

# Fetal Testosterone and Sex Differences in Typical Social Development and in Autism

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## ABSTRACT

Experiments in animals leave no doubt that androgens, including testosterone, produced by the testes in fetal and/or neonatal life act on the brain to induce sex differences in neural structure and function. In human beings, there is evidence supporting a female superiority in the ability to read nonverbal signals, specific language-related skills, and theory of mind. Even more striking than the sex differences seen in the typical population is the elevated occurrence of social and communicative difficulties in human males. One such condition, autism, occurs four times more frequently in boys than in girls. Recently, a novel theory known as the “extreme male brain” has been proposed. It suggests that the behaviors seen in autism are an exaggeration of typical sex differences and that exposure to high levels of prenatal testosterone might be a risk factor. In this article, we argue that prenatal and neonatal testosterone exposures are strong candidates for having a causal role in sexual dimorphism in human behavior, including social development, and as risk factors for conditions characterized by social impairments, particularly autism spectrum conditions. (*J Child Neurol* 2006;21:825–845; DOI 10.2310/7010.2006.00213).

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Experiments in animals leave no doubt that androgens, including testosterone, produced by the testes in fetal and/or neonatal life act on the brain to induce sex differences in neural structure and function. In human beings, sex differences are apparent in both brain structures and cognitive skills.<sup>1–5</sup> In addition, many neurodevelopmental disorders occur more often in male individuals than in female individuals, including autism, dyslexia, attention-deficit hyperactivity disorder (ADHD), and early-onset persistent antisocial behavior.<sup>6</sup> Autism in particular has been

described as an extreme manifestation of some sexually dimorphic traits or an “extreme male brain.”<sup>7</sup> Despite the dramatic sex ratios reported for these conditions, research on the potential causes of biased sex ratios and on sex differences in phenotypic expression that would be informative regarding potential causes is extremely limited. In this review article, we argue that the prevalence and expression of neurodevelopmental disorders might be related to sex differences in brain development and that prenatal and neonatal testosterone exposures are strong candidates for having a causal role in sexual dimorphism in behavior, including social behavior, and as risk factors for conditions such as autism.

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## TESTOSTERONE AND THE SEXUAL DIFFERENTIATION OF THE BRAIN

Endocrine (hormonal) systems are involved in every aspect of pregnancy, including implantation, formation of the placenta, maternal adaptation, embryonic and fetal development, parturition and birth, and fetal adaptation to life outside the womb. Hormones have a range of functions involving reproduction, growth and development, maintenance of the internal environment, and the production, use, and storage of energy. Experiments in animals show that gonadal hormones are essential to the sexual differentiation of both the body and the brain. These include the androgens (eg, testosterone, dihydrotestosterone), estrogens (eg, estradiol, estrone, estriol), and progestins (eg, progesterone).

Hormone effects are usually classified as organizational (those that are permanent and happen early in development) or activational (those that occur later in development, are transient, and are superimposed on the early organizational effects). These later hormonal actions are often essential to allow the tissue or organ in question to perform its function. For example, the tissues of the genetic male individual are organized prenatally for male adult reproductive behavior. However, the male adult will not display such behavior unless adequate sex hormones are present in adulthood. This article focuses primarily on organizational effects. However, the dichotomy between organizational and activational, although useful, is oversimplistic.<sup>8</sup> When studying the organizational effects of hormones on the developing fetus, it is important to remember that later activational effects can be essential to the function in question. For some hormones, such as estrogen, the distinction is particularly problematic because estrogen appears to exert organizational effects for a very long period of time.<sup>9,10</sup>

Organizational effects often occur during a sensitive (or critical) period. This is a specific period of time in which a tissue can be modified by environmental influences. They are adaptive because development cannot be influenced outside the sensitive period, protecting the animal from disruptive influences. This means, for example, that circulating sex hormones necessary for adult sexual functioning do not cause unwanted alterations to tissues, even though the same hormones might have been essential to the development of those tissues. In contrast, if development is disrupted during the critical period, the relevant tissue can be altered irrevocably, whereas the rest of the embryo remains essentially unaffected. Different behaviors can have different sensitive periods for development. For example, androgen exposure early or late in gestation has differential effects on male-typical juvenile behaviors in the female rhesus macaque.<sup>11</sup>

It has been recognized since the 1940s that castration of males during neonatal or prenatal life prevents the development of masculine genitalia, whereas prenatal or neonatal treatment of females with androgen masculinizes their genitalia.<sup>12</sup> Phoenix et al reasoned that similar experiments would affect the development of the brain, producing differences in sex-typical behavior.<sup>13</sup> They exposed female guinea pig fetuses to testosterone and found that, as adults, these female animals showed more male and fewer female copulatory behaviors. Similar experiments have been conducted in a wide range of mammals, comparing castrated male, normal male, normal female, and female mammals treated with androgens on a range of sexually dimorphic features. Castrated male mammals usually show feminized neural development, cognition, and behavior, whereas female mammals treated with androgen show masculinized neural development, cognition, and behavior. Fetal testosterone has been shown to affect the anatomy of the brain, including the hypothalamus, limbic system, and neocortex<sup>4,5,14</sup>; sexually dimorphic behavior, such as aggression and activity level<sup>15</sup>; and sexually dimorphic cognitive skills, such as spatial navigation.<sup>16</sup>

Human beings also show sex differences in both brain structures and cognitive skills.<sup>1-5</sup> For example, male subjects tend to outperform female subjects on some spatial and mathematical tasks, whereas female subjects are superior at

some language tasks.<sup>3</sup> However, the effects of prenatal hormones on these skills have been difficult to study because it is patently unethical to manipulate hormone levels in human fetuses. Certainly, the differentiation of the male and female phenotypes in the developing human fetus follows a pattern similar to that in other mammals, although exact timings vary by species. Gonadal hormones appear to play the primary role in this process,<sup>3,4,17,18</sup> although direct genetic influences on sexual differentiation of the brain are increasingly recognized.<sup>19-21</sup>

Under most circumstances, the genetic sex of a human being is determined at the moment of conception by the presence of an X (female) or Y (male) chromosome in the fertilizing sperm cell. The karyotype of the normal female is 46,XX and that of the normal male is 46,XY. Each is made up of 44 autosomes and 2 sex chromosomes. The male chromosome can be described as heterogametic and the female chromosome as homogametic. There are few differences between the genes of male and female human beings, except for the Y chromosome in the male human being.<sup>22</sup> The critical gene for starting the process of phenotypic differentiation of the sexes is the *Sry* (sex-related Y) gene, located on the Y chromosome. It is possible for the *Sry* gene to be translocated to another chromosome, resulting in a child who might have a normal female karyotype but develops as a phenotypic male individual. Studies of individuals who appear to be male but who have the XX karyotype confirm that this is because a section of the Y chromosome containing the *Sry* gene has been integrated into one of the X chromosomes.<sup>23</sup> Experiments such as these isolated the *Sry* gene as critical for male gonadal development.

Genetically, male and female fetuses have undifferentiated gonads during early development, that is, there is no difference in their reproductive structures. Around week 6 of gestation, the *Sry* gene on the Y chromosome initiates testicular differentiation in the male fetus. This is thought to be the major function of the Y chromosome. The Leydig cells of the testis are capable of testosterone synthesis by the end of week 8. Further development of the Leydig cells means that testosterone secretion is high between weeks 10 and 20. Fetal synthesis of testosterone is probably controlled by human chorionic gonadotropin and luteinizing hormone from the fetal pituitary gland. In addition, male fetuses are exposed to testosterone from the fetal adrenal glands.

In the female fetus, differentiation of the ovaries begins around week 7 of gestation. The fetal ovary is generally considered inactive until late in development<sup>23</sup> but can produce a small amount of estrogen.<sup>24</sup> The female fetus is also exposed to low levels of androgens. A small proportion can come from the fetal adrenal glands (a by-product of the production of corticosteroids), and some comes from the maternal adrenal glands, ovaries, and fat.<sup>25,26</sup>

The secretions of the gonads determine the phenotypic sex. If male sex hormones and the appropriate receptors are present, the male genital phenotype will develop, and if sufficient male sex hormones or functioning receptors are not present (i.e., in female fetuses), the female genital phenotype will develop.<sup>27-31</sup>

The gonadal hormones are also thought to be involved in the sexual differentiation of the brain, determining the neuronal sex, can refer to male- or female-type gonadotropin secretion, sexual

orientation, gender role behavior, and gender identity.<sup>32</sup> In animal models, the general critical period for sexual differentiation of the brain usually occurs when sex differences in serum testosterone are highest.<sup>33</sup> Therefore, it is likely that this is an important period for masculinization of the brain in humans as well. The onset of testosterone biosynthesis occurs at about 9 weeks.<sup>23</sup> The maximal sex difference in serum levels occurs between weeks 12 and 18 (mean [SD] = 249 [93] ng/dL and mean [SD] = 29 [19] ng/dL for male and female fetuses, respectively).<sup>34</sup> Testosterone levels in male fetuses are initially elevated in response to placental human chorionic gonadotropin and remain high under the influence of luteinizing hormone. However, human chorionic gonadotropin and luteinizing hormone receptors might not appear until weeks 10 to 12 of gestation, raising the possibility that the earliest secretion of fetal testosterone is controlled by other, unknown factors. Levels of both gonadotropins, luteinizing hormone and follicle-stimulating hormone, are controlled by the hypothalamic gonadotropin-releasing hormone pulse generator, which is insensitive to the negative feedback of gonadal sex steroids in early pregnancy. The pulse generator has matured by the third trimester and becomes sensitive to the high levels of estrogen and progesterone produced by the placenta. As a result, levels of gonadotropin etc fall.<sup>35–37</sup> After 24 weeks, plasma testosterone levels are low (in the early pubertal range).

