the direction opposite to expectation. These inconsistencies are most parsimoniously explained by methodological limitations: most behaviors studied did not show large sex differences, and sample sizes were very small.

It is also important to consider whether hormones obtained from amniotic fluid truly represent the fetus's exposure to these hormones. Amniotic fluid is obtained from amniocentesis conducted for purposes of diagnosing fetal anomalies. This means that the samples studied are selected in several ways that may influence the offspring's outcome as well as the generalizability of the results. The pregnant women may be anxious about the result, and, as shown in other papers in this special issue, anxiety and stress can affect behavior, although probably not human sex-typed behavior [156–158]. Women referred for amniocentesis tend to be above average in age and education, and, this may restrict both range and generalizability, because maternal androgens appear to decrease with age [159,160], and high socioeconomic status is associated with nontraditional sex-typed attitudes [161]. Nevertheless, amniocentesis itself

appears not to have any negative effects on the offspring's behavior [162].

A major advantage of amniocentesis for examining hormone-behavior relations concerns its timing. It is done during the second trimester of pregnancy, during a relatively narrow time window (usually 14–20 weeks of gestation) which fortunately coincides with the serum testosterone peak period in male fetuses. This peak is also apparent in amniotic fluid: several studies have documented a large sex difference in amniotic androgens [132,141,145,147,163–165]. It is interesting to note that amniotic fluid was used to index fetal hormonal levels well before it was recognized as an opportunity by behavioral scientists [147].

The origins of androgens in amniotic fluid are not fully understood, but the main source seems to be the fetus itself. Hormones enter the amniotic compartment in different ways: via diffusion through the fetal skin in early pregnancy, and via fetal urine in later pregnancy [163,166]. Given the risk of obtaining blood from the fetus, there are very limited data directly comparing testosterone in amniotic fluid to that from fetal blood. One study at 15–23 weeks of gestation [165] reported no significant correlations among testosterone levels obtained from fetal plasma and amniotic fluid, but it is unclear whether there would be significant associations if assessments were done earlier in development or if multiple measures were used. Androgens in amniotic fluid are also unrelated to androgens measured in maternal blood in the same period, as shown in studies in early- and mid-gestation [145,165]. The origin of estradiol and progesterone levels in amniotic fluid is still speculative. The placenta is probably the principal source, but the fetal ovary may also contribute, given that some investigators (but not all) have found higher amniotic estradiol levels in female fetuses than in male fetuses in midpregnancy [145, 164; but see 141].

Thus, testosterone obtained in amniotic fluid appears to be a good reflection of the levels in the fetus, and thus represents an alternative to direct assay of fetal serum. Further, it is obtained during an important developmental period and thus provides an opportunity to examine the behavioral effects of prenatal hormones present during a key sensitive period. The value of other sex hormones in amniotic fluid is not as clear.

3.5. Recommendations for prospective studies in typical populations with direct measures of prenatal hormones

There are three significant issues to consider in designing a prospective study to examine the behavioral effects of prenatal hormones: method of obtaining hormones (amniotic fluid or maternal serum), timing of collection of hormone data, and methods for behavioral assessment (number and age of participants and specific behaviors to be studied). With respect to the first issue, the evidence reviewed above suggests that amniotic fluid provides a better reflection of

fetal hormones than does maternal serum and is therefore probably the best choice for studying the behavioral effects of variations in prenatal androgen exposure [53,145]. Nevertheless, there may well be value to studying maternal serum, in light of the fact that results have been more encouraging with maternal serum than with amniotic hormones, although it is important to note that studies showing testosterone in serum to be related to sex-typed behavior [51,135,136] had large samples (and thus power to see small effects), and these studies are not without limitations, as discussed above. Further, maternal serum, unlike amniocentesis, is relatively easy to collect and can be measured repeatedly throughout pregnancy in order to assess the possibility of multiple sensitive periods and stability of hormone levels. The ideal study would combine hormones from amniotic fluid and maternal serum to assess the similarities and differences in their relation to postnatal behavior. It would also provide an opportunity to study factors that account for differences in hormone levels in maternal serum vs. amniotic fluid, e.g. prenatal stress, maternal age.

It is also important to develop methods for direct assessment of hormone levels in fetal blood during sensitive periods of brain sexual differentiation. A potential method might involve data obtained from cordocentesis due to suspicion of fetal abnormality or alloimmunization, which is usually done between weeks 18–22 of gestation [167]. This method has already demonstrated independent production in mother and fetus of pregnenolone sulfate, a progesterone precursor [168]. Of course, it is also limited to selected cases, raising questions about generalizability.

It is ethically and practically difficult to obtain repeated samples of hormones during gestation, so it is important to determine the most likely sensitive period and to obtain hormone samples during that time. Given the reported time course of testosterone secretion in males [23], the most promising times are prenatal weeks 8–24. But this is still a wide range. Most studies of hormones from amniotic fluid and maternal serum have considered gestational age in analyses, but this cannot correct for assessments that are not made during the appropriate sensitive period. Further, studies in primates also suggest the importance of the later prenatal period [9]. Practical considerations further limit the ability to choose the optimal timing of collection of hormones, because both amniocentesis and sampling of maternal blood are based on medical factors and convenience. There should also be more attention to the fact that hormones fluctuate within a day and across days, even in fetuses [169,170]. The representativeness of a single sample of hormones is unclear.

With respect to the third issue regarding design of prospective behavioral follow-up, it is essential to have studies that are maximally sensitive to behavioral effects of hormones. This seems obvious, but is not always easy

to implement. Behaviors studied should show reasonable size sex differences and ideally already have been found to relate to prenatal hormones in other studies (e.g. of girls with CAH). The best candidates here are sex-typed interests (e.g. childhood toy preferences, adolescent sex-typed activity interests, and adult hobbies), spatial ability, interest in babies, and aggression. Further, samples need to be large enough to detect what are likely to be small-to-moderate effects (given the multiple influences on behavior and the effects found in other studies), and this often involves more participants than are easy to recruit.