Although weeks 8 to 24 have been considered the most important in human sexual differentiation, this does not mean that they are the only period for differentiation. Male infants are born with elevated testosterone levels (approximately 200 ng/dL) as a result of the sudden drop in inhibitory estrogen levels produced by the placenta. Testosterone rapidly decreases in the first day of life and then begins to rise again after the first week.<sup>35</sup> Levels remain high for the first year of life, peaking around the third to fourth month of life. Median levels are equivalent to those in the second stage of puberty (200–300 ng/dL)<sup>35,38</sup>; this is referred to as the neonatal surge. The function of the neonatal surge is not fully understood in humans but is likely to be related to the preparation of tissue for subsequent androgen-mediated growth. In monkeys, disruption of the neonatal surge is known to lead to disrupted testicular function at puberty.<sup>39</sup> As male infants have had a testosterone surge at this time but female infants have not, the same amount of subsequent testosterone exposure will have very different effects on each sex.<sup>4</sup> Female infants have a postnatal surge in estradiol production, which is thought to come from the ovaries.<sup>40</sup> The postnatal surges in both sexes are stimulated by surges in gonadotropin levels.<sup>40</sup>

During childhood, the gonads are quiescent. The gonadotropin-releasing hormone pulse generator is inhibited by the very low levels of sex steroids present. This feedback system is 10 times more sensitive to sex steroids than the adult feedback mechanism. Puberty occurs when the sensitivity of the pulse generator drops and the hypothalamic-pituitary axis is released from inhibition. Levels of gonadotropins rise, causing the gonads to enlarge, mature, and secrete increased amounts of gonadal steroids. The pubertal surge allows the secondary sexual characteristics to develop. By the end of this stage, the organism is prepared for reproduction.<sup>35</sup> In addition to these potential periods for steroid-induced sexual differentiation, sexual

differentiation under direct genetic control can occur when no sex differences in testosterone are apparent.<sup>21,41,42</sup>

This article focuses primarily on the prenatal stage of sexual differentiation. Testicular hormones, particularly testosterone, play a major role in this process. The default mammalian sex is female, and in the absence of very high levels of testosterone and anti-mullerian hormone, female structures will develop. It has been assumed that no special hormonal environment is required for the formation of the female phenotype.<sup>23</sup> However, this traditional model is now being replaced by a more complex one that recognizes that small amounts of ovarian hormones can be required for active feminization of the female brain.<sup>9,10</sup> Still, there are many stages that must be successfully completed for the male phenotype to develop, and these rely to a large extent on the existence of the right hormonal environment. This implies that there are a number of stages at which the normal development of the male could potentially be disrupted.

Not all animals have female as the default sex. In birds, for example, the default homogametic sex is male, and differentiation of the female bird depends on exposure to ovarian hormones. In mammals, fetuses are exposed to high levels of female hormones from the mother, so it is adaptive for the default sex to be female. In species that do not develop in the womb (i.e., egg layers), this reasoning does not apply, so having one sex as the default sex over the other does not necessarily confer the same advantages as in mammals.<sup>43</sup> It is interesting to note that feminization of the brain in mammals by ovarian estrogen is thought to occur at a later period than masculinization (in female rats, this can extend from the late neonatal to the pubertal period and perhaps even into adulthood).<sup>9</sup> This would mean that ovarian estrogen-mediated feminization takes place after the individual is free from the maternal hormonal environment of the womb.

## HUMAN SEX DIFFERENCES

The psychologic study of sex differences has traditionally focused on spatial, mathematical, and verbal ability.<sup>3</sup> However, there is increasing interest in potential sex differences in social relationships. Geary argued that sexual selection can provide a unifying framework for incorporating hormonal, experiential, and evolutionary influences on human cognitive sex differences and laid out a specific rationale for why sex differences should be seen in social cognition.<sup>44,45</sup>

Sexual selection involves both competition with members of the same sex and species (intrasexual competition) over mates and the processes associated with choosing mates (intersexual choice).<sup>46</sup> It can be expressed as male-male competition, female-female competition, female choice, male choice, or a combination thereof.<sup>44</sup> The actual expression of sexual selection in a given species depends on the degree to which male and female animals focus their reproductive effort on mating or parenting, and this, in turn, is determined by sex differences in reproductive rates and the operational sex ratio (the ratio of sexually active male animals to sexually active female animals in a given breeding population at a given point in time).<sup>47</sup>

Humans, like other mammals, have internal gestation and obligatory postpartum female care (i.e., suckling). This means

that the rate of reproduction in female humans is much lower than that of male humans.<sup>48</sup> This leads to female humans putting relatively more “effort” into parenting. In contrast, male humans benefit by putting relatively more “effort” into mating. The difference in reproductive rates also biases the operational sex ratio so that there are usually more sexually active male mammals than sexually active female mammals in a population. This bias creates conditions that lead to intense male-male competition over access to a limited number of mates. In humans, this competition is focused on social dominance and resource control and often involves a physical component, as it does in many other species.<sup>44</sup>

The importance of parenting effort to female reproductive success has significant implications for female social behavior. Unstable social relationships have negative effects on the well-being of children,<sup>49,50</sup> including elevated or highly variable cortisol levels (cortisol is an important stress hormone). Children in such environments are ill more frequently and weigh less than children in more stable social environments. Social instability can also increase child mortality rates,<sup>51</sup> especially in societies that might be more similar to human ancestral conditions. Even for those who survive until adulthood, a socially unstable environment appears to increase mortality risks at all ages and thus shorten the lifespan.<sup>44,52</sup> This might explain why women appear to value reciprocal social relationships more than men and why their relationships with other women are more consistently communal than those among men, exhibiting greater empathy, nurturance, intimacy, and emotional support. In contrast, men’s relationships with each other are more often instrumental or agentic.<sup>53</sup> Concern for others might, in some cases, actually be disadvantageous for men because their reproductive success might have depended on dominating others, sometimes through physical aggression.<sup>44,54</sup>

The importance of reciprocal social relationships for female reproductive success might have also been a selection factor because female humans have historically migrated to the social group of their mate, whereas male humans remained in their birth group (male humans are thus termed the “philopatric” sex). This is also true for other great apes.<sup>55–57</sup> The probable result is that female humans, more than male humans, were forced to form social alliances with nonkin during evolutionary history. Arguably, to be accepted into a new nonkin social group requires greater social skill and social sensitivity compared with being accepted by a kin group into which one is born.

However, women’s social relationships are not always cooperative. Throughout primate species, the female members compete with each other over access to high-quality food. Because men often control resources that women need to raise their children successfully, women are expected to compete over these resource-holding men. Male humans show greater paternal investment compared with closely related primates, and both direct and indirect paternal investment appears to improve children’s well-being. In harsh environments, the absence of a father increases mortality,<sup>51</sup> and in less harsh environments, paternal investment seems to improve children’s physiologic, social, and psychologic functioning.<sup>58</sup> In contrast to men, for whom competition is often direct and physical, when women do compete with each other, they use indirect methods, such as gossip and social exclusion,<sup>59</sup> in an attempt to damage the social

networks crucial for female success and reduce their competitors’ desirability as mates. In addition, indirect aggression can be less apparent than physical aggression, thus maintaining the individual’s appearance of empathy and kindness.<sup>54</sup>

Based on these evolutionary arguments, Geary suggested three sociocognitive abilities that should show a female superiority: the ability to read nonverbal communication signals (i.e., body posture and facial expressions), language, and the theory of mind.<sup>44,45</sup> We propose that these three abilities might not be independent of one another but instead reflect that women, on average, are better at “empathizing”.<sup>7,54,60–63</sup> This is defined as the drive to identify another’s mental states and respond to these with an appropriate emotion. This encompasses what is referred to as using a theory of mind but includes an affective reaction as well.

Several studies have shown a female advantage in reading nonverbal signals. A meta-analytic study by Hall showed that women are, on average, better than men at interpreting body language, vocal tone, and facial expression.<sup>64,65</sup> In a more recent study, women were better at attributing subtle mental states to a person when interpreting the eye region of the face.<sup>66</sup> However, not all studies show this effect.<sup>67</sup> Part of this variation might depend on the specific emotions being examined. For example, one study showed that whereas women were better at identifying emotions overall, men were superior to women at recognizing male anger.<sup>68</sup>

Although a female superiority for language-related skills is commonly accepted, actual results vary considerably across studies. This is not surprising given that language consists of a number of subsystems, including phonology, morphology, the lexicon, semantics, syntax, pragmatics, and discourse.<sup>69</sup> There are well-replicated female advantages for verbal memory, spelling ability, and verbal fluency in adulthood, although women do not have a larger vocabulary than men.<sup>3</sup> Developmentally, a number of studies have reported greater vocabularies and faster rates of language acquisition in girls.<sup>70–74</sup>

Theory of mind is the ability to make inferences about the intentions, beliefs, and emotions of other people to predict and explain their behavior. Research into sex differences in theory of mind has been limited because many of the associated tests are not sensitive enough to detect subtle individual differences, such as sex differences.<sup>66</sup> Several studies, however, suggest that theory of mind may develop earlier in females and that girls and women are, on average, better at making inferences about people’s mental states and adjusting their behavior accordingly.<sup>66,75–77</sup> These differences can arise, in part, from differences in social interest. Young girls, even at 12 months old, show a preference for dyadic interactions,<sup>78</sup> spend more time watching a film of a face than a film of a car,<sup>79</sup> and make more eye contact.<sup>80</sup> They are more interested in facial than spatial or mechanical stimuli, even at birth.<sup>81</sup> It has recently been suggested that autism represents an exaggeration of these typical sex differences in social development.