A prospective study is a challenge for both the researcher and the participant, requiring years of investment before meaningful results are available. There are also considerable methodological challenges to a prospective study. There is a risk of participant dropout, and it is especially important to guard against *selective* attrition (especially people with atypical behavior dropping out in higher numbers than those with typical behavior). In order to maximize the benefit of a prospective study, it is desirable to conduct behavioral assessment when the children are very young, but behavioral sex differences have not been as well-studied in infants as in older children and adults (so there are fewer good measures available), and it is important to determine if behavioral effects of hormones change across development (which seems likely because sex differences do). A longitudinal study of participants measured on more than one occasion allows the investigation of important questions regarding the nature and causes of behavioral change (e.g. do prenatal androgens exert increasing or decreasing effects across age?), but this requires comparability of measures across age.

4. Studies in typical populations: co-twin sex as an indirect indicator of prenatal hormones

4.1. Opposite-sex twins

4.1.1. Background

Most evidence for behavioral and physiological effects of early hormones comes from nonhuman studies in which hormones are directly manipulated. Interestingly, however, there is good evidence that behavior and physiology are influenced by naturally occurring variations in hormones that result from an animal's position in the uterus, particularly the sex of its littermates (intrauterine position, IUP) [17,171,172]. Female rodents that developed between male fetuses in utero are less female-typical in postnatal behavior (e.g. aggression, attractiveness to males), anatomy (e.g. anogenital distance, an aspect of genital morphology), and reproductive characteristics (pubertal maturation, reproductive life) than are female animals that developed between female fetuses in utero. This effect extends beyond rodents. For example, female

swine surrounded by male in utero are more likely to participate in and to win fights than female swine positioned between female swine in utero [173]. The masculinizing effect on females of gestating close to males is attributed to the transfer of testosterone from the male fetus to the adjacent female fetus [174]. In some species, intrauterine position affects postnatal behavior, anatomy, and physiology in male animals too, presumably by the same mechanism. For example, male gerbils that developed between two females are less masculine than those that developed between two males in reproductive characteristics and sexual behavior [17]. The studies of intrauterine position effects are consistent with studies in which hormones are manipulated directly and shown to affect later behavior [6,8] and confirm the importance for sex-typed behavior of exposure to sex hormones early in development.

As suggested by Resnick [62] and Miller [175], human twins might also be affected by the sex of the co-twin, providing a parallel to the intrauterine position effect in animals, and an opportunity to examine the human behavioral and physiological effects of prenatal exposure to higher-than-average-female (or lower-than-average-male) levels of testosterone. The female member of an opposite-sex (OS) twin pair is assumed, as a result of sharing the womb with a male co-twin, to be exposed to higher levels of testosterone during prenatal development than is a female member of a (dizygotic) same-sex (SS) twin pair. Thus, OS females should be more male-typical and less female-typical than SS females. Similarly, the male member of an OS twin pair might be exposed to lower levels of testosterone than a male member of a SS twin pair, making OS males less male-typical and more female-typical than SS males. It is important to note, however, that twins also share a postnatal environment, and that children with opposite-sex siblings may be exposed to a different gender-related social environment than children with same-sex siblings [176,177]. This complicates interpretation of differences between OS and SS twins, as considered below. In this section we briefly review human studies of opposite-sex twins designed to investigate effects of prenatal sex hormones on physical and behavioral characteristics, interpreting findings in light of the strengths and limitations of the method, and making recommendations for future studies.

4.1.2. Findings: physical and behavioral traits in opposite-sex female twins

Twin studies designed to investigate the possible masculinizing effect on behavioral and physical traits of prenatal exposure to testosterone in females have yielded contradictory results.

4.1.2.1. Physical characteristics. The majority of twin studies have focused on *physical* or *psychophysical* characteristics, possibly because they are thought to be less likely to be influenced by postnatal environmental factors (although clearly that is not always true). Most studies failed to find differences between OS and SS females on a variety of measures of reproductive characteristics and handedness. Exceptions concerned tooth size [194] and spontaneous otoacoustic emissions, an auditory characteristic that shows sex differences and is related to hearing sensitivity [178]: in both cases OS females were masculinized relative to SS females. In contrast, click-evoked otoacoustic emissions, another auditory characteristic that shows sex differences and is related to hearing sensitivity [179], did not differ significantly between OS and SS females, although OS females were considered by the investigators to show 'masculine' changes and the overall results were interpreted to suggest that prenatal exposure to testosterone leads to a more masculinized pattern of otoacoustic emissions [180]. Two large-scale studies have failed to show differences between OS and SS females in reproductive characteristics [181,182]. In a recent study of cerebral lateralization assessed with a verbal dichotic listening task in pre-adolescent 10-year-old twins, OS girls had a more masculine pattern of cerebral lateralization than did the SS girls, reflected in a larger right ear advantage. The result was interpreted to reflect an effect of prenatal testosterone on hemispheric specialization [183]. It is interesting to note, however, that the effect was no longer present when the girls were retested at age 13 [289], and another study failed to find effects of co-twin sex on handedness [185].

4.1.2.2. Cognitive abilities. The only twin study to examine this domain found OS females to have higher spatial ability than SS females [186]. Although the results are consistent with prenatal testosterone effects, they are also consistent with effects of environmental factors associated with having an opposite-sex sibling, such as the availability of toys that might facilitate spatial ability [177,187]. Further, the effect has not been subject to replication.