## EXTREME MALE BRAIN THEORY OF AUTISM

Autism spectrum conditions are characterized by impairments in reciprocal social interaction, impairments in verbal and nonverbal communication, lack of imaginative play, and repetitive,

stereotypical behaviors and interests. Autism, high-functioning autism, Asperger syndrome, and pervasive developmental disorders (not otherwise specified) are all classified as autism spectrum conditions. As many as 1 in every 200 people can have such a condition.<sup>82</sup> Although the conditions are thought to have a strong neurobiologic and genetic component,<sup>83</sup> the factors causing autism spectrum conditions are still unclear, with many competing hypotheses discussed in the literature.

The starting point of the extreme male brain hypothesis is that factors during fetal life shape the brain as either a male brain type or a female brain type. In the initial formulation of this theory, the male brain type was held to be more developed in terms of folk physics than in terms of folk psychology. The female brain type was held to be more developed in terms of folk psychology than in terms of folk physics. Folk physics is our everyday understanding of objects in terms of physical causality and spatial relations,<sup>84</sup> whereas folk psychology is our everyday understanding of people in terms of their mental states, sometimes referred to as “theory of mind.”<sup>85</sup> The theory has been reformulated, defining the male brain as being more developed at “systemizing” and the female brain as being more developed at “empathizing.”<sup>84</sup> Systemizing refers to the ability to understand systems in terms of rules. This includes not only some traditional spatial tests but also abstract mathematical systems, technical systems such as tools, natural systems such as the weather or biologic phylogenies, and structured social systems (e.g., dominance hierarchies). Empathizing includes appropriate emotional responding to another person but also includes the cognitive ability to model and predict people’s emotions, mental states, and behavior using a theory of mind and the ability to read people’s emotions and mental states in their face, voice, and body language.

In this framework, autism occurs when an individual’s systemizing ability is intact or superior, whereas their empathizing ability is impaired.<sup>86</sup> Evidence includes the fact that individuals with an autism spectrum condition perform poorly on tests in which female subjects are usually superior to male subjects, such as “Reading the Mind in the Eyes,” in which subjects have to look at an individual’s eyes and choose which of four emotions the person is experiencing,<sup>87</sup> but perform better than people without an autism spectrum condition on tests in which male subjects usually outperform female subjects, such as the Embedded Figures Task.<sup>88,89</sup> Some individuals with Asperger syndrome (a subgroup on the autism spectrum) have achieved extremely high levels of success in fields that require systemizing ability, such as mathematics and engineering.<sup>90</sup> The restricted interests seen in autism might also be the result of this high drive for systemizing. In a study of obsessions in autism, obsessions were significantly more likely to focus on systems than on emotions or relationships.<sup>91</sup> If autism is an exaggeration of typical sex differences, then normally developing male individuals should show more restricted interests compared with female individuals. Is this the case?

Intriguing evidence comes from a study of the Autism-Spectrum Quotient. This self-administered instrument measures autistic traits and consists of five subscales (communication, social, imagination, local details, and attention switching). Male subjects score slightly but significantly higher on the scale as a whole and on all subscales except local details. Higher

male scores on the imagination and attention switching scales suggest that male individuals are more likely to have restricted interests than female individuals.<sup>92</sup> Another source of evidence comes from tests of systemizing,<sup>62,93</sup> which, by definition, involves a very narrow focus of attention on each variable in the system. Male subjects score higher on both the Systemizing Quotient<sup>93</sup> and on an experimental test of systemizing.<sup>86</sup>

Lines of evidence implicating testosterone as a candidate mechanism in the etiology of autism include the following:

1. Autism is much more common in male individuals than female individuals: 3:1 for classic autism and 9:1 for Asperger syndrome.<sup>94,95</sup>
2. Normal male individuals develop socially more slowly than do normal female individuals, and people with autism are even more delayed in social development.<sup>76</sup>
3. Left-handedness is more common among male individuals and in individuals with autism.<sup>96–98</sup>
4. In the normal population, the male brain is heavier than the female brain, and people with autism have even heavier brains than normal male individuals.<sup>99</sup>
5. A subset of adolescents with autism show elevated androgen levels and precocious puberty.<sup>100</sup>
6. The ratio of the second to the fourth digit (2D:4D ratio), an index of prenatal testosterone exposure,<sup>101</sup> is lower in male individuals than in female individuals and is even lower in individuals with autism compared with population normative values.<sup>102</sup>

Social and environmental factors certainly play a significant role in the development of sex differences in social development, but biologic contributions are also suggested by genetic and neurobiologic studies. Social skills, social reciprocity, and empathy have a heritable component.<sup>103–105</sup> Several brain regions appear to subserve social cognition, and theory of mind, in particular. This neural network comprises the amygdala, superior temporal sulcus, and the orbital and medial prefrontal cortices.<sup>106,107</sup> In addition, autism is considered to have a neurobiologic basis and a strong genetic component.<sup>83,108</sup>

## STRATEGIES FOR STUDYING FETAL TESTOSTERONE IN HUMANS

The effects of prenatal hormones on human sex differences have been difficult to study because it is clearly unethical to manipulate hormone levels in human fetuses. A role for fetal testosterone in human development is suggested by studies of the following:

1. Individuals with disorders of sexual development
2. Individuals who have been exposed to chemicals that mimic or block endogenous hormones
3. Opposite-sex dizygotic twins
4. Individuals whose fetal testosterone has been measured in umbilical cord blood
5. Maternal testosterone levels during pregnancy
6. Individuals whose fetal testosterone has been measured in fluid obtained at amniocentesis

Each of these lines of evidence is reviewed.

### Disorders of Sexual Development

Disorders of sexual development can be divided into two broad categories: disorders of sex determination and disorders of sex differentiation. Disorders of sex determination are most often caused by sex chromosome abnormalities or gene abnormalities affecting gonadogenesis, and disorders of sex differentiation are often characterized by an abnormal hormonal environment. This can be due to genetic or environmental factors.

Congenital adrenal hyperplasia is a family of genetic disorders, each characterized by a specific enzyme deficiency that impairs cortisol production by the adrenal cortex, and can lead to sexual ambiguity in both genetic male individuals and female individuals. The most common biochemical cause is 21-hydroxylase deficiency (making up 90% to 95% of cases). When there is a complete or almost complete absence of a functioning enzyme, classic congenital adrenal hyperplasia occurs. This occurs in 1 in 15,000 live births and results in a significant overproduction of androgen beginning in the third month of fetal life.<sup>109</sup>

Although it can affect both male and female individuals, the most interesting psychologic findings have come from studies of female subjects, who are usually compared with unaffected female relatives. Most girls with the condition are recognized at birth or during early childhood, at which point, the hormonal abnormalities can be ameliorated through cortisone replacement therapy.<sup>110</sup> This means that for individuals who received early neonatal diagnosis and treatment, androgen is elevated only in the prenatal and early neonatal periods. If testosterone affects human brain development, girls with congenital adrenal hyperplasia should have masculinized cognition and behavior. As predicted, studies of girls with congenital adrenal hyperplasia report that they have superior spatial reasoning<sup>111</sup>; prefer masculine toys and activities<sup>112,113</sup>; draw pictures with masculine characteristics<sup>114</sup>; are more likely to report the use of physical aggression in conflict situations<sup>115</sup>; are less interested in marriage, motherhood, and physical appearance<sup>116,117</sup>; and are less likely to engage in heterosexual activity and more likely to fantasize about other women.<sup>118,119</sup>

In contrast, in most male fetuses with congenital adrenal hyperplasia, testosterone appears to be in the high normal range, and affected male infants are born with normal male external genitalia.<sup>23,120,121</sup> Prenatal levels of the relatively weak androgen androstenedione appear to be elevated. Neonatally, there is some evidence that treatment to reduce corticosteroid exposure can also produce abnormally low testosterone levels in boys with congenital adrenal hyperplasia.<sup>122</sup> Despite these abnormalities, the majority of studies of cognition and behavior in male subjects with congenital adrenal hyperplasia show no differences from control male subjects.<sup>1,121,122</sup> Boys with congenital adrenal hyperplasia showed decreased "rough-and-tumble" play compared with control boys in one study,<sup>123</sup> and there are two reports of reduced mental rotation ability in male individuals with the condition.<sup>111,124</sup> In contrast, two recent studies found that the 2D:4D ratio is lower in both male and female individuals with congenital adrenal hyperplasia, suggesting that they are exposed to elevated fetal testosterone levels.<sup>125,126</sup>

Hypermasculinization of boys with congenital adrenal hyperplasia has also been reported for activity level and

preference for right-handed writing.<sup>127</sup> There are two potential explanations for these paradoxical findings. First, it is possible that an initial elevation in androgen is detected by neural feedback mechanisms, leading to reduced testicular androgen secretion. This reduction in testicular androgen could normalize androgen levels for most of the period of sexual differentiation but could also cause levels that are lower than normal at some points (e.g., when testicular androgen would normally peak or when cortisol treatment is initiated to reduce adrenal androgen production). Androgenization can be spatially and temporally specific: a concept McFadden referred to as "localized effects."<sup>128</sup> For example, digit length might be affected during early pregnancy before negative feedback reduces testosterone levels. Second, the effect of testosterone on certain behaviors can be nonmonotonic. That is, increasing testosterone exposure can produce increasing masculinization up to a certain dose, but an increase beyond this dose could cause a reversal toward the original state (demasculinization). There is support in the animal literature for nonmonotonic effects.<sup>129,130</sup> In the human literature, it has been suggested that male humans are overrepresented at the extremes (both high and low) of abilities that show mean sex differences favoring male humans, although this idea is controversial.<sup>131</sup> Grimshaw et al, in their study of fetal testosterone and mental rotation, found an inverted U-shaped relationship, with the best scores in the low male range.<sup>132</sup> The levels of circulating prenatal testosterone in male humans with congenital adrenal hyperplasia are clearly complex, making it substantially more difficult to test specific hypotheses about testosterone-behavior relationships in this group.