4.1.2.3. Personality and gender-role behavior. Some studies have found OS females to be more masculine than SS females, on traits such as sensation-seeking [62], rule-breaking [188], and social attitudes [189], although differences are not always seen [182,184,188]. There is some suggestion of an age effect in one study [188], with differences between twin groups in younger females (mean 23.4 years) but not in older ones (mean 41.2). Although the age division does not reflect clear developmental distinctions, it is possible that testosterone effects diminish with age, as influences of other factors, such as circulating hormones and social environmental events, become salient. This suggests an important need to consider age in these studies.

Other studies showed mixed results in comparisons of OS and SS females on a variety of traits. In a large Australian study, very few traits differentiated the twin groups, with the exception of retrospectively-recalled sex-typed childhood behavior [190]. This stands in contrast to three studies in children, in which sex-typed behavior was measured and found not to differentiate OS and SS females [191–193], suggesting the possibility of Type I error with many comparisons in the Australian study. A recent study [184] of aggression and sensation-seeking also revealed mixed results: compared to SS girls, OS girls reported more verbal aggression and less withdrawal behavior in aggressive situations (and these were not related to current circulating testosterone levels), but *less* experience seeking, contradictory to expectations and to other results [62]. Inconsistencies across studies might reflect differences in age.

4.1.3. Findings: physical and behavioral traits in opposite-sex male twins

A few studies examined co-twin effects in males, hypothesizing, on the basis of rodent studies, that OS males would be demasculinized/feminized compared to SS males or non-twin males. These studies were originally focused on co-twin effects in females, but also studied males. Therefore, they examined the traits described above in studies of females. Most studies failed to find demasculinization in males with a female co-twin on physical traits, such as tooth size [194], psychological traits, such as social attitudes [189] and sensation-seeking [62], spatial ability [186], sex-typed toy play [193], and handedness [185]. Interestingly, however, there is some suggestion that males with a female co-twin might be demasculinized or feminized on gender-role behavior [190,191], but it is not possible to know whether this reflects effects of gestating in proximity to a female or being reared with one.

4.1.4. Interpretation of findings in opposite-sex twins

The results of the studies summarized above show a mixed picture regarding effects for females of having an opposite sex co-twin and not a lot of information regarding effects for males. The positive effects in females primarily concern masculinized tooth size, spontaneous otoacoustic emissions, lateralization, and spatial ability in female OS twins, but it is important to note that these positive findings have not been replicated. This masculinization (if replicated) is difficult to explain by gender-socialization factors. Moreover, if OS females are raised in a more male-typical environment than SS females, and this affects behavior, then there should be many and pronounced differences between OS and SS females. This is clearly not the case. Nevertheless, twin studies need to have better control for the role of the social environment. This would involve a comparison group of singleton girls with an older brother very close in age; only one twin study used such a comparison group

[192]. Although this is the best comparison currently available, it is not perfect, because same-aged siblings, as in twin pairs, might affect each other differently than siblings of different ages.

The lack of differences between OS and SS females is open to several interpretations. First, of course, it is possible that prenatal sex hormones do not affect behavior within the normal range, suggesting that effects in clinical populations cannot be generalized to sources of normal within-sex variability. But the consistency of results across clinical conditions and with other methods (as described elsewhere in this paper) would appear to make this unlikely.

Second, twins may not provide a good model for assessing behavioral effects of prenatal sex hormones. There are only limited data demonstrating the transfer of testosterone in human multiple pregnancies. Early in pregnancy, there may be transfer of androgens from one twin to the other, as steroids readily cross the fetal membranes (and placenta) and the fetal skin is permeable to hormones dissolved in the amniotic fluid [195,196]. Later in pregnancy, changes in the fetal skin prevent the simple diffusion of amniotic fluid constituents [195], but hormones from one twin may reach the other twin via trans-membrane transport and the maternal–fetal circulation [140]. If these mechanisms of transfer differ in their effectiveness, and if the brain is more sensitive to androgens during some periods than during other periods, then androgens from a male co-twin might not be equivalent in their effects across pregnancy. Further, females must be protected in some ways by the separate placenta from possible exposure to testosterone from their male womb mate; the genitalia of female OS twins are not obviously masculinized, although exposure from a male co-twin may not be high enough to do so. Even if female OS twins are exposed to testosterone from a male co-twin, the levels might not be very high or they might be counteracted by other hormones or the exposure might not occur during the key sensitive periods. Females in other species are probably exposed to relatively higher levels than are human female twins, because the former have multiple littermates. Intrauterine position effects in rodents primarily reflect differences between females that gestate between two males and those that gestate between two females; effects are weaker for females that gestate next to only one male. In addition, androgen exposure in twins may be affected by other factors at play in twin pregnancies, such as left-right position in the uterus (which influences how much blood is received from the mother and from the co-twin), placentation (whether the placentas are separated or fused), number of chorions between the fetal compartments, and twin differences in growth. Some of these factors may differ in monozygotic (MZ) vs. dizygotic (DZ) twin pairs and, unfortunately, not all studies restricted their samples of same-sex twins to the latter.

Third, behavioral effects of intrauterine position may be small, and samples have generally not been large enough to detect such effects. In other species, effects are moderate to large, but behavior is also affected by other environmental factors, such as housing conditions and maternal stress [181]. Many of the twin studies described above used measures that show small to moderate sex differences, and most studies did not have sufficient power to see what would be even smaller within-sex twin differences.

4.1.5. Recommendations for studies of opposite-sex twins as reflections of prenatal hormones

Existing studies are not particularly encouraging in terms of twin studies revealing support for behavioral effects of prenatal hormones, but it is unclear whether it is time to give up on this method. Existing studies had limitations (e.g. relatively low statistical power, some combined MZ and DZ twins) that make it difficult to draw strong inferences about the lack of effects. The method has several advantages compared to others for studying hormone effects, such as the relatively high frequency of fraternal twinning (about 1 in 150 births worldwide), the availability of twin registries, and the problems with other methods (e.g. the difficulty obtaining amniotic hormones). Nevertheless, personal communications with other scientists involved in twin studies suggest that many have failed to find differences between OS and SS twins, but the bias against reports of null findings in peer-reviewed journals means that results are not published. This makes it difficult to evaluate fully the utility of the twin method for studying behavioral effects of prenatal hormones and may result in misdirected research efforts. Twin studies remain an interesting paradigm that is well-grounded in studies in other species, but its further use should depend on data validating its utility, for example, by studying whether the genitalia of females with a male co-twin are masculinized in subtle ways compared to females with a female co-twin (e.g. a larger clitoris).