No study has directly examined whether autism is more common in girls with congenital adrenal hyperplasia, although there is a case report of a boy born with congenital adrenal hyperplasia and an XYY chromosomal karyotype who was described as having autism.<sup>133</sup> A recent study, however, has found that girls with congenital adrenal hyperplasia have a higher Autism-Spectrum Quotient score than their unaffected sisters (Knickmeyer RC et al, submitted for publication). In addition, girls with congenital adrenal hyperplasia score lower than control girls on measures assessing empathy, intimacy, and the need for close social relationships.<sup>134,135</sup> In reference to other developmental conditions, Plante et al reported that individuals with congenital adrenal hyperplasia showed higher levels of language and learning difficulties than unaffected family members.<sup>136</sup> The authors reported that their results are consistent with an androgen effect but could also be explained by direct genetic factors. In addition, the study did not separate the sexes for analysis. Given the possibility that prenatal androgen levels are not elevated in boys with congenital adrenal hyperplasia, separate sex analyses are crucial. Nass and Baker found that female subjects with congenital adrenal hyperplasia had a discrepancy between their Wechsler Verbal IQ and Performance IQ, which was considered indicative of a learning disability.<sup>137</sup> However, this result might reflect the enhanced spatial ability often reported in congenital adrenal hyperplasia as opposed to a learning disability.<sup>138</sup> It is interesting that children with classic autism often show a discrepancy between their Wechsler Verbal IQ and Performance IQ in the same direction. However, individuals with Asperger syndrome often have better verbal scores than performance scores.<sup>139</sup> Further study is

clearly needed to determine whether there is a raised incidence of developmental disabilities in congenital adrenal hyperplasia. Such studies will require large sample sizes because the population-based rate of diagnosable learning disabilities is relatively low. Future studies should also assess whether learning disabilities are related to androgen excess or to other characteristics of the disease.

Whereas many studies have supported the idea of masculinization in female individuals with congenital adrenal hyperplasia, others have found no such effect. One study, for example, reported that diagnosed female individuals did not differ from controls on hand preference or dichotic listening asymmetry,<sup>140</sup> and another reported no difference on tests of visuospatial ability, as well as noting deficits in quantitative ability.<sup>141</sup> They do not appear to have atypical gender identity or playmate preference.<sup>113</sup> In addition, diagnosed female individuals were found not to differ from their unaffected sisters with regard to assertiveness, dominance, acceptance in peer groups, and energy expenditure.<sup>117</sup> These negative findings do not disprove the androgen theory of sexual differentiation in humans; rather, they suggest that testosterone plays a role only in a subset of sex differences.

Research needs to differentiate within the category of 'visuospatial' skills because a male superiority does not exist for all visuospatial skills. Most of these show no sex difference. Remembering an object in a busy environment shows a female superiority.<sup>142</sup> Even for those skills that do show a male superiority, this varies in strength. Mental rotation shows a very strong male superiority, whereas finding an embedded figure shows a moderate effect.<sup>3</sup> Timing of exposure must also be considered. Hines et al found that girls with congenital adrenal hyperplasia performed better than unaffected girls on a targeting task but not on a mental rotation task.<sup>124</sup> In contrast, boys with congenital adrenal hyperplasia performed equally well as unaffected boys on a targeting task but performed more poorly on mental rotation. The authors speculated that prenatal testosterone levels influence the development of targeting ability but not mental rotation. They suggested that mental rotation might be affected by testosterone in the first 6 months of life (note that once treatment is begun, androgen levels can be suppressed, so boys in their sample might have had lower testosterone levels than normal during the neonatal testosterone surge).

Studies of individuals with congenital adrenal hyperplasia provide some of the most compelling evidence that prenatal androgens have a lasting affect on human behavior. However, interpretation of the results could be confounded by difficulty in differentiating between the effects of elevated prenatal androgens and other characteristics of the condition. Individuals with congenital adrenal hyperplasia are also exposed to high levels of adrenocorticotropic hormone, and treatment can result in glucocorticoid excess.<sup>138</sup> In contrast to the androgen hypothesis, there is no clear theoretic reason why these changes would masculinize behavior. In addition, masculinization is correlated with the degree of androgen excess.<sup>143,144</sup> An additional potential confound is that parents might treat daughters with congenital adrenal hyperplasia differently because they are virilized at birth.<sup>145</sup> There is currently no experimental support for this position,<sup>11,112,117,138,144</sup> but it has yet to receive a strong test.<sup>138</sup>

Complete androgen insensitivity occurs when there is a complete deficit of androgen receptors. Complete androgen insensitivity is an X-linked recessive disorder and hence occurs more often in genetic male individuals. Prevalence is estimated at between 1 in 20,000 and 1 in 60,000 live male births. At birth, genetic male infants with complete androgen insensitivity are phenotypically normal female infants, despite an XY complement, and are usually raised as girls, with no knowledge of the underlying disorder. At puberty, the breasts develop under the influence of estrogen derived from testicular androgens. Diagnosis usually takes place when menarche fails to occur.<sup>23,146</sup> Quality of life (self-esteem and psychologic general well-being), gender-related psychologic characteristics (gender identity, sexual orientation, gender role behavior in childhood and adulthood), marital status, personality traits that show sex differences, and hand preferences do not differ between genetic male individuals with complete androgen insensitivity and control women.<sup>147,148</sup> Therefore, these male individuals have been demasculinized. They also perform in a female-typical fashion on tests of visuospatial ability.<sup>149</sup> Findings argue against the need for two X chromosomes or ovaries to determine feminine-typical psychologic development in humans and reinforce the important role of the androgen receptor in influencing masculine-typical psychologic development.

Idiopathic hypogonadotrophic hypogonadism occurs when an individual has low levels of pituitary gonadotropins or their hypothalamic releasing factors. As a result, their gonads lack sufficient stimulation to produce normal levels of hormones. The disorder can occur after puberty or congenitally. Male individuals with congenital idiopathic hypogonadotrophic hypogonadism are usually diagnosed when they fail to undergo normal puberty. These individuals have normal genitalia at birth, so it cannot be assumed that their prenatal testosterone levels were lower than normal. This is consistent with the important role of human chorionic gonadotropin in stimulating the human fetal testis. Possibly, maternal gonadotropins also stimulate the testes to produce hormones prenatally. Cappa et al found no deficits on the block design subtest of the Wechsler Adult Intelligence Scale (WAIS) (which shows a small sex difference, eg,  $d = 0.34$ ) in a group of men with idiopathic hypogonadotrophic hypogonadism.<sup>150,151</sup> In contrast, Hier and Crowley found that men with idiopathic hypogonadotrophic hypogonadism performed significantly worse on the block design subtest, the Embedded Figures Test, and the space relations subtests from the differential aptitude tests when compared with normal men and men with acquired hypogonadotrophic hypogonadism after puberty.<sup>152</sup> This would be consistent with androgens exerting a permanent organizing influence of the brain before or at puberty.

Individuals with Turner syndrome and Klinefelter syndrome have also been studied. Both of these conditions are associated with abnormal sex chromosomes and extensive clinical problems, so their study is less relevant to the relationship between prenatal hormones and normal psychologic development than the study of some other individuals. However, they might suggest direct genetic affects on sexual differentiation.

Individuals with Klinefelter syndrome have a 47,XXY karyotype. The condition occurs in 1 per 800 to 1000 males and is the most common human chromosomal abnormality. They develop as phenotypic males, confirming that a single Y

chromosome expressing the *Sry* gene is sufficient to cause formation of the testis and male sexual differentiation. Seminiferous tubule dysgenesis results in testosterone levels that are variable but usually decreased; it is not known if prenatal levels are low, but they are not so low as to cause ambiguous genitalia at birth. Individuals have a lower than normal Wechsler Verbal IQ, delayed emotional development, poor motor control,<sup>23</sup> abnormal cerebral asymmetry,<sup>153</sup> and an impairment in inhibitory skills.<sup>154</sup> They are at greater risk of developing schizophrenia.<sup>155,156</sup> They might have particular strengths in spatial planning, organization, and memory.<sup>152</sup> The observed relationships presumably reflect X-linked effects, but their relevance to normal sex differences is unclear.