5. Studies in typical populations: biological markers as indirect indicators of prenatal hormones

Three relatively new methods for investigating behavioral effects of prenatal exposure to sex hormones involve use of morphological indices, specifically otoacoustic emissions (reflecting auditory function), finger ratio (the relative lengths of the index and ring fingers), and dermatoglyphics (fingerprints). These markers are assumed to reflect prenatal exposure to sex hormones, and are examined in relation to postnatal behavior, with associations between these markers and behavior taken to reflect influences of prenatal sex hormones on the behaviors studied. In this section, we describe the rationale behind these methods, briefly review the studies investigating

associations between the markers and various behaviors, interpreting findings in light of the strengths and limitations of the methods, and making recommendations for future studies.

5.1. Otoacoustic emissions

5.1.1. Background

There have been a number of interesting studies examining the possibility that characteristics of the auditory system, called otoacoustic emissions (OAEs), provide a window into prenatal hormone exposure. OAEs are sounds *produced* by the ear; they are of cochlear origin and recorded with a miniature microphone inserted into the external ear canal [197] (for review, see [198, 199]). Although they are not fully understood, OAEs seem to be caused by the motion of the sensory hair cells. The displacements of the basilar membrane that are produced by weak sounds are amplified by the cochlea, a mechanism called cochlear amplification [200], with OAEs occurring as a by-product. OAEs are related to hearing sensitivity [201].

There are two types of OAEs. Spontaneous OAEs (SOAEs) represent sounds that are spontaneously and continuously produced by most normal-hearing ears. Click-evoked OAEs (CEOAEs) are sounds produced in response to a brief acoustic stimulus and vary in strength (amplitude). There is substantial variability across people in OAEs, with the correlation between CEOAE amplitude and SOAE number 0.76 [202]. There are some interesting properties of OAEs that make them good candidates for understanding behavioral effects of hormones. OAEs vary across ears: there are more SOAEs and stronger CEOAEs in right than left ears [203,204]. OAEs are stable from an early age [205,206], although they can be influenced by postnatal events, including, e.g. hormonal fluctuations associated with the menstrual cycle [207,208], and exposure to loud noise. Importantly, OAEs show sex differences across the life span (from infancy through adulthood), with females having more SOAEs and stronger CEOAEs than males [179,202,209]. The differences are moderate-to-large in size: mean differences are about 0.6–1 standard deviation [210]; about 75–85% of females but only 45–65% of males have at least one SOAE [202].

McFadden and collaborators have studied the origin of the sex difference in OAEs [179,206]. A substantial proportion of the variation in OAEs appears to be under genetic control, with heritabilities about 0.6 to 0.8 [180, 211]. Some variation is hypothesized to reflect prenatal exposure to androgens, which is suggested to diminish OAE strength through suppression of cochlear amplifiers [178–180]. The evidence in support of prenatal hormone effects is indirect, coming from opposite-sex twin pairs: females with a male co-twin have a more masculine OAE pattern, with fewer SOAEs and weaker CEOAEs than

other females. It should be noted that this effect is inconsistent, perhaps because it is not large. For example, when the data of opposite-sex dizygotic females were directly compared with those from same-sex dizygotic females, the difference was statistically significant only for SOAEs [178], and marginally significant for CEOAEs [180].

5.1.2. Findings: OAEs in relation to behavior

The findings from opposite-sex twins raise the possibility that OAEs represent a window into prenatal hormones, and this has been pursued in studies by McFadden and colleagues examining associations between OAEs and sex-typed behaviors hypothesized to be influenced by prenatal hormones. Thus, OAEs have been studied in relation to sexual orientation [202,209] and a variety of sex-typed traits [210].

5.1.2.1. Sexual orientation.
OAEs have been found to relate to sexual orientation in women, but not in men. In women, as hypothesized, homosexuality was associated with masculinized OAEs: compared to heterosexual women, lesbians had weaker CEOAEs ($d = 0.37$–0.44) and fewer and weaker SOAEs ($d = 0.30$). There was no relation in males between sexual orientation and OAEs. Positive findings in women but not in men may be explained by a number of factors: statistical power, different paths to sexual orientation in men vs. women, or differences in timing of androgen effects on OAEs and male sexual orientation [202,209]. These alternatives demonstrate the limitations of OAEs as markers of prenatal androgen exposure; failures to find relations between OAEs and specific behaviors do not rule out prenatal androgen effects, and documented relations do not necessarily implicate prenatal androgens.

5.1.2.2. Other sex-typed traits.
Relations between OAEs and 23 sex-typed traits were examined in the same samples described above [210]. Traits were categorized as physical variables, spatial abilities, sexual activity, sex atypicality, and miscellaneous. Although several traits showed differences related to sex and to sexual orientation (consistent with demasculinization in homosexual males and masculinization in homosexual females), the traits were not related to OAEs. It is not clear whether failure to find associations reflects a true lack of relation in the population or methodological limitations, such as the nature of the sample, the nature of the measures (all were self-report, even some of the spatial abilities), or statistical power.