Turner syndrome usually refers to individuals with a 45,X karyotype. The condition occurs in approximately 1 in 2000 to 1 in 5000 live female births. The condition is usually apparent at birth owing to a number of somatic stigmata. Gender identity and attitudes are female; however, because of gonadal dysgenesis, individuals have extremely decreased estrogen levels.<sup>23,157</sup> Individuals often have cognitive deficits in Wechsler Performance IQ, visuomotor skills, visuospatial processing, visuospatial memory, and mathematics with preserved or enhanced verbal skills.<sup>23,158,159</sup> Collaer et al specifically examined skills that showed sex differences and concluded that individuals with Turner syndrome are impaired on both male-superior and female-superior skills (as opposed to showing an exaggerated female pattern) and should be considered neutral or less differentiated.<sup>157</sup> Impairments in social functioning are also common among individuals with Turner syndrome, and they have significant impairments in face and emotion processing.<sup>160–165</sup>

Turner syndrome, like Klinefelter syndrome, is a genetic disorder, and, hence, at a simple level, all of its characteristics correlate with the fundamental genetic abnormality. The challenge is to discover which outcomes are caused directly by genetic factors and which are mediated by other causal factors (such as steroid hormones) that might correlate with genetics. Researchers can attempt to separate hormonal and genetic effects on theoretic grounds by extrapolating from animal research<sup>166</sup> and by comparing individuals with full Turner syndrome with those who are mosaic for the disorder and testing for X-chromosome dosage effects.<sup>167</sup> Skuse et al reported that the social difficulties in Turner syndrome are mainly attributable to individuals who have inherited their X chromosome from their mother as opposed to their father.<sup>168</sup> They suggested that there is an imprinted locus on the short arm of the paternally derived X chromosome, which is responsible for the female superiority on sociocognitive abilities and for the greater vulnerability of male individuals to developmental disorders, including autism.<sup>168–170</sup> Any study on individuals with Turner syndrome should check for imprinting effects as well as X-chromosome dosage and hormonal effects.

### Exposure to Chemicals That Mimic or Block Endogenous Hormones

Diethylstilbestrol is a synthetic estrogen. Beginning in 1946, it was marketed for numerous gynecologic conditions, including prevention of threatened miscarriage in high-risk pregnancies. It became widely prescribed even during normal pregnancies, until

1971, when a report associated diethylstilbestrol with a rare form of vaginal cancer in the daughters of women who took the drug. Diethylstilbestrol was shown to cross the placenta and have a direct effect on the developing fetus.<sup>171</sup> Initial predictions were based on rodent models. In rodents, estrogen is a potent masculinizing agent. In normal rodent development, testosterone from the gonads is carried to the brain, where the enzyme aromatase converts it to estrogen. It is estrogen and its receptor that actually affect gene transcription. Therefore, exogenous estrogen masculinizes rodents when administered at the correct time. Human females are protected from maternal and placental estrogen by binding proteins.<sup>9,171</sup> Some studies of exposed female subjects are consistent with this model.<sup>172,173</sup> However, in another study, no group differences were seen for exposed women on a range of abilities at which female subjects excel on average or for abilities at which male subjects excel on average.<sup>174</sup>

Clark strongly argued against extrapolating from rodent models where estrogen is concerned.<sup>175</sup> A study of prenatal diethylstilbestrol exposure in rhesus monkeys suggested that estrogen has little or no effect on behavioral masculinization in primates (with the possible exception of rough-and-tumble play).<sup>176</sup> It has also been suggested that estrogen has demasculinizing or feminizing effects that occur at a later period than the testosterone-initiated effects discussed above.<sup>9</sup> Studies of exposed male humans offer some support for this position (laterality and spatial ability).<sup>177</sup> When administered to rats, diethylstilbestrol (and 4-octylphenol, a putative environmental estrogen) exposure results in decreased expression of CYP17 messenger ribonucleic acid (CYP17 codes for P450c17, an enzyme in the testosterone synthesis pathway). Suppression of CYP17 could have an adverse effect on fetal masculinization, suggesting an alternative explanation for the feminizing effects seen in the above study.<sup>23</sup> Given that estrogen can play a masculinizing role (or none at all) in early pregnancy and a feminizing role in later pregnancy or after birth, the timing of exposure can be a serious confounding variable in studies of diethylstilbestrol. Additional problems are that diethylstilbestrol can have different effects from endogenous estrogen and that whatever factor made the pregnancy high risk might also affect cognition. However, these studies are one of the few that can examine the role of estrogen in sexual differentiation.

Progestins have also been routinely prescribed to pregnant women. These occur in two types. Progestational progestins (derived from natural progesterone) interfere with the actions of androgens; these include medroxyprogesterone acetate and hydroxyprogesterone caproate (Delalutin). Natural progesterone itself has also been prescribed. Progesterone has been shown to act as an antiandrogen in female rodents, providing protection against the masculinizing effects of testosterone.<sup>178–181</sup> Androgenic progestins mimic the action of androgen and include danazol and 19-nortestosterone derivatives, such as norethindrone and ethisterone. Thus, we would predict progestational progestins to impair masculine-typical development and androgenic progestins to promote it.<sup>148</sup>

Reinisch et al reviewed 19 studies on the behavioral effects of prenatal exposure to hormones administered as treatment for at-risk pregnancies.<sup>182</sup> In general, the findings suggested that natural and synthetic progesterone-based progestins feminize



behavior, whereas androgen-based synthetic progestins masculinize behavior, as predicted. However, there were numerous nonsignificant findings, and many of the reported findings are, in fact, trends ( $.05 \leq P \leq .10$ ). Because sample sizes were small and nonparametric tests with limited power were used, one study used a statistical alpha criterion of 0.10 instead of the more conventional 0.05.<sup>183</sup> None of the studies examined social development or other variables relevant to autism.

Overall, the reports give little support for the effects of progesterone and synthetic progestins on sex-differentiated behavior. However, sample sizes were small in all studies (5–18), raising the possibility that small or moderate effect sizes might be missed. This represents the difficulty in finding large groups of individuals with similar treatment characteristics. Accessing and reviewing medical records can be expensive and time-consuming, and often records contain insufficient information. The dosage, duration of treatment, and combination of hormones administered varied widely even when using patients from the same physician. Seldom was a patient exposed to one type of progestin; they were more often treated with a mix of different progestins and often estrogens as well. In one study, 71 of the mothers in the study received at least one medication, 59 at least two, 43 at least three, 20 at least four, and 8 five different medications.<sup>184</sup> Many studies have used individuals exposed to a mix of synthetic hormones and attempted to separate them into groups, for example, based on the ratio of progestin to estrogen exposure, as in Reinisch and Karow and Reinisch, which found that individuals with exposure primarily to progestins were more independent, sensitive, individualistic, and self-sufficient than those exposed primarily to estrogens, who were more group oriented and group dependent.<sup>184,185</sup>

In addition to the difficulty arising from differing treatment variables, as with diethylstilbestrol, an association between consumption of a drug during pregnancy and a behavioral effect on the fetus does not necessarily imply causation. It might be impossible to differentiate a possible effect of illness on the fetus from an effect of the drug used to treat the illness. Well-matched controls are crucial, with sibling controls being the gold standard.<sup>184</sup> One of the benefits of studies using individuals exposed to drugs that mimic or block endogenous hormones during pregnancy is that, in contrast to congenital adrenal hyperplasia, the external genitalia is not usually affected (although some degree of masculinization is present in 2.75% of female infants whose mothers receive synthetic progestins of various types<sup>23</sup>). Therefore, parents could not have treated their exposed children differently on the basis of their external genitalia, and it is unlikely that they would have known that the drug might affect the sexual differentiation of the child's mind. In fact, several studies have reported that mothers did not even remember taking any medication during pregnancy.<sup>184,186</sup>

### Opposite-Sex Twins

Study of opposite-sex twins has the benefit that the children involved do not have a genetic abnormality or exposure to a synthetic substance. The rationale for this strategy comes from experiments in mice: female mice adjacent to male mice in utero are masculinized,<sup>187</sup> possibly by testosterone diffusing across the amniotic membrane<sup>188</sup> or being carried through the maternal circulation.<sup>189</sup> Human twin pregnancies are very different from

multiple offspring pregnancies in rats, but fetal testosterone can transfer from the male to the female fetus through amniotic diffusion in humans.<sup>190</sup> The fetal skin is permeable to fluid, and some dissolved solutes up to week 18 of gestation and amniotic fluid moves through the entire fetoplacental unit.<sup>191–193</sup> Human female individuals with male cotwins are masculinized with regard to sensation seeking<sup>189</sup> and spatial ability on a mental rotation task.<sup>194</sup> Further, female individuals with a male cotwin are masculinized with regard to spontaneous otoacoustic emissions, which are continuous, tonal sounds produced by most normal-hearing cochleas. Spontaneous otoacoustic emissions are more common in female than in male individuals.<sup>128,195</sup> This observation is important as it is unlikely to reflect the social effects of having a twin brother. It must be kept in mind when evaluating opposite-sex twin studies that individuals with opposite-sex twins can be different, not because of exposure to hormones but because they have grown up with a member of the opposite sex. This potential problem can be addressed by comparing twins with controls with an opposite-sex sibling of a similar age.