5.1.3. Interpretation of findings: OAEs in relation to behavior

Although there is some limited evidence that prenatal hormones play a role in the development of OAEs, there is much that is still not known. A particular question concerns the timing of androgen effects on OAEs. As discussed above, prenatal weeks 8–24 are considered to be most critical to brain and behavioral sex differentiation because of a surge in serum testosterone in male fetuses. The auditory system has its origin in the cells of the neural crest, which emerge at about weeks 3–4 of gestation, although the development of cochlear structures related to OAEs occurs later in gestation. Histological data on the development of the outer hair cells is incomplete, but it appears that they are not developed along the entire cochlear epithelium before 24 weeks of gestation. In preterm neonates, SOAEs can be recorded at 30 weeks of gestation [212], and they are comparable to full-term neonates in prevalence, peak number and acoustic frequencies. But, OAEs in preterm neonates do not show typical effects of laterality (more SOAEs in right than left ears) or sex (more SOAEs in females than males), suggesting that a substantial part of the maturation of the peripheral auditory system occurs around birth [212,213]. Overall, then, the current evidence makes it difficult to use OAEs as a measure of prenatal androgen exposure, although its promise should be explored, especially because it is unlikely that OAEs are influenced by aspects of the sex-typed social environment.

5.1.4. Recommendations: OAEs as reflections of prenatal hormones

It is important to obtain direct data on androgen effects of OAEs, including information about the specific levels necessary to masculinize them and the timing of the sensitive period. There are several ways to obtain this information. First, OAEs can be examined in relation to hormone manipulations in nonhuman animals. For example, the macaque appears to be a good model for studying SOAEs [214]; in fact, effects of prenatal lead exposure on auditory function have been investigated with OAEs in monkeys [215]. Second, it would be interesting to study OAEs in individuals with known alterations in prenatal androgen exposure, such as CAH. Third, OAEs might be included in studies of prenatal hormones in typical samples in which participants are followed prospectively, as described above.

At first glance, it seems relatively simple to conduct these studies because OAE measurement is fairly straightforward, non-invasive, and objective. Data can be collected by a trained technician, although support from a specialist is required in design and interpretation, and calibration of the equipment. But, data collection requires more tightly controlled conditions than is usually possible in studies of hormone effects: advanced OAE techniques require a sound-attenuated chamber, although useful OAEs can be made in a quiet office environment [199]; body movements interfere with recording, a particular concern in studies of children; reliable measurement requires 20 min of recording, and it is often difficult to have participants remain still for such a long period of

time; timing of data collection needs to be controlled to adjust for diurnal rhythms in SOAEs (the frequency is lowest at night and in the early morning) [207,208]. These limitations explain why some attempts to conduct studies in patients with CAH have not been realized. A potential opportunity for collecting data on OAEs comes from their widespread use in hearing screening for newborns, although it is unclear whether such programs collect sensitive enough data to obtain reliable individual differences or whether they only detect hearing loss.

5.2. Finger ratio (2D:4D)

5.2.1. Background

The ratio of the length of the second digit (index finger) to the length of the fourth digit (ring finger), the 2D:4D ratio, is assumed to be fixed by week 14 of fetal life [216]. The development of the ratio has been hypothesized to be affected by testosterone, which is high from prenatal weeks 8–24 [217]. If the 2D:4D ratio does, in fact, accurately reflect prenatal sex hormones, it would provide a simple and widely-available method for examining hormonal effects on human behavior. The evidence supporting this assumption is mostly indirect. The 2D:4D ratio is lower in men than in women, resulting from men showing longer fourth digit relative to the second digit, and women showing longer second digit relative to the fourth digit. (Other digit ratios have been investigated and some also show sex differences [218], but we concentrate on the 2D:4D ratio because it has been the most extensively studied.) This sex difference was noted more than 125 years ago [219] and a standard method for measuring it was developed 55 years later [in 220]. The finger ratio appears to be stable from age 5 [221,222] and shows a sex difference across races [223, 224]. In fact, the 2D:4D ratio shows larger ethnic than sex differences [225]. The sex difference in the 2D:4D ratio is found on both hands, although it is somewhat larger on the right than the left hand, *d*'s of 0.85 and 0.74, respectively [226].

The details of the theory and evidence regarding 2D:4D have been extensively discussed in recent reviews [217, 227]. Our intent is not to provide another review, but instead to consider the value of the finger ratio as a marker of prenatal sex hormone exposure and therefore its utility in behavioral studies. Therefore, we focus on the major studies examining the relation between 2D:4D and prenatal hormones, on the one hand, and sex-typed behaviors, on the other hand.

The 2D:4D ratio is thought to be negatively related to testosterone levels in men and positively related to estrogen levels in both men and women [221]. Testosterone has been suggested to stimulate the prenatal growth of the fourth digit, and estrogen to promote the growth of the index finger [217]. Prenatal exposure to sex hormones has been considered important in

the development of the ratio because the sex difference is apparent in childhood and the ratio appears not to be affected by the changing levels of sex hormones during puberty. The sex difference is suggested to arise about week 8 of gestation, when the fetal testes are formed in males. The homeobox genes, especially the HOXA and HOXD genes, are responsible for the differentiation of the urogenital system (including the gonads) [227,228]. These genes also influence the formation of human toes and fingers [230], and a mutation in the HOXA gene leads to the hand–foot–genital syndrome, which is characterized by malformation of the digits, toes, and genitals [231]. This relation between the formation of the gonads and the fingers has led to the hypothesis that finger differentiation, as measured by the 2D:4D ratio, may reflect prenatal testosterone production in humans during the first trimester of gestation [221].

There is limited direct evidence to support these assumptions. Testosterone and estradiol in amniotic fluid measured in the second trimester were studied in relation to finger ratio in 2-year-old children [232]. Results showed a negative association between the finger ratio and the ratio of fetal testosterone to estradiol, regardless of sex but more strongly for the right than the left hand. Given that finger ratio may change later in childhood, the meaning of this relation is not clear [221,222]. Further, this relation was documented later in gestation than the presumed critical period for 2D:4D development (second vs. first trimester), although it may be explained if there is stability of individual differences in sex hormones across prenatal development. Studies in patients with CAH are inconsistent, with some finding a more masculine finger pattern in females and males with CAH than in controls [233,234], but some finding no difference [235]. Some of the inconsistencies may relate to differences in method: most studies in normal populations examine the finger ratio of the right hand, measured from photocopies, and this was the method used in the two studies finding differences between CAH and controls [233,234]. The study failing to find differences used X-rays of the left hand [235].

5.2.2. Findings: finger ratio associations with behavioral and physical traits

5.2.2.1. Physical traits.
Finger ratio has been examined in relation to a number of physical traits that show sex differences and that have been suggested to be influenced by the prenatal environment, although it is important to note that there is no direct evidence for the latter. Given the hypothesized association between prenatal testosterone and finger ratio, and the hypothesized slower prenatal growth in males than in females, the 2D:4D ratio was studied as an indicator of fetal growth [236]. Results are not easy to interpret. In males, a high (demasculinized) 2D:4D ratio was associated with shorter birth length and bigger head circumference relative to

birth length, but the association was found only in those whose placenta was heavier than the median. No association was found in females. A higher (less masculine) 2D:4D ratio has also been associated with early presentation of cardiovascular disease and with prognosis after myocardial infarction [237].

Adult sexually dimorphic physical traits have also been examined in association with 2D:4D. In general, these traits are used to make inferences about the effects of prenatal hormones on physical characteristics that develop at puberty or about the relation between prenatal hormone levels and adult hormone levels. Traits such as body mass index, waist-to-hip ratio, and waist-to-chest ratio, were expected to be associated with low 2D:4D, i.e. masculine body form associated with masculine finger ratio. Results showed body shape to be more strongly associated with finger ratio in women than in men, and were taken to reflect an effect of prenatal androgens on body shape [238]. For example, in women there was a negative correlation between finger ratio and waist-to-hip ratio and waist-to-chest ratio, whereas in men there was a positive correlation between left-hand finger ratio and body mass. Adult sperm number and circulating testosterone levels [221] were found to be negatively associated with right-hand finger ratio. Facial asymmetry in students was found to be associated with finger ratio, negatively in men and positively in women, and was interpreted to reflect prenatal hormone effects on facial attractiveness [239]. Skiing performance was associated with finger ratio, with skiers having lower 2D:4D ratios than sex- and age-matched non-skier controls, and faster skiers having lower ratios than slow skiers [240].

It is difficult to interpret these reports in light of the limited theoretical basis for the studies. They would be more convincing if the traits could be clearly tied to prenatal development and if they were replicated by independent researchers; all studies described above were conducted by the same research group.

5.2.2.2. Sexual orientation. Findings of finger ratio in relation to sexual orientation are complex. Studies in men have been motivated by two conflicting hypotheses. On the one hand, homosexual men were hypothesized to be exposed to *high* levels of testosterone in utero, which would be associated with a lower 2D:4D ratio than that found in heterosexual men. Two studies found this to be the case [241,242], but one did not [243]. On the other hand, homosexual men have been hypothesized to have *low* prenatal testosterone exposure, and data from two studies are consistent with that hypothesis [244,245], showing homosexual men to have a higher finger ratio (on both hands) than heterosexual men; one study was large and considered effects of ethnicity [244]. There is no direct evidence that would favor one or the other hypothesis about prenatal testosterone exposure in gay men, but indirect evidence supports the idea that they have low exposure,

given their demasculinized/feminized interests and spatial ability [246–248; but see 249]. Alternatively, the inconsistency may reflect population differences in 2D:4D and the fact that studies sample different populations, in this case the United Kingdom and the United States [250]. Mean 2D:4D appears to be constant across populations of homosexual individuals, but variable across populations of heterosexual individuals (higher in the UK than in the US), and the data indicating hypermasculinization come from the UK and those indicating hypomasculinization from the US [250].

Studies in women are generally motivated by the hypothesis that homosexual women have been exposed to higher prenatal testosterone than heterosexual women, consistent with their masculinized interests [246]. One study failed to find a difference in finger ratio between heterosexual and homosexual women after correction for ethnicity [244], but two other studies found that lesbians had a lower (masculinized) finger ratio [242,243], and another found a difference for butch-type, but not femme-type lesbians [251]. A study of finger ratio in female mono-zygotic twins provided an innovative control for genetic and environmental background factors [252] and showed a relation between finger ratio and sexual orientation. In female twins discordant for sexual orientation, the lesbian twin had a significantly lower (masculinized) finger ratio in both hands compared to her heterosexual co-twin; in twins concordant for sexual orientation, there was no difference in finger ratio.

Thus, the data suggest that masculinized finger ratio is associated with homosexuality in women. The evidence in men is inconsistent. Given that differences in other sex-typed traits between homosexual and heterosexual individuals are equivocal (except for interests), the inconsistency in finger ratio is not surprising.

5.2.2.3. Sex role. The development of sex role has been related to prenatal exposure to androgens (as described above for CAH), and it is reasonable to hypothesize that 'masculinized' role would be associated with lower finger ratio in women. One study is consistent with this: the 2D:4D ratio was lower in women who had more 'masculine' scores on a sex role questionnaire [253].

5.2.2.4. Personality. Finger ratio has been examined in relation to a number of psychological traits that show sex differences, with most findings based on a single study and most conducted by a single research group. Dominance and masculinity were negatively related to the 2D:4D ratio, but circulating testosterone levels were not related to any variable [254]. A variety of personality traits were examined in association with 2D:4D, with some, such as sensation-seeking, psychoticism, and neuroticism, associated as expected but only in women, and others, such as extraversion, depression, and aggression not associated in either sex [255]; it is difficult to interpret both positive and negative

findings given the number of traits examined and the relatively small sex differences in some of them. Assertiveness and competitiveness in women were associated with low (masculine) finger ratio [256].