Some behaviors, such as play preferences, do not show an opposite-sex twin effect,<sup>196,197</sup> even though play preferences are dramatically affected by congenital adrenal hyperplasia. A further study of handedness in opposite- and same-sex twins found no association between the sex of the cotwin and handedness.<sup>198</sup> It is not clear why this should be the case, but one limitation of opposite-sex twin studies is that they involve no quantitative measure of hormone levels. It is possible that the amount of hormone transfer in humans is not sufficient for masculinization or that a mechanism is in place to protect female fetuses from excess androgen exposure. For example, progesterone has been shown to act as an antiandrogen in female rodents, providing protection against the masculinizing effects of testosterone,<sup>179–181</sup> and might serve a protective role in female humans, as discussed previously.<sup>183,199</sup>

Timing of exposure could also play a role. Henderson and Berenbaum argued that exposure to androgen from a twin brother should not occur after the 18th week of gestation.<sup>194</sup> If the critical period for a particular sexually dimorphic behavior occurs after week 18, one would not expect to see an opposite-sex twin effect. Miller argued that testosterone could be passed from a male fetus to a female fetus throughout pregnancy via the bloodstream.<sup>200</sup> He cited a study that reported that maternal blood testosterone levels depend on the sex of the fetus and that unbound testosterone crosses the placenta via a diffusion gradient.<sup>201</sup> The placenta is rich in aromatase, the enzyme that converts testosterone to estrogen. This should protect the fetus from maternal androgens and vice versa. In fact, when there is a deficiency in placental aromatase, for example, caused by mutations in the *CYP19* gene, both the female fetus and the mother virilize during pregnancy.<sup>23</sup> However, this protection system is not perfect. Women with medical conditions causing elevated androgen during pregnancy or those taking androgenic hormones can give birth to female infants with ambiguous (somewhat masculinized) genitalia, suggesting that maternal hormones can cross the placenta into the fetal system.<sup>202–204</sup> It does not appear that hormones pass from fetus to mother. Several studies found no differences in maternal testosterone levels during pregnancy in women carrying male versus female

fetuses (but see Klinga et al),<sup>205–210</sup> and women carrying fetuses with congenital adrenal hyperplasia do not show elevated androgen levels.<sup>23,209</sup>

Current evidence suggests that dizygotic twins are no more concordant for autism than other siblings.<sup>211</sup> This does not necessarily contradict the theory that prenatal testosterone levels and autism are related, given the issues regarding dosage and timing of exposure discussed above.

### **Umbilical Cord Blood**

Jacklin et al measured five steroid hormones, including testosterone, in umbilical cord blood at birth.<sup>212</sup> They assessed timidity in home and laboratory observations using a set of novel toys. Girls had higher mean timidity scores in two of the three samples tested. Timidity in girls was not predicted by any of the hormones measured. In boys, testosterone and progesterone increased boldness, whereas estrogen decreased boldness. The relationship to testosterone is in the expected direction (although sex differences in timidity are small and not always present). It is difficult to interpret the findings for progesterone and estrogen. The authors stated that their findings are consistent with the study of Reinisch and Karow.<sup>184</sup> However, in that study, women were exposed to synthetic progestins (some of which were androgen based), whereas in the Jacklin et al study, only natural progesterone levels were measured.<sup>212</sup> Their results are certainly not consistent with natural progesterone protecting against the effects of testosterone.

Jacklin et al examined cognitive abilities at 6 years of age.<sup>213</sup> In girls, testosterone was inversely correlated with spatial ability, but there was no correlation in boys. The testosterone result in girls was in the opposite direction to that predicted. At full term, there is considerable overlap between male and female testosterone levels measured in umbilical cord blood, and significant sex differences are reported inconsistently,<sup>34,36,38,122,206,209,214,215</sup> so this might not be the best time to look for hormone correlates for sexually dimorphic behaviors. In addition, the abilities measured in Jacklin et al did not show any sex differences and therefore are less likely to relate to gonadal hormone levels.<sup>213</sup> Jacklin et al also suggested that the onset of labor and the stress of labor might affect the levels of hormones found in cord blood.<sup>213</sup>

### **Maternal Testosterone During Pregnancy**

Udry et al examined a broad questionnaire measure of gendered behavior in women in relation to maternal testosterone levels.<sup>216</sup> Total testosterone and sex hormone-binding globulin were measured in maternal blood samples. The study focused exclusively on women because, as discussed previously, testosterone can pass from the mother to the female fetus along a diffusion gradient, but it does not appear that testosterone can pass from the male fetus to the mother. Only free testosterone can pass through the placenta, so total testosterone and sex hormone-binding globulin are proxies for actual testosterone exposure. Androgen exposure (as indicated by sex hormone-binding globulin levels) in the second (and no other) trimester of fetal life, combined and in interaction with adult androgens, masculinized women's behavior, explaining 16% to 18% of the within-sex variation in scores.

Hines et al related levels of testosterone and sex hormone-binding globulin in maternal blood samples to children's gender-role behavior as assessed by the Pre-school Activities Inventory.<sup>209</sup> Gender-role behavior was correlated with testosterone in girls but not in boys. One potential explanation for this finding is that maternal testosterone levels pass to the female fetus and promote masculine-typical development. Male fetuses will usually have higher endogenous testosterone levels than their mothers, and it does not appear that testosterone produced by male fetuses passes to their mothers. Thus, maternal serum samples do not indicate the testosterone exposure of their male offspring. Given the protective role of placental aromatase discussed earlier, Hines et al also suggested as an alternative or additional explanation that mothers with a relatively high testosterone level have daughters with a relatively high testosterone level because of a genetic predisposition passed from mother to daughter.<sup>209</sup> In either case, studies using this strategy can be applied only to female subjects. Levels of postpubertal testosterone are determined, in part, genetically, with heritability estimates around 40% to 60%.<sup>217,218</sup> The second study included only male subjects, but the first included both sexes and provided some evidence that the genetic connection is clearer in female individuals. In contrast, Sakai et al found that variation in testosterone levels at birth was accounted for primarily by environmental variation, not genetic variation.<sup>219</sup> However, sexes were not examined separately, so different relationships in boys and girls could not be detected. No one has evaluated whether testosterone levels at midgestation are heritable.

Hines et al also drew attention to the lack of a consistent relationship between prenatal hormone variability and gender-role behavior in boys when other research strategies were used, including studies of congenital adrenal hyperplasia and synthetic progestins.<sup>209</sup> They suggested two reasons why consistent relationships are less common in boys than in girls across studies. First, because fetal testosterone levels are much higher in male fetuses than in female fetuses, normal variability might be insignificant in relation to the high levels seen in the majority of male fetuses. Second, hormone-related predispositions in boys might be reduced or eliminated by other forces. For example, boys are more strongly encouraged to behave in a sex-typical way and are more likely to be discouraged from behaving in a cross-gendered way.<sup>220</sup> Although this strategy might be applicable only to girls, it does have several major advantages. It involves quantitative measures of hormone levels, it is possible to collect very large sample sizes, and it looks at normative variations in hormone levels.

### **Measuring Fetal Testosterone via Amniocentesis**

Both male and female fetuses produce some testosterone. In male fetuses, the main source is the testes. Female fetuses are exposed to small amounts of testosterone from the fetal adrenal glands and from the maternal adrenal glands, ovaries, and fat.<sup>25,26</sup> Testosterone can be measured in amniotic fluid collected during midtrimester amniocentesis.<sup>221</sup> Testosterone is thought to enter the amniotic fluid via diffusion through the fetus's skin in early pregnancy and later from fetal urination.<sup>222–224</sup> Although the exact correlation between testosterone levels in the fetal serum and the amniotic fluid is unknown, the maximal sex

difference in amniotic testosterone between male fetuses and female fetuses occurs between weeks 12 and 18, closely paralleling peak serum levels.<sup>221</sup> In animal models, the general critical period for sexual differentiation of the brain usually occurs when sex differences in serum testosterone are highest.<sup>33</sup> Therefore, it is likely that this is an important period for sexual differentiation of the human brain as well. This is supported by the study reviewed previously, which found that only prenatal androgen exposure in the second trimester related to adult gendered behavior,<sup>216</sup> and the study that measured testosterone in maternal blood during pregnancy at a mean gestational age of 16 weeks.<sup>209</sup>

The first study to use this methodology was carried out by Finegan et al.<sup>225</sup> They reported relationships with language comprehension, classification abilities, counting, number facts, and block building, but the results were not consistent with the predictions of androgen theory. This might be because the abilities studied did not show a sex difference in their or other samples.<sup>226</sup> Later studies by the same group have produced results more consistent with predictions. At age 8 years, girls with higher levels of amniotic testosterone performed a mental rotation task faster than girls with lower levels.<sup>132</sup> At age 10 years, girls with higher levels of amniotic testosterone showed a more masculine pattern of cerebral lateralization.<sup>227</sup>

The amniocentesis design has several strengths. As with the measurement of testosterone in maternal blood, it involves quantitative measures of hormone levels and measures normal variability. The majority of studies showing that variations in fetal testosterone are related to gendered behavior have used groups with large abnormalities in prenatal endocrine conditions owing to genetic flaws or fetal exposure to synthetic progestins. This can be a weakness as well as larger sample sizes might be needed to show an effect than in studies in which exposure is very high. Sample sizes are generally smaller than in studies measuring maternal testosterone levels because only a selection of women will be advised to have amniocentesis. However, amniocentesis is carried out routinely, and children who underwent amniocentesis are far more common than those with prenatal endocrine conditions. For example, Addenbrooke's Hospital, which analyzes amniocentesis samples from six hospitals in East Anglia, United Kingdom, processes approximately 1000 samples a year.<sup>228</sup> Amniocentesis takes place in midgestation, which is thought to be an important period for sexual differentiation of the human brain, and unlike studies using maternal blood, testosterone exposure can be measured in both boys and girls. A significant limitation of research using this method is that a truly random sample cannot be collected because one can include in a study only those individuals who have decided or been advised to have amniocentesis owing to late maternal age or other factors that increase the risk of fetal abnormality.

Each of the above methodologies has strengths and weaknesses (see Table 1 for a synopsis), but taken together, they indicate that fetal testosterone does play a role in the sexual differentiation of the human brain. A role for fetal estrogen appears to be much less likely but should not be ruled out. Each strategy could, potentially, be applied to studying whether fetal testosterone is a risk factor for autism. In the next section, we review the results of a longitudinal study examining the

relationship between amniotic testosterone, social development, and other traits relevant to autism.

### CAMBRIDGE FETAL TESTOSTERONE PROJECT

Anyone who is interested in the role of prenatal hormones in the development of autism is immediately faced with a problem. Although published prevalence rates for autism have increased significantly over the past decades, it is still a relatively rare condition. Autism spectrum conditions can occur as often as 1 in every 200 people.<sup>82</sup> Only a small proportion of pregnant women will be asked to undergo amniocentesis. To add to the difficulties, autism is seldom diagnosed before age 3 years, so there is a considerable lag between the time when an amniotic fluid sample is collected and a child is old enough for a diagnosis to be made with confidence. However, we can begin investigating possible links between fetal testosterone and autism spectrum conditions in a less direct way. It is increasingly suggested that autism is part of a spectrum of conditions that blend into the "normal" population. If "autistic" traits are continuously distributed in the population, then it is possible that factors that are related to variation in those traits in nonclinical groups are also important in the clinical population. At Cambridge University, we have been following up a group of approximately 100 children whose fetal testosterone level had been measured in amniotic fluid. Their mothers had undergone amniocentesis in the Cambridge region between June 1996 and June 1997 and had given birth to the healthy singleton infants between December 1996 and December 1997.<sup>229</sup>

The children were first seen at 12 months of age, when the infants and parents were filmed and the amount of eye contact made by the infant to the parent was recorded. Eye contact is of major importance in normal social development.<sup>230-232</sup> Infants as young as 2 months of age spend more time looking at the eye region of the face than any other part of the face.<sup>233</sup> This might also be relevant to autism, which is defined by marked social impairment, including abnormal eye contact.<sup>234-236</sup> Girls made significantly more eye contact than boys. The amount of eye contact varied quadratically with amniotic testosterone level when data from both sexes were examined together and when the data for the boys were examined alone. This suggests that testosterone might shape the neural mechanisms underlying social development.

The children were next followed up 18 and 24 months after birth, and their vocabulary size was assessed using the Oxford Communicative Development Inventory.<sup>237</sup> Girls were found to have a significantly larger vocabulary than boys at both ages. This replicates previous findings of a female advantage in language ability but reveals this sex difference at the earliest point of development. Additionally, amniotic testosterone was an inverse predictor of vocabulary size when data from both sexes were examined together, but not within sex. The lack of a significant correlation between testosterone and vocabulary within each sex might reflect the relatively small sample size. However, the significant correlation between testosterone and vocabulary when the sexes were combined suggests that testosterone might be involved in shaping the neural mechanisms underlying communicative development.

**Table 1. Available Research Strategies for Studying Fetal Sex Hormones and Later Behavior: Strengths and Weaknesses**

<i>Research Strategy</i>	<i>Pros</i>	<i>Cons</i>
Congenital adrenal hyperplasia	High dosage of FT in female infants means that effects can be observed with small sample sizes Genotype and sex of rearing are female, but exposure to FT was high Degree of FT exposure correlates well with genotype Treatment of female infant usually begins early in neonatal life: restricting FT exposure to prenatal period	Nature of FT exposure in male infants unclear; can be high, low, or normal Virilization of genitalia can affect parents' response to affected girls Also exposed to high levels of adrenocorticotrophic hormone during prenatal period With treatment, girls can become hypoandrogenic and also risk glucocorticoid excess Condition rare, so sample sizes are limited No quantitative measure of hormone exposure, although disease severity can be used as a proxy Does not measure normal variability
Complete androgen insensitivity	Good comparison with congenital adrenal hyperplasia as genotype is male but exposure to FT is absent	Hormonal sex and sex of rearing confounded Condition rare, so sample size is limited Does not measure normal variability in FT
Idiopathic hypogonadotropic hypogonadism	Sex of rearing is male, but testosterone levels are low Could reveal effects of neonatal and pubertal testosterone as the condition is not usually diagnosed until puberty	Not clear if FT levels are low prenatally; maternal hormones can stimulate the fetal testes Condition rare, so sample size is limited
Turner syndrome	Can examine direct genetic effects of having only 1 X chromosome Can examine effects of low estrogen levels	Direct genetic effects and hormonal effects confounded Extensive clinical problems Condition rare, so sample size is limited
Klinefelter syndrome	Can examine direct genetic effects of having 2 X chromosomes and 1 Y chromosome Low testosterone levels	Direct genetic effects and hormonal effects confounded Not known if FT levels are low Extensive clinical problems Condition rare, so sample size is limited
Exposure to diethylstilbestrol	Can examine effects of high estrogen exposure Genitalia normal	Unclear what role estrogen has in human sexual differentiation Different researchers have predicted both masculinizing and feminizing effects Dosage and timing of exposure vary widely and can be critical in determining the drug's effect Effects of drug and effects of disease state are confounded Sample size often small
Exposure to progestins	Can act as antiandrogens, providing a good comparison with congenital adrenal hyperplasia studies Roughly comparable to animal experiments Genitalia normal in most cases	Some progestins are virilizing, whereas some act as antiandrogens Patients often given a mix of progestins of different types (also estrogens) Dose and timing of exposure vary widely Effects of drug and effects of disease state are confounded Sample size often small
Opposite-sex twin studies	No genetic disorder, maternal disease, or drug exposure Genitalia normal	Unclear how hormones might be transferred between twins No quantitative measure of hormone exposure Effects mainly restricted to female infants Results can be confounded by the experience of growing up with an opposite-sex twin
Umbilical cord blood	No genetic disorder, maternal disease, or drug exposure Measures normal variability in hormone level Quantitative measure of hormones	Large overlap in male and female testosterone levels; significant sex differences reported inconsistently Can miss the critical period for sexual differentiation Hormone levels can be affected by the stress of labor

Table 1. Continued

Research Strategy	Pros	Cons
Maternal blood	No genetic disorder, maternal disease, or drug exposure Measures normal variability in hormone level Quantitative measure of hormones Large sample sizes possible Can assess FT levels throughout pregnancy	Might be applicable only to female samples
Amniotic fluid	No genetic disorder, maternal disease, or drug exposure Measures normal variability in hormone level Quantitative measure of hormones Moderate sample sizes possible Occurs during a suspected critical period for sexual differentiation Applicable to both male and female fetuses	Cannot select a truly representative sample Will probably not reveal effects on behaviors that are organized late in pregnancy

FT = fetal testosterone.

The children were next followed up at 48 months. Their mothers completed the Children's Communication Checklist,<sup>238</sup> a questionnaire assessing language, the quality of social relationships, and restricted interests. Amniotic testosterone was negatively correlated to quality of social relationships and directly correlated with restricted interests, taking sex differences into account. Testosterone was also positively correlated with restricted interests when boys were examined separately. Children also completed a laboratory test of social cognition where they were asked to describe cartoons with two moving triangles whose interactions with each other suggested social relationships and psychological motivations. Females used more mental and affective state terms to describe the cartoons than males. FT was not associated with the frequency of mental or affective state terms. Females also used more intentional propositions than males. FT was negatively correlated with the frequency of intentional propositions, taking sex differences into account. FT was also negatively correlated with the frequency of intentional propositions when males were examined separately.<sup>239</sup> These findings implicate testosterone in both social development and attentional focus.

Since the publication of these results an additional 400 children have been recruited into the study to be followed up at age 5 and beyond. Our newest results demonstrate that FT is related to the number of autistic traits a child shows (as measured by the Childhood Asperger Syndrome Test [CAST]), their scores on the Empathising Quotient (EQ) and Systemising Quotient (SQ), and their ability to identify subtle emotional expressions using only the eye region of the face.<sup>240–242</sup>

Causal interpretations are, of course, unjustified from these correlational studies, but the results suggest that high levels of fetal testosterone could produce a behavioral profile relevant to that seen in autism.