5.2.2.5. Cognitive abilities. The finger ratio has also been examined in association with cognitive abilities, with inconsistent results. Most studies reporting support for hypothesized associations were again conducted by a single research team. Rightward pegboard performance was positively associated with the right-hand ratio [257]. There was a complicated relation between lateralized hand performance and finger ratio: a high ratio in the left hand and a low ratio in the right hand were associated with faster performance with the left hand. Spatial ability has been negatively associated with finger ratio (high performance, low ratio) in men [217,245,258], but not consistently [255,259].

5.2.2.6. Developmental disabilities. Given the sex difference in autism and the suggestion that it is caused by exposure to high levels of testosterone in utero [260], autism has been examined in relation to finger ratio [261]. The 2D:4D ratio was lower in families with autistic individuals than in the general population. Children with Asperger syndrome, considered a mild form of autism, had higher ratios than children with 'pure' autism, with both having ratios lower than normals. The 2D:4D ratio has also been found to be lower in girls with hyperactivity and poor social cognition and higher in boys with emotional problems [222].

5.2.3. Interpretation of findings regarding associations with finger ratio

The studies discussed above assume that the 2D:4D ratio reflects the fetus's exposure to prenatal sex hormones early in gestation. It is very important to note that the ratio is an indirect measure of sex hormones and is contaminated by other factors. For example, the finger ratio appears to relate more to ethnicity than to sex, and is affected by handedness. Effects are more pronounced for the right hand, and a similar asymmetry has been seen in mice [220].

A very recent review of the literature on the 2D:4D ratio [227] addressed some of the methodological issues in the published studies (e.g. Type I error) and concluded that some evidence supports an association between prenatal exposure to sex hormones and finger ratio, but raised some interesting questions, most prominently how 2D:4D could be unrelated to other sexually dimorphic traits which are known to be influenced by sex hormones (although many have not even been investigated in relation to 2D:4D). The reviewers suggested a role for developmental timing, that is, all traits depend on hormonal influences, but they may differ in developmental timing and therefore be uncorrelated with each other. It is

also reasonable to consider that different traits are also influenced by additional different factors.

It is also difficult to understand how prenatal exposure to estrogen is related to the development of the finger ratio because exposure to this hormone comes largely from the mother and is identical for all fetuses, regardless of their sex. Prenatal estrogen appears to have little effect on early human development, perhaps because both males and females are exposed to high levels of estrogen from the mother [5]. Thus, it is difficult to explain results showing associations between 2D:4D and estrogen [221,232].

5.2.4. Recommendations regarding finger ratio as a reflection of prenatal hormones

Studies of finger ratio are promising, if complicated. The main issues are the limited support for the major assumption underlying the method—that it reflects prenatal exposure to sex hormones—and the lack of clear theoretical motivation for many of the measures used. The attractions of the method are clear: it can be used with children (the finger ratio is stated to be stable from age 5) and it is easy to use. Given the substantial literature on behavioral sex differences [161] and the evidence from CAH, it might be wise to focus studies of finger ratio on traits already suggested to be influenced by prenatal androgens (such as sex-typed interests, spatial ability, aggression). If those studies confirm findings in CAH, then it might be reasonable to extend studies of finger ratios to additional sex-typed traits. It is also important to continue to seek ways to validate the method. For example, findings of low 2D:4D (masculinized) ratio in females with CAH and high 2D:4D (female-typical) ratio in CAIS would confirm the role of androgens in the development of relative finger lengths.

5.3. Dermatoglyphics

5.3.1. Background

Dermatoglyphics (fingerprints) represent another morphological measure suggested to reflect prenatal exposure to sex hormones, particularly testosterone. There are two indices used: total finger ridge count and asymmetry of ridge count on the two hands. The number of dermal ridges is fixed by about the fourth month of gestation and is largely under genetic control, although prenatal environmental events can perturb their development [262]. There are sex differences in total finger ridge count (males have more total ridges than females) and in asymmetry (although both sexes have more ridges on the right hand than on the left hand ($R > L$), the reverse asymmetry (left greater than right, $L > R$) is more common in females than in males). Total ridge count is associated with the number of X chromosomes (lower count with increasing numbers), but not necessarily as a reflection of prenatal hormones (e.g. individuals with low hormones due to Turner Syndrome have higher ridge

count than typical males) [263]. Total ridge count has been suggested to reflect the rate of early fetal growth, with a higher count reflecting rapid cell division in prenatal weeks 8–16 [264,265]. These findings and associated indirect evidence about fetal development, e.g. regarding the role of homeobox genes in development of urogenital system and digits (as described above), the common endoderm derivatives of skin and nervous system [266], and the role of testosterone in stimulating production of epidermal and nerve growth factors [267,268], have led to the suggestion that dermatoglyphics can serve as a window into prenatal development.

Nevertheless, direct evidence regarding testosterone effects on fingerprints is not compelling. Evidence regarding *total* finger ridge count is inconsistent: prenatal testosterone treatment in monkeys affected total count, but only if it was given early and, opposite to expectation, was associated with lower ridge count [269]; females with CAH had higher ridge count than controls in one study [270], but not in another [271], but both had small samples. There is no direct evidence for testosterone effects on fingerprint *asymmetry*: several measures of asymmetry were reported to be related to circulating testosterone in 39 adult males, and taken to provide indirect support for effects of prenatal testosterone, but there are no direct data showing that postnatal levels of sex hormones reflect prenatal levels of those hormones. (It is important to note here that, if a relation between prenatal and postnatal sex hormone levels could be documented, circulating hormones would then provide a simple and elegant ideal window into prenatal development.)