#### WHERE IN THE BRAIN MIGHT FETAL TESTOSTERONE EXERT AN EFFECT ON AUTISM SPECTRUM CONDITIONS?

Owing to its small size, circulating testosterone can easily cross the blood–brain barrier. It is lipophilic and can therefore pass through cell membranes and enter the cytoplasm of cells. The androgen receptor is a classic steroid receptor found in the

cell cytoplasm or nucleus. Once bound to testosterone (or dihydrotestosterone, which is synthesized within the target cell from testosterone by 5 $\alpha$ -reductase), the receptor-hormone complex binds to DNA and thereby affects transcription. Testosterone can also be aromatized to estrogen within the target cell, where it can bind to the estrogen receptor and influence transcription in a similar fashion. Testosterone can affect neural development in multiple ways, which include rescuing cells from programmed cell death, altering patterns of interconnections among neurons, and specifying the neurochemicals used by cells.<sup>20</sup>

In the fetal primate brain, significant androgen receptor binding is observed in the cerebral cortex, cerebellum, medio-basal hypothalamus, amygdala, corpus callosum, and cingulate cortex of both sexes. Detectable levels of 5 $\alpha$ -reductase and aromatase are also found in these regions.<sup>243–245</sup> Androgen receptors are present as early as the first trimester of gestation, with some areas, including the temporal cortex, showing a transient elevation in expression at this time.<sup>244</sup> Sex differences are seen in the distribution of androgen receptors in the developing cortex. Androgen receptor binding is higher in the right frontal lobe and the left temporal lobe of male individuals compared with the contralateral side. Female individuals do not show the same asymmetry.<sup>246</sup> These findings support the hypothesis that androgens can act to differentiate the brain in a site-specific fashion. Information on the distribution of androgen receptors in the human fetal brain is extremely limited. However, the distribution in monkeys is similar in many respects to that described in the male rat, suggesting that receptor distribution might be conserved across species. The human fetal hypothalamus does take up radioactively labeled testosterone during the midtrimester,<sup>36</sup> but estrogen, androgen, and progesterone receptors were not found in a study of midtrimester fetal brains.<sup>247</sup> However, this is only a single study, and the results have not been replicated. In addition, it is also possible that testosterone during this time period affects brain development through mechanisms other than classic steroid receptors.

There is a great deal of evidence for sexual dimorphism in the central nervous system, and many of these features are thought to depend on early sex hormones<sup>1,4,248</sup>; however, it must be kept in mind that genetic factors other than those mediated by

hormonal factors can also be involved. The hypothalamus is the brain area most widely recognized as sexually dimorphic and is important in mediating the sexual behavior of male and female individuals. A subregion of the preoptic area of the hypothalamus is larger in male than in female rats and enlarges under the influence of testosterone. This region has been labeled the sexually dimorphic nucleus of the preoptic area. The human analogue of this nucleus is thought to be located in the interstitial nuclei of the anterior hypothalamus, but there is no absolute consensus on which of the four interstitial nuclei is actually analogous to the sexually dimorphic nucleus in the rat.<sup>3</sup> Several studies have reported that parts of the interstitial nuclei of the anterior hypothalamus are smaller in women than in men.<sup>249,250</sup> The bed nucleus of the stria terminalis, another hypothalamic structure involved in sexual behavior, is also larger in women than in men.<sup>3</sup>

Sex differences have also been reported in the human corpus callosum. Although the literature includes multiple studies either confirming or failing to find a sex difference in this area, the consensus appears to be that there is a small difference in size favoring women. This difference might be restricted to the posterior region (the splenium), and several reports suggest that the difference is apparent only when a correction is made for the larger size of men's brains.<sup>3,251</sup> Women also have a larger cross-sectional area of the anterior commissure, another system that connects the right and left hemispheres. The massa intermedia, which connects the two sides of the thalamus, is absent more often in men than in women.<sup>252</sup> Overall, this suggests that women have greater interhemispheric connectivity than men, but the functional significance of this can vary for different cognitive skills.<sup>3</sup>

The amygdala can also be sexually dimorphic. Caviness et al found that the amygdala was smaller in girls than in boys aged 7 to 11 years (based on a sample of 15 girls and 15 boys) using magnetic resonance imaging (MRI).<sup>253</sup> Goldstein et al also found that the amygdala was smaller in female than in male adults in a community sample of 48 normal adults using MRI,<sup>254</sup> but note that Murphy found no sex difference when examining 17 postmortem brains from gestation through age 94 years.<sup>255</sup>

Sexual dimorphism is also present in the cortex. The planum parietale (part of the parietal lobe at the posterior end of the sylvian fissure) shows an overall rightward-larger asymmetry that is greater in right-handed men than in right-handed women. The pattern is reversed in left-handers.<sup>256</sup> The degree of leftward asymmetry in the planum temporale (the area

on the upper surface of the temporal lobe behind the primary auditory area) has also been reported to be greater in men than in women, but studies in large groups suggest that the difference is fairly trivial.<sup>3</sup> Goldstein et al found that women had larger cortical volumes relative to cerebrum size, particularly in the frontal and medial paralimbic cortices, than men, whereas men had larger volumes of frontomedial cortex relative to cerebrum size.<sup>254</sup>

Areas of the brain that show gross anatomic differences between the sexes are certainly of interest, but it should be kept in mind that differences in cognitive functioning between the sexes can arise in the absence of gross visible structural variations. Differences can occur at the cellular level (eg, in the size, number of branches, parts of neurons, or distribution of neurotransmitters) and in variations in fiber connections between neurocognitive systems. Any functional differences between the sexes are likely to reflect parallel physiologic differences, even if they are not visible to the naked eye.

If fetal testosterone does play a role in the development of autism spectrum conditions, we would expect a significant degree of overlap between brain areas that show sex differences and/or are known targets of androgen and brain areas that are implicated in autism.<sup>257</sup> Brambilla et al reviewed all original MRI research articles involving autistic patients published from 1966 to May 2003.<sup>258</sup> They found that increased total brain, parieto-temporal lobe, and cerebellar hemisphere volumes were the most frequently reported abnormalities, whereas recent findings suggested that the amygdala, hippocampus, and corpus callosum can also be abnormal. Although they noted conflicting evidence for whether the frontal lobes were anatomically abnormal in autism, they argued that several well-controlled functional reports support their involvement in these conditions. Table 2 compares brain areas consistently implicated in autism with brain areas that contain the androgen receptor during development and with sexually dimorphic brain areas. As predicted, there is a substantial degree of overlap. However, this list cannot be considered complete. As noted previously, there are doubtless physiologic differences between the sexes that are not observable at a gross anatomic level. Equally, there can be physiologic differences in autism that are not observable at a gross anatomic level. Also, it is increasingly recognized that steroid hormones might not exert all of their effects through classic receptors. The existence of a novel membrane-bound androgen receptor has been postulated by a number of authors. In addition, androgens can also stimulate rapid, nongenomic effects through second-messenger cascades via the sex hormone-binding globulin

**Table 2. Comparison of Brain Regions Implicated in Autism With Those Showing Gross Anatomic Sex Differences and Those Expressing Androgen Receptors**

<i>Autism</i>	<i>Androgen Receptors</i>	<i>Sexually Dimorphic (Gross Anatomic Level)</i>
Parietotemporal lobe	Temporal lobe	Parietal and temporal lobes
Cerebellum	Cerebellum	
Amygdala	Amygdala	Amygdala
Hippocampus		
Corpus callosum	Corpus callosum	Corpus callosum
Frontal cortex	Frontal cortex	Frontal cortex
	Hypothalamus	Hypothalamus
	Cingulate cortex	

Adapted from Kimura D,<sup>3</sup> Sholl SA et al,<sup>243</sup> Handa RJ et al,<sup>244,245</sup> Goldstein JM et al,<sup>254</sup> and Brambilla P et al.<sup>258</sup>

receptor. Testosterone can also act as a functional antagonist at the 5-hydroxytryptamine receptor.<sup>259</sup> Nongenomic effects could also potentially be induced directly by androgens in the absence of a receptor. Dihydrotestosterone has been found to alter membrane fluidity in some cell types lacking the classic androgen receptor but only at extremely high levels, which might not be relevant in vivo.<sup>260</sup>

## SUMMARY AND CONCLUSIONS

Although it is widely accepted that autism is a biologic condition, there is no consensus on what biologic factors are involved. The higher incidence of autism in male individuals might provide important clues to the etiology of the condition. The studies reported in this article suggest that prenatal testosterone could be involved in the sex differences in social behavior in the general population and in the male vulnerability to autism, but further research is required. Several important questions remain, including (1) Do variations in fetal testosterone relate to variations in cognitive functioning and symptom severity within individuals with autism spectrum conditions? Our longitudinal study suggests that variations in fetal testosterone are related to social cognition and attentional focus in typically developing children, but one should be cautious about extrapolating these results to individuals with autism. (2) Do individuals with autism show evidence of high fetal testosterone exposure? (3) Is fetal testosterone specifically involved in the development of autism, or does it promote a general male vulnerability?<sup>261</sup> Rutter et al noted that male-biased sex ratios largely occur in early-onset disorders that involve some kind of neurodevelopmental impairment, including autism, dyslexia, ADHD, and early-onset persistent antisocial behavior.<sup>6</sup> In contrast, female-biased sex ratios largely occur in emotional disorders with a peak age at onset in adolescence. Although they acknowledge that there can be diagnosis-specific factors relevant to the sex differences in individual conditions, they suggest that the type of causal influence will be similar within these two groups of disorders that differ in both sex ratio and age at onset. (4) Is fetal testosterone relevant to most cases of idiopathic autism or only to a specific subset of cases? Gillberg and Coleman argued that autism is a syndrome or series of syndromes caused by many different, separate, individual conditions, all of which affect the same final common pathway in the brain that causes individuals to present with autistic symptoms.<sup>139</sup> Exposure to elevated levels of fetal testosterone could be an important component of one or several of these individual conditions that lead to the behavior profile called autism.

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