5.3.2. Findings: behavior in association with fingerprint asymmetries and total finger ridge count

Given the sex difference in fingerprint asymmetry ($R > L$ more common in males than in females, $L > R$ more common in females than in males), several investigators have looked to see if sex-atypical asymmetry is associated with sex-atypical behavior. Thus, studies have looked to see if, regardless of sex, the female-typical pattern ($L > R$) is associated with female-typical patterns of behavior (cognition and sexual arousal have been studied), and the male-typical pattern ($R > L$) is associated with male-typical patterns of behavior.

5.3.2.1. Sexual orientation. In men, fingerprint asymmetry was found to be associated with sexual orientation in one study, with homosexual men found to have the female-typical $L > R$ pattern more often than heterosexual men [272], but this was not replicated in two studies of homosexual men [273,274] or in two studies of transsexual men [275,276]. In women, fingerprint asymmetry has not been found to relate to sexual orientation, with two studies failing to find differences between homosexual and heterosexual women [234,274]. Total ridge count has generally not been found to be associated with sexual orientation in men or in women [272,274–276], with one

exception (in monozygotic female twin pairs, lesbians had lower ridge counts than heterosexual twins [277]).

5.3.2.2. Cognitive abilities. In several studies, dermatoglyphic asymmetry was found to relate to abilities that show sex differences but not in a simple way. In adults, both men and women with $L > R$ were found to be better at tasks on which females generally excel, a composite of measures of perceptual speed and fluency [278–280]. Those with $R > L$ were found to be better at some tasks on which males generally excel, but the specific tasks differed across study, including different aspects of spatial ability [278,280], or math reasoning [279]. A similar pattern has been reported in children, with $L > R$ associated with female-superior tasks and $R > L$ associated with male-superior tasks [281].

5.3.3. Interpretation of findings regarding associations with dermatoglyphics

The studies described above suggest that dermatoglyphic asymmetry is unlikely to be related to sexual orientation, although it may be related to cognitive pattern. Nevertheless, it is unclear whether asymmetry reflects prenatal testosterone, given inconsistencies in the direct evidence for testosterone effects on dermatoglyphic asymmetry. It is important to remember, however, that asymmetry is likely to be several steps removed from testosterone, so it is perhaps surprising to find any associations at all, given the relatively small sex differences in cognition.

5.3.4. Recommendations dermatoglyphics as a reflection of prenatal hormones

As with the 2D:4D ratio, studies using dermatoglyphics are promising, if complicated. The main issue here is also the limited support for the major assumption underlying the method—that it reflects prenatal exposure to sex hormones. Studies of dermatoglyphics have been better motivated than studies of 2D:4D in terms of measures used, but this may be because it is more difficult to collect and evaluate dermatoglyphics than finger ratios (and, thus, it is easy to collect 2D:4D in an ongoing study and relate it to whatever is being measured). It is also important to continue to seek ways to validate the method, e.g. additional studies in CAH and other clinical samples, and to examine dermatoglyphics in relation to other measures of sex-typed behavior, especially interests.

5.4. Other potential markers

There are a variety of other markers that have been proposed to reflect prenatal exposure to sex hormones, and some have been examined in relation to behavior. Although we have not reviewed those studies in detail, we comment on them to indicate the variety of methods that are available to those interested in studying the behavioral effects of prenatal hormones.

5.4.1. Pubertal onset

The sex difference in physical maturation—females have earlier puberty than males—is suggested to reflect hormonal programming of the hypothalamic–pituitary–gonadal axis during gestation [282]. Thus, pubertal timing has been considered a possible candidate marker for typical variations in prenatal hormones, and has been studied primarily in association with two traits, cognition and sexual orientation. The first major study in this area tested the intriguing hypothesis that cognitive sex differences reflect a sex difference in maturation rate, which is mediated by pubertal effects on the maturation of cerebral lateralization [283]. Initial data supported the hypothesis: regardless of sex, early maturers had better verbal than spatial abilities, whereas late maturers had the opposite pattern [283]. Subsequent studies have sometimes supported the hypothesis, but often have not, and the consensus is that the effect is small at best [56,284, 285]. Pubertal onset has been associated with sexual orientation in men [286,287], with homosexual men having an earlier puberty than heterosexual men, but not in women [288].

5.4.2. Body asymmetry

The human body is asymmetrical in many ways. Psychologists and neuroscientists have primarily focused on the asymmetry in the functions of the left and right cerebral hemispheres, but there has also been interest in body asymmetries, and some of these have been related to prenatal sex hormones [58]. We described above the use of fingerprint asymmetries. A study of asymmetry in sexual characteristics (comparison of breast or testis size on the left and right sides of the body) suggested a relation between body asymmetry and cognitive pattern [58].

6. Summary and conclusions

As is apparent, there is a lot of interest and work dedicated to understanding the human behavioral consequences of prenatal exposure to sex hormones. Although there is still work to be done, there is increasing convergence of evidence across methods showing the masculinizing effects of prenatal androgens, especially at high doses of androgens and especially for sex-typed interests, spatial ability, and aspects of personality. Thus, it seems likely that androgens are responsible for some of the differences between the sexes in these traits, although it is not as clear how much they contribute to variations within males and within females. There is a need to continue to develop and validate methods for assessing the issue in typical samples, e.g. direct evidence about androgen effects on the 2D:4D finger ratio, clarification about the mechanism accounting for an association between testosterone in maternal serum and childhood sex-typed activity interests.

Our ability to study this topic in multiple ways will also enable us to understand the details of the human behavioral effects of hormones (as has been done in other species), such as the key sensitive periods (which may differ across behavior), the forms of androgen most important for different aspects of behavioral masculinization, the importance of variations in receptor sensitivity, and the extent to which androgen effects are modified by other hormones. It will also enable us to study the developmental pathways by which hormones affect behavior, including their mediation and moderation by the social environment, and their neural underpinnings. In fact, studies of the behavioral effects of prenatal hormones provide a rich opportunity to unravel and document the oft-cited but poorly studied transactions between biology and the social environment.

